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Antimicrobial Resistance Profile of Bacteria Indicators of Contamination in Ready-to-Eat Retail Fish Foods

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Abstract

The increasing global production of aquaculture as one of the major sources of food calls for developing effective food safety measures to protect consumers from exposure to pathogens through the consumption of fish and seafood. This study reports the detection of bacterial indicators of contamination in fish sold in retail markets. The Gram-negative indicator bacteria identified were dominated by members of the family Enterobacteriaceae. They included *Klebsiella pneumoniae*, *Klebsiella variicola*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Citrobacter* sp., *Escherichai coli*, *Acinetobacter iwoffi* and *Acinetobacter* sp. while the Gram-positive indicator bacteria detected comprised *S. aureus*, *S. pseudintermedius*, *S. dysgalactiae*, *A. viridans*, and *A. urinaequi*. Overall, the coliform group accounted for the largest proportion of the indicator bacteria of contamination identified in the fish examined.

Of the 12 antibiotics examined for resistance in the indicator bacteria isolates using the disc diffusion test, the highest resistance prevalence was against ciprofloxacin (62%) followed by colistin (52%), erythromycin (48%), rifampicin (43%), amoxicillin (38%), cefoxitin (33%), cephalothin (33%), sulphonamide (33%), trimethoprim (29%), nitrofurantoin (24%), tetracycline (24%), and gentamicin (5%). Thus, there was resistance at different prevalence levels against all 13 antibiotics tested. On the contrary, only five of the 13 AMR genes were detected by PCR in the same bacteria isolates examined using the disc diffusion test indicating that although the selection of AMR genes used in the PCR was matched with antibiotics used in the disc diffusion test, PCR results showed a lower resistance prevalence than the disc diffusion test. The detection of the $bla_{\text{CTX-MA}}$, bla_{SHV}, and bla_{TEM} genes by PCR is indicative of the presence of the extended spectrum of beta-lactamases (ESBLs) and carbapenems in the indicator bacteria examined. Given that infections by ESBLs or carbapenem-resistant bacteria are among the most difficult to treat and often lead to high death rates due to lack of response to treatment, there is a need to develop stringent food safety control measures for fish foods to prevent the spread of AMR from fish to consumers.

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Acronymyms

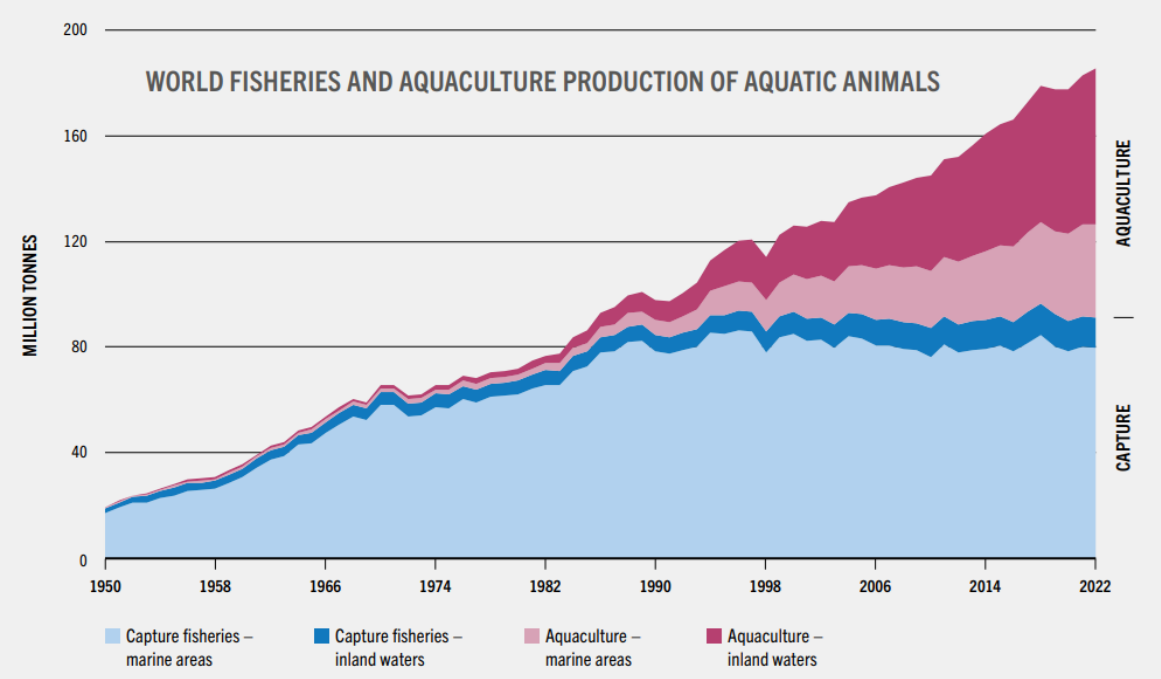
ABR	-	Antibiotic resistance
AMR	-	Antimicrobial resistance
ARE	-	Antibiotic-resistant *Enterococcus*
ARG	-	antibiotic resistance groups
bla$_{CTX}$	-	Cefotaximase beta lactamases
bla$_{NDM}$	-	New Delhi metallo-beta-lactamases
bla$_{OXA}$	-	Beta lactamase oxacillinase
bla$_{SHV}$	-	Sulf-hydryl variable beta lactamases
bla$_{TEM}$	-	Temonierabeta lactamases
CARD	-	Comprehensive Antimicrobial Resistance Database
CAT1	-	Cationic amino acid transporter 1
CFU	-	Colony forming unit
CLSI	-	Clinical and Laboratory Standards Institute ()
CPE	-	Ccarbapenem producing Enterobacteriales
CRA	-	Carbapenam resistant Acinetobacter
DNA	-	Deoxyribonucleic acid
ESBL	-	Extended spectrum of beta lactamases
FAO	-	Food and Agriculture Organization
FC	-	Fecal coliform
FIB	-	Fecal indicator bacteria
gDNA	-	Genomic Deoxyribonucleic acid
GIT	-	Gastrointestinal tract
HMW	-	High molecular weight
KPC	-	Klebsiella pneumoniae carbapenem
LMIC	-	Low medium income country
Mbp	-	Mega base pair
MDR	-	Multidrug resistance
MGE	-	Mobile genetic element
MEGA 7	-	Molecular Evolutionary Genetic Analysis version 7
MRSA	-	Methicillin-resistant Staphylococcus aureus
n-3 PUFA	-	n-3 polyunsaturated fatty acids
NCBI	-	National Centre for Biotechnology Information
NDM-1	-	New Delhi metallo-1
OXA	-	Oxacillinase
PCR	-	Polymerase chain reaction
rpoB	-	RNA polymerase subunit beta
RTE	-	Ready-to-eat
SCCmec	-	Staphylococcal cassette chromosome *mec*
Sul2	-	Sulfonamide resistant dihydropteroate synthase 2
TBE	-	Tris Borate
TC	-	Total coliform
TCC	-	Total coliform count
TFCC	-	Total fecal coliform count
TPC	-	Total Plate Count
TSB	-	Tryptic Soy Broth
TVC	-	Total viable counts
UK	-	United Kingdom
UN	-	United Nations

US FDA	-	United Stated Federal Drug Agency
USA	-	United States of America
UV	-	Ultraviolet
WGS	-	Whole genome sequencing
WHO	-	World Health Organization
WWTP	-	Wastewater treatment plant

1.0 Aquaculture

1.1 Expansion of the aquaculture industry

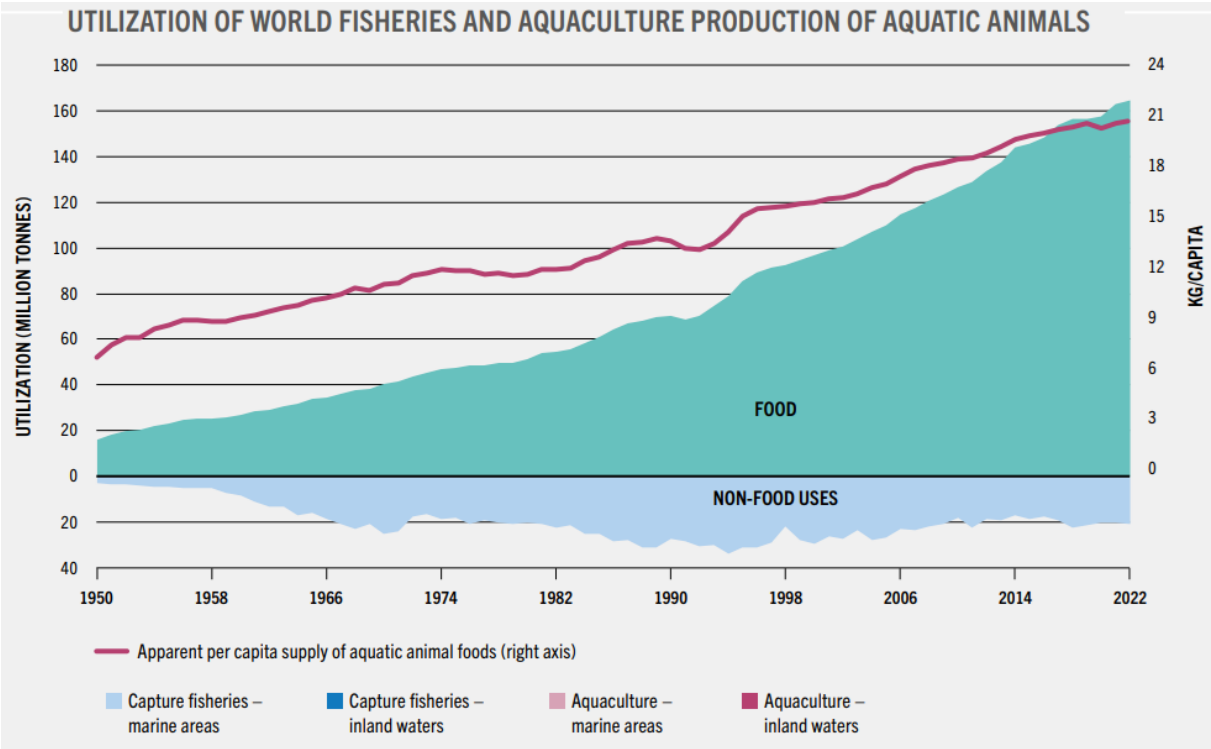
The human population is expected to reach 8.5 billion by 2030 and, aquaculture, as one of the leading protein producers, is expected to play a significant role in reducing the chronic undernourishment, which is currently estimated to affect about 600 million people [1]. In 2015 Kobayashi et al [2], estimated global fish production to increase from 154 million tons in 2011 to 186 million tons by 2030, with aquaculture being the main key player accountable for the increase. By 2022, the Food and Agriculture Organization (FAO) of the United Nations (UN) reported that the production of fisheries and aquaculture reached 223.2 million tonnes leading to an estimated 20.7 kg per capita of aquatic animal foods [1] (Figure 1). This corroborates with earlier observations indicating that since 2020 global aquaculture production that has increased by 6.6% accounting for more than 57% of aquatic animal products used for human consumption [1]. The strategic framework for the period 2022 to 2031 adopted by FAO in 2021 [1] on the blue transformation, whose priority program aims at maximizing the opportunities presented by aquatic foods to enhance food security, improve nutrition, eradicate poverty and support the environment of the 2030 Agenda for sustainable development goals promotes the expansion of aquaculture.



Figur 1. World fisheries and aquaculture production of aquatic organisms

In 2017, Anderson et al [3] showed that the ongoing increase of aquaculture products exceeds all other types of meat production of which seafood is expected to account for 34.5% of the global animal production by 2030. In their study, Anderson et al [3] observed that more than 90% of the world's aquaculture production is done in developing countries, where it contributes to food security by consumption of aquaculture products and it also serves as a source of income to the low and medium income countries (LMIC).

The average annual global fish food consumption between 1961 and 2016 was estimated at 3.2% which translated from 9 kg per capita in 1961 to 20.5 kg per capita in 2017 [4], which implies that the increase in global food consumption exceeded the human population growth of 1.6 % during the same period. This increase in per capita fish consumption is attributed to the rapid expansion of global aquaculture production since the 1980s. Currently, it is estimated that aquaculture supplies 50 % of the global food used for human consumption [5]. It is also estimated that aquaculture products will increase by 10% by 2032 of which 90% will be used for human consumption, at a rate of 21.3 kg per capita [1] (Figure 2). Despite so, aquaculture remains dominated by LMIC and not able to exploit the full potential of the industry.



Figur 2. Utilization of world fisheries and aquaculture production of aquatic organisms

1.2 Challenges in fish processing and retail supply of aquaculture products

Consumption of aquaculture products, compared to animal protein sources, is linked to several health benefits like high-quality protein, valuable lipids, minerals, and vitamins. It is presumed that fish foods are healthy for human consumption [1]. They have been linked to lower risk of diseases like coronary heart disease [6] while other benefits include the presence long-chain n-3 polyunsaturated fatty acids, eicosapentaenoic acid, and docosahexaenoic acid. Also, the fatty acid content of fish food is considered favorable compared to terrestrial animal fatty acid [6]. The universal perception that fish foods have low fat and cholesterol content makes fish and seafood preferred food products for human consumption given that most people are concerned about their weight and also that the current trend of consumption of healthy and natural products favors the fish sector. Despite so, a large proportion of the fish products are consumed without

stringent food safety inspection given that most of the fish products are processed and sold to consumers directly after harvest from aquaculture.

A study by Pieniak et al showed that [7] consumers from European countries raised quality and food safety concerns when buying fish. Girard and Paquotte [8] reported that consumers believe that the fish they buy originates from the ocean or aquaculture farms and was still alive the day before purchase rendering it more nutritious and taste given that it directly sold without adding artificial preservatives. Ferfolja et al [9] showed that the growing consumer awareness about the impact of food on health has led to an increasing trend towards fresh fish consumption. Nerrie [10] pointed out that in the United States, many food items including raw fish are marketed directly to consumers through the Internet. Consumers perceive buying fish and seafood products directly from aquaculture suppliers as satisfying because it is reflected to be obtaining high-quality products directly from the farm [10]. Similarly, Chase and Otts [11] observed that the consumer demand for locally produced fish and seafoods in several countries in the world. This is because the interest to buy food produced within close proximity to people's homes is considered to be a supply of freshly produced foods without artificial preservations. And as such marine foods are considered to be a local safe supply in most coastal communities[11]. In Although this marketing strategy has expanded tremendously, there is a general lack information the safety of fish and seafood sold directly to consumers . In LMIC, the processing fish and seafoods is often done under poor sanitary conditions, which may occasionally entail health risks that include exposing the consumers to pathogenic organisms, pollutants, and feed additives like antibiotics [12]. As mentioned by Grema et al [13] food safety risks include the lack of appropriate biosecurity control measures, lack of effective cold chain, poor processing, poor hygiene by fish handlers, and lack of qualified fish inspection personnel. Thus, there is a need to develop similar to methods used to assess microbial contamination in the production, processing and supply chain of terrestrial animal products for the aquaculture industry especially in LMICs. It is important to develop microbiological quality assessment methods using indicator microorganisms to ensure safety before selling to consumers.

1.3 Microbial indicators of food contamination

Much as aquaculture products freshly sold in retail outlets are considered safe, taste and highly nutrition, it is likely that they contain different microorganisms that include pathogens and other contaminants [14, 15]. In most cases, pathogens coexist with microorganisms that can be used as indication of contamination. As defined by Mossell et al [14], indicator microorganisms are microorganisms or a group of microorganisms indicative of the possible presence of pathogens whose presence in given numbers points to inadequate safety in processing or the use of unhygienic conditions during processing. For example, the presence of organisms like *Escherichia coli*, commonly found in the intestinal tract of terrestrial animals, in processed or retail fish which does not cause disease in fish might entail that fish were exposed to fecal contamination. Given that fish and shellfish are not natural habitats of microorganisms found in the mammalian microflora, like *E. coli* and other faecal coliform, the presence of these microorganisms in processed fish and shellfish food is indicative of contamination from the terrigenous source [15, 16]. Thus, indicator microorganisms are most often used to assess sanitation conditions, and hygiene during the entire production, processing, preservation, transportation, and supply chain [15, 17].

1.4. Source of microbial contamination of aquaculture products

1.4.1 Environmental sources

Different bacteria species that serve as indicators of human and animal fecal contamination are used to assess the quality of water used in aquaculture production. The commonly used bacteria species include the coliform, *Escherichia coli*, *Streptococcus* spp., *Acinetobacter* spp., and *Enterococci* spp. [18]. The microorganisms are considered to be fecal indicator bacteria (FIB). The FIBs have been detected in different aquatic environments including aquaculture facilities. Leung et al [19] found fecal *streptococci*, and fecal coliforms in water sediment, and fish samples collected from aquaculture ponds in Alabama in the USA while Shafai et al [20] found fecal-contaminated ponds that had tilapia, which had tissue samples contaminated with fecal coliform in Egypt . Ko et al [21] found a higher abundance of FIBs in fish ponds than in water bodies located in the delta region in Myanmar. They found a significant association between the multidrug-resistant Enterococci spp. in the chicken excreta-fed fishpond. Boufafa et al [22] found high FIB levels and pathogenic bacteria in seawaters and *Perna perna* mussels in Northeastern Algeria of which they attributed the high FIB levels to the location of mussel farms close to industrial and coastal areas that had urban, agricultural, and industrial pollutants. The bacteria species isolated included the genera *Escherichia*, *Salmonella.*, *Staphylococcus spp.*, *Klebsiella*, *Pseudomonas*, and *Proteus.* Altogether these studies show that FIBs are contaminants in different aquatic environments and that they have been found in different aquaculture products as indicator contaminants.

Shin et al [23] investigated the prevalence of FIB in seawater samples and aquatic animals collected from aquaculture farms along the Korean coast and found that *E. coli* followed by *Enterococcus* spp. were most dominant. Mok et al [24] found variations in fecal coliform (FC) in an area close to shellfish aquaculture facilities in Korea whose abundance was highly influenced by the rainfall pattern. Leight et al [25] found FCs in water samples from a shellfish aquauclture area in the Chesapeake Bay, USA where they observed the Enterobacteriaceae increased in abundance after rainfall suggesting that the detected FCs were allochthonous organisms washed in from land. This corroborates with Mitch et al [26] who observed that during the summer, wastewater effluents were not significant contributors to FC loading in the Quinnipiac River in the USA but in winter, the FCs accumulated along the river predominantly due to loadings from successive wastewater outfalls. Likewise, Maipa et al [27] observed that seasonal fluctuation influenced the FIBs mainly concerning *E. coli* and FBs had a significant influence of the relationships between the coliforms, fecal coliforms, *E. coli*, fecal streptococci, location, time, and seasonal factors in marine environments in Greece. Overall, these studies show that FIBs, FCs, and other indicator bacteria can be used to monitor contamination levels caused by seasonal rainfall variations, which can be used to predict the potential increase in the risk of exposureto pathogens of fecal origin.

1.4.2 Fish and Seafood Processing Facilities and retail markets

The processing of fish to produce edible products like fillets involves skinning, eviscerating, and cutting which can lead to contamination of fillets with different microorganisms.Fish and seafood products are unacceptable when high quantities of indicator contaminant bacteria or certain pathogens are found [10, 16, 28]. Assessing microbial exposure at retail outlets before fish products reach consumers is vital to ensure that the food is safe for consumption [29]. Contact surfaces are a major source of contamination of fish and shellfish foods in the processing facilities and retail markets [30-32]. The US Food and Drug Administration has classified food contact surfaces to include all equipment, utensils, and facilities used during

processing and storage, as well as worker clothing, hands, and packaging material[33]. A study done by Ananchaipattana et al [34] reported that more than 90% of fish or seafood collected from retail markets in Thai markets was contaminated with coliform bacteria of which the contamination rate of *Staphylococcus* spp. was higher in fish or seafood samples bought from open markets than those from supermarkets. Sousa et al [35] found high counts of aerobic mesophiles and FCs in samples collected from surfaces of skinning machines, polyethylene cutting boards, polypropylene cases, baskets, and trays, the plastic material used to cover the trays, packaging tanks, knives, and stainless steel sorting and packaging tables, and gloves used by handlers in fish industries. Prasai et al [36] found high Total Plate Count (TPC), Total Coliform Count (TCC) and Total Fecal Coliform Count (TFCC) in *Labeo rohita, Cyprinus carpio* and *Clarias batrachus* collected from retail markets of Kathmandu valley in Nepal. They TPC, TCC and TFCC in all fish samples. The pathogens isolated from the samples were *Escherichia coli, Staphylococcus aureus*, S*taphylococcus spp.*, *Vibrio cholerae, Salmonella* Typhi and *S. paratyphi*. Their findings showed high bacterial load in all fish above the acceptance level and presence of TPC, TCC and TFCC suggesting that the microbial quality of the fish available in the market is not satisfactory. These studies show that fish sold in retail markets might be contaminated by different bacteria species and can source as a source of infection of different pathogens to humans

1.4.3 Contamination from fish and seafoods by handling personnel

The indicator contaminants from fish handling personnel are some cases different from FCs and FIBs. For example, the detection of *Staphylococcus aureus* in fish and seafood, which is dominantly found on skin surfaces and the upper respiratory tract in humans, could be directly associated with the hygiene of fish handling personnel [37-42]. Apart from *S. aureus*, other bacteria species have also been detected from fish handling personnel. For example, Roy et al [43] collected 150 swab samples from mobile phones of veterinarians, students, laboratory attendants, meat and fish handlers in the Kashmir valley in Nepal of which 96.66% mobile phones were found to be contaminated with different bacteria species that include *E. coli*, *Bacillus cereus*, *Proteus* spp., *Streptococcus* spp., *Staphylococcus* spp., and *Klebsiella* spp.. The highest percentage of *E. coli* and *B. cereus* was from the fish and meat handlers. This study showed that mobile phones can serve as an important mode of contamination of fish and serve as vehicle for transmission of bacteria to humans. In Nigeria, Grema et al [44] isolated *S. aureus*, *Streptococcus* spp., *E. coli*, *Klebsiella* spp., and *Proteus* spp. from fish handlers . Abou Elez et al [45] found a high prevalence of *Pseudomonas euroginosa* and other *Pseudomonas* spp. from fish handlers in different fish markets in Egypt. Lues and Tonder [46] found a high (98%) presence of total viable counts (TVC), TCs on the hands and the apron's samples of food handlers that consisted of a high presence of , *E. coli*, Enterobacteriaceae and *S. aureus* in a prominent South African retail group. Altogether, these studies show that fish handlers can serve as a source of contamination to fish during processing and retail.

1.4.4 Contamination of water used for fish processing

The use of water during processing to clean fish after removing the viscera followed by cutting to produce fillet is one of the critical stages in the production cycle when the risk of bacteria contamination is high [47]. In most LMICs, clean water may not be available for the fish vendors to degut the fish. Mhango et al [48] observed that fish vendors in Botswana carry water in containers to selling sites where they use the same water several times to keep the fish wet. They noted that unsanitary water used during the gutting of tilapia on the streets influenced the increase in microbial levels in the gutted compared to the ungutted fish. They isolated different

bacteria species from the gutted fish of which the most highly isolated was Staphylococcus spp. although were also isolated from in street vended fish. Other bacterial species isolated included *Citrobacter brackii, Citrobacter freundii, Enterobacter* spp., *Proteus* spp., *Klebsiella pneumoniae, Salmonella arizonae* and *Salmonella paratyphi* . Dissasa et al [49] found a high prevalence of different bacteria that included *S. paratyphi, S. flexneri*, *V. parahemolyticus C. freundii* and *K. pneumonia, E. coli*, *P. aeruginosa*, and *S. dysenteriae* in water from Lakes Hawassa, Ziway and Langanoo in Ethiopia while Inoue et al [50] found Acinetobacter spp., Pseudomonas spp., and Legionella spp. in water samples collected consisted of samples from commercial jar water, groundwater from shallow dug wells and deep tube wells, spring water, and river water from the Kathmandu valley in Nepal. Motsepe and Warwick [51] isolated *S. aureus* as an indicator of pollution in different water bodies in Durban in South Africa. Altogether, these studies show the presence of diverse bacteria species in different water sources some of which were used for the processing fish.

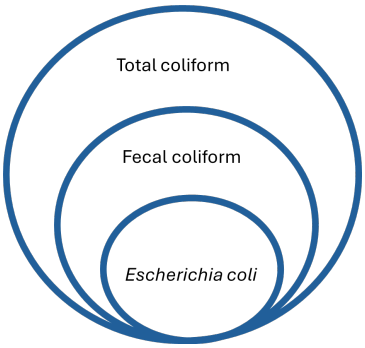
1.5 Types of Indicator Organisms

Total coliform, fecal coliform, *E. coli*, and *Enterococcus* are the microbial groups used for assessing fecal contamination in the environment or food[52].

1.5.1 Coliforms

The coliforms are Gram-negative rod-shaped non-spore forming bacteria that ferment lactose when grown at 35 ^{0}C to 37 ^{0}C. Much as they are found in different environments that include soil, vegetation, and aquatic habitats, they are principally found in fecal matter of all warm-blooded animals including humans. As such, they are used as indicators of the sanitary quality of water and food products as indicators of contamination by fecal matter from terrestrial warm-blooded animals. Generally, they are divided into three categories with each category having its own risk level indicator of food or water quality (Figure 3).

- Total coliforms
- Fecal coliforms (Subgroup of total coliforms)
- *Escherichia coli* (Subgroup of fecal coliforms)



Figur 3. Total coliforms, fecal coliform and Escherichia coli

1.5.2 Total coliform

Total coliforms (TCs) are Gram-negative bacteria that are rod-shaped and are facultative anaerobic lactose fermentors that produce acid and gas within 48 h when cultured at 35°C. They include *E. coli*, *Citrobacter freundii*, *klebsiella pneumoniae*, and *Enterobacter aerogenes*. Given that coliforms are habitats of the intestinal tract of warm-blood animals including humans, they are used as indicators of sanitation conditions during food processing and as a standard for assessing fecal contamination [53].

1.5.3 Fecal coliform

Fecal coliforms (FCs) are a sub-group of the TCs (Figure 3). They inhabit the intestinal tract of warm-blooded animals as part of the natural microflora of the gastrointestinal tract (GIT). Similar with the TCs, they ferment lactose to produce different gaseous forms when cultured at 35 °C. They have ability to grow at elevated temperatures like 44^{0}C. When passed to the

external environment from the GIT through fecal excretion, they are used in the sanitary quality assessment of fecal pollution of water and food [54-58].

1.5.4 Escherichia coli

Escherichia Coli is a sub-group of the TCs and FCs (Figure 3). Nevertheless, several studies point to *E. coli* as a more specific indicator of fecal contamination than TCs and FCs [59, 60]. This is because *E. coli* is the most universal bacteria species among the coliforms found in the GIT of most warm-blooded animals including humans specifically associated with fecal material. Thus, the presence of *E. coli* in water and processed foods signifies a more positive risk of fecal contamination than the presence of other coliforms[61, 62].

1.5.5 Fecal Streptococci and Enterococci species

Similar to *E. coli*, fecal *Streptococci* are part of the normal microflora of the GIT of most warm-blooded animals including humans. The most common fecal *streptococci* species with potential application as FIBs include *S. faecalis, S. faecium*, *S. bovis*, *S. equinus*, and *S. avium*. Although *Streptococci* are not able to replicate in aquatic environments, they have been show to survive for a long time outside the GIT of host animals rendering them to serve as good indicators of fecal contamination. For example, a study by Elkayam et al [63] showed that Streptococci was an ideal indicator for evaluating the effectiveness of soil aquifer treatment in removing microbial contaminations. A study by Lorincz and Incze [64] showed that although *E. coli* can be used as an indicator of contamination of preserved meat products, its presence in meat products is rare. Thus, they proposed replacing *E. coli* with *S. faecalis* as an indicator of faecal contamination in meat products although they pointed out that *Aerococcus viridans* occurs more ubiquitously in preserved meat products than the other Aerococcus species. Thus it is likely that *S. faecalis* and *A. viridans* can be used as fecal indicators in preserved fish products.

Several studies point to the enterococci as better indicators of fecal contamination than the FCs because *Enterococcus* spp. survive longer in marine environments and wastewater treatment facilities than FCs [65-67]. Moreover, the numbers *Enterococci* spp. in marine and freshwater environments have been shown to correlate with the risk of human pathogens and disease, which is unlike the fecal coliforms [65, 66].

1.5.6 Other bacteria indicators

1.5.6.1 *Staphylococcus aureus*

Staphylococci are commonly found on skin surfaces and the upper respiratory tract of different warm-blooded animals including humans and are used as indicators of contamination of food or water by humans or warm-blooded animals. The presence of Streptococci in foods is indicative of contamination by handling personnel or contaminated equipment or utensils [68, 69]. Different studies have pointed to *S. aureus* as a major component of the bacterial flora of swimming pools, coastal areas, and other recreational waters due to human contamination [70, 71]. El-Shenawy [72] found a strong association between *S. aureus* counts and increase in recreational activities in coastal areas in Egypt indicating that *S. aureus* can be used as an indicator microorganism of contamination in recreational water bodies. Gabutti, Giovanni, et al [73] compared the survival of FC, faecal *Streptococci*, *Salmonella* spp., and *S. aureus* grown in sea and brackish water and showed that *S. aureus* had a higher survival rate than fecal streptococci followed by *Salmonella* spp. while FCs had the highest sensitivity to salinity. Thus,

their results showed that *S. aureus* could be a better indicator of human contamination in salinity waters than the other indicator organisms.

1.5.6.2 *Klebsiella* and *Citrobacter* species

Although Klebsiella and Citrobacter are part of the TCs that ferment fructose to produce different gases when cultured at 35 ºC, they are mostly used as indicators of industrial waste and vegetation. For example, several studies have pointed out that *Klebsiella pneumoniae* is a less common intestinal inhabitant and is present in industrial wastes or nature [74-76], while *Citrobacter freundii* is also not commonly associated with the GIT but it is mostly associated with vegetables [77, 78].

1.5.6.3 *Acinetobacter* species

Acinetobacter has been linked to contamination of a wide range of foods that include vegetables, fruits, meats like bacon, chicken, and fish [79, 80]. They are mostly found in aquatic environments like wetlands, ponds, wastewater treatment facilities, freshwater and marine environments [81]. Shrestha et al [82] showed that Acinetobacter was the most isolated from water samples collected from different sources including shallow-dug wells, deep tube wells, springs, and rivers. Similarly, Adewoyin and Okoh [83, 84] found a high prevalence of *Acinetobacter* species in water samples collected from the Great Fish, Keiskamma and Tyhume rivers in the Eastern Cape Province, South Africa. Bergogne-Berezin and Towner [85] pointed out that *Acinetobacter* spp. are nosocomial pathogens while some medically important species like *A. lwoffii, A. nosocomialis, and A. pittii*, have been detected in vegetables, meat, dairy products, human skin, and fish [86, 87].

1.5.6.4 Salmonella species

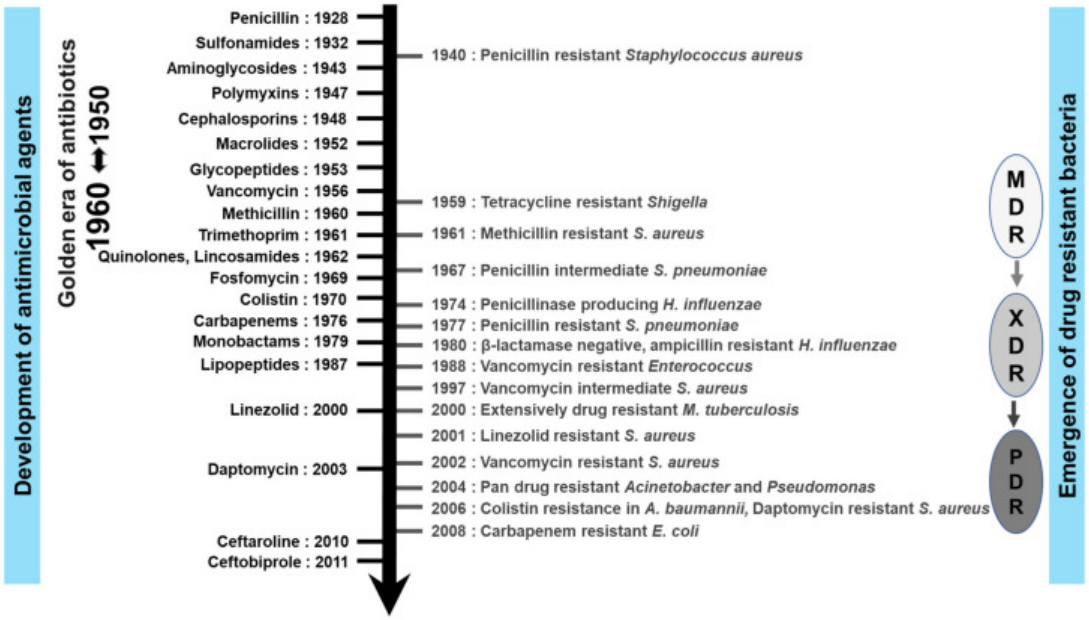
Salmonella spp. are pathogens of terrestrial animals including humans that do not naturally occur in aquatic environments whose presence in food and aquatic environments points to contamination from terrestrial animals[40, 88]. Despite so, *Salmonella* spp. have been isolated from different aquatic environments [37, 89], fish and shellfish [90, 91], farmed molluscs[92], seafoods in retail markets [91, 93], and ready-to-eat (RTE) fish products [94-96].

1.6 Antimicrobial resistance in aquaculture

Since the discovery of the first antibiotics by Fleming in 1928 (Figure 4), antibiotics have been widely used in humans and different animals[97] species including farmed aquatic organisms for the treatment of different diseases. The prohibitive cost of vaccination in low-income countries coupled with the increase of diseases in aquaculture in LMIC [98], antibiotics are widely used for the control of diseases in aquaculture in LMICs. This has contributed to the increase of antimicrobial resistance in aquaculture which has been exacerbated by presence of resistance plasmids and horizontal gene transfer using plasmids and mobile genetic elements [99-101].

Done et al [102] used a metadata analysis of more than 650 publications to show that 51 antibiotics were commonly used in aquaculture and agriculture (livestock and poultry) of which 39 (76%) were of importance to human medicine. They also noted that six classes of the antibiotics used in both agriculture and aquaculture were included in the World Health Organization (WHO) list of critically important antimicrobials. In addition, they also observed that the resistance profile seen in bacteria from both sectors share the same resistance mechanisms demonstrating that aquaculture is contributing to similar resistance problems like

terrestrial agriculture. Similarly, Schar et al [103] used a systematic meta-analysis of 749-point prevalence surveys reporting antibiotic resistance from aquatic animals in Asia to show that the percentage of resistance to medically important antimicrobials in foodborne pathogens is very high. In another study, Schar et al [104] estimated the global human, terrestrial, and aquatic food animal antimicrobial use in 2030 at 236,757 tons, of which aquaculture carries the highest use intensity per kilogram of biomass. Thus, it is evident that the use of antibiotics in aquaculture in increasing on a global scale and that farmed aquatic organisms are bound to play an important role in the spread of antimicrobial resistance (AMR).



Figur 4. History of antimicrobial resistance[100]

1.7 Antimicrobial resistance in Indicator microorganisms

It is noteworthy that some of the microorganisms serving as indicator bacteria like members of the family Enterobacteriaces as well as members of the genera *Staphylococci*, *Streptococci*, *Acinetobacter*, and *Enterococci* are among the microorganisms classified to be in the WHO top priority list of pathogens encoding antibiotic resistance classified as critical, high and medium as shown in Table 1 [105]. Thus, indicator bacteria can also serve as indicators of AMR for specific antibiotic resistance (ABR).

1.7.1 Enterobacterales

Enterobacterales are among the top in list of pathogens classified in the critical priority list of the WHO list of antibiotic resistance groups (ARG). Table 1 shows that they are important for the resistance against the carbapenems and extended spectrum of β-lactamases (ESBLs). Thus, there has been extensive surveillance of the Carbapenemase-producing Enterobacterales (CPE) in nosocomial infections, animal and aquatic foods, and the environment. Among the members of the family Enterobacteriaceae, the CPE have been detected in indicator bacteria species like *E. coli*, *Klebsiella* spp, and *Citrobacter freundii*. The monitoring and reporting of ESBL-/AmpC-/carbapenemase-producing *E. coli* recovered from food animals has become mandatory in most European countries [106].

Nakayama et al [107] reported CPEs harbouring the bla_{NDM-1}, bla_{OXA48} and bla_{KPC} genes from river fish in Vietnam. Dwivedi et al [108] carbapenem-resistant *Enterobacter cloacae complex*, *K. pneumoniae*, and *Serratia plymuthica* isolated from farmed freshwater fish. Similarly, Adibe-Nwafor et al [109] reported the distribution and Antibiotic Resistance Profile of ESBL producing *E. coli* from fish pond farms in Abakaliki Metropolis. Das et al [110] detected CREs that harbored bla_{NDM} gene in the Enterobacteriaceae in seafood of which the major species involved *K. pneumoniae*, and *E. coli*. Similarly, Sola et al [111] reported high prevalence of EBSL- and CPEs from Tunisian Seafood. Brahmi et al [112] found a high prevalence of ESBL producing *Enterobacteriaceae* in wild fish from the Mediterranean Sea in Algeria while Singh et al [113] found the ESBL producing Enterobacteria in seafood that included fish, shrimps, clam, and squid in Mumba in India of which *E. coli* was the most dominant followed by *K. oxytoca* and *K. pneumoniae*. Brahmi et al [112] found high prevalence of ESBL producing *Enterobacteriaceae* in wild Fish from the Mediterranean Sea in Algeria that included *E. coli*, *K. pneumoniae*, and *Citrobacter freundii*. whereas Nakayama et al [107] reported CREs harbouring bla_{NDM-1}, bla_{OXA48} and bla_{KPC} genes in retail outlets and supermarkets in Vietnam. Altogether these studies show that the ESBLs and CPEs have detected and isolated from wild and farmed aquatic organisms. As for environmental detection, several studies have reported the detection of the CPEs have been identified from different aquatic environments that include river, river sediments, lakes, well waters, and coastal waters [114-123].

Tabell 1. World Health Organization priority pathogens list new antibiotics[105]

Priority level	Pathogens	Antibiotic resistant
Critical	*Acinetobacter baumannii*	carbapenem-resistant
	Pseudomonas aeruginosa	carbapenem-resistant
	Enterobacteriaceae	carbapenem-resistant, ESBL-producing
High	*Enterococcus faecium*	vancomycin-resistant
	Staphylococcus aureus	methicillin-resistant, vancomycin-intermediate and resistant.
	Helicobacter pylori	clarithromycin-resistant
	Campylobacter spp.	fluoroquinolone-resistant
	Salmonellae	fluoroquinolone-resistant
	Neisseria gonorrhoeae	cephalosporin-resistant, fluoroquinolone-resistant
Medium	*Streptococcus pneumoniae*	penicillin-non-susceptible
	Haemophilus influenzae	ampicillin-resistant
	Shigella spp.	fluoroquinolone-resistant

1.7.2 Methicillin resistant Staphylococcus aureus

An important challenge in the control of methicillin resistance associated with *S. aureus* is the integration of a staphylococcal cassette chromosome *mec* element into the chromosome that converts drug-sensitive *S. aureus* into the notorious methicilin-resistant *S. aureus* (MRSA), which is resistant to all β-lactam antibiotics [124]. As a consequence, surveillance of MRSA in food animals and aquatic environments has become an important public health safety globally [106, 125-128]. Gilmore [129] also detected MRSA in the Washington State marine beaches whereas Louse et al [130] found MRSA in the nearshore waters in the Hilo Bay and its rivers in Hawaii brought about by road runoff water, sewage, and soils. Similarly, Gerken et al [131] found MRSA in Hawaiian streams and estuaries. The also found MRSA in soil and observed

that bacteria concentrations were highest in urban soils and lowest in forest soils. Identifying high-risk environmental areas is critical for the design of control measures that prevent the spread of MRSA to water bodies used for aquaculture. In farmed aquatic organisms, MSRA has been isolated from fish [132], shrimp[133], seafoods [134] and RTE foods [135, 136].

1.7.3 Acinetobacter

The Carbapenam resistant Acinetobacter spp. (CRA) are among the top list of critical pathogens enlisted by the WHO (Table 1). They have been detected various ready to eat foods including fish and seafoods [137, 138]. They have also been detected in sources and aquatic environments. For example, Shrestha et al [82] found the CRAs encoding the $bla_{\text{OXA23-like}}$ genes in different water sources in the Kathmandu Valley in Nepal while Serwecińska et al [139] found the CRA bacteria in the effluent wastewaters of all tested municipal waste water treatment plants (WWTPs), and in most of the Pilica River water samples in central Poland.

1.6.4 Other indicator bacteria

The indicator bacteria used as FIBs like *Streptococcus faecalis* and *Enterococcus* spp., which are also enlisted as top priority pathogens by the WHO (Table 1). These bacteria species have been isolated from farmed aquatic organisms and aquatic environments shown to encode the vancomycin, penicillin and carbapenems resistance genes. For example, Asode et at [140] found cabapenem-resistant Streptococcus spp. in the sewage of fish tanks market. Inzar et al [141] found *Enterococcus faecalis* and *Streptococcus faecalis* isolates in Romanian aquatic fishery lowland salted lakes that exhibited resistance to vancomycin and macrolides like erythromycin. They also found high resistance levels to β-lactams like cefuroxime, cefazolin, ceftriaxone, ceftazidime, and aztreonam. Hammad et al [142] isolated *Enterococcus faecalis, and E. faecium* from RTE fish in the Japanese retail outlets that encoded *tet*(M), *tet*(L), *tet*(K), *erm*(B), *msr*(A/B), *aph*(3′), and *blaZ* genes. These findings are in agreement with observations made by Korajkic et al [143] who pointed out that antibiotic-resistant Enterococci (ARE) are among the leading causes of nosocomial infections worldwide ubiquitously found in several waterways like rivers, lakes, and oceans.

2. Rationale

Fish foods are increasingly becoming important as a source of protein for humans. The demand for fresh foods strain from fish farms to retail outlets is also increasing because of people's interest to consume fresh foods directly from the fish farm or the river or sea is perceived health. Moreover, the global production of meat products from livestock like beef, pork or mutton is decreasing given the increasing demands of large nutrient sources needed to feed terrestrial animals to produce enough protein from food animals. Thus, aquaculture becomes a better alternative for nutrient for humans. However, food safety testing procedures have not been well optimized for aquaculture products as done for terrestrial animal products. For the example, the pasteurization of milk is well optimized in most countries. Meat inspection as part of the food safety protocols used in many countries. On the contrary, aquaculture products often enter the food chain up to retail outlets with less rigorous infection procedures as done for terrestrial animal foods. Moreover, the testing of aquaculture products for the presence of microbial contaminants is less documents as done for livestock products milk in which pasteurized milk products are tested for the presence of coliforms and standard procedures for coliform tests have been optimized are widely used globally. Given, that fish foods are often sold directly to consumers, in this study we wanted to determine the presence of indicator bacteria in RTE fish food sold in retail market in Mangalore, in India.

Another challenge is that most aquaculture facility are vulnerable to contamination through wastewater effluents from homes, urban areas, and hospitals as well as runoff water from different places that could be carriers of different microorganisms into aquaculture facilities. Moreover, aquaculture products are at risk of contamination during processing, unhygienic handling and poor sanitary handling conditions. Thus, aquaculture products are vulnerable to contamination by different bacteria from harvesting, processing, handling and point of chain. The contaminants can vary from those acquired from culture facilities, processing, handling during transportation and unhygienic conditions at selling outlets. Thus, it is a good idea to carry out a comprehensive analysis of the type contaminants found in the fish foods to identify the potential source of contamination in the production and supply chain of fish foods as they would help develop effective control measures to ensure fish food safety. Thus, the rationale for findings different bacteria indicators of contamination in this study was to identify the sources of the contamination given that presence of some indicator bacteria, like E. coli and Enterococci spp. could point to fecal contamination in the production facilities of water used for processing while detection of other indicator bacteria like the *Staphylococci* spp. could point to contamination by fish food handlers.

Another important challenge faced by consuming aquaculture products is the limited knowledge of AMR phenotypes that are associated with microorganism contaminants. The WHO has listed the top priority pathogens that are associated with the difficult to treat AMR genes like the carbapenems, and ESBLs [105]. The extent to which indicator organisms play an important role in the spread of AMR genes like the ESBL, carbapenems and the third generation cephalosporins have not been widely investigated in RTE fish foods sold on retail markets as done for other terrestrial foods of animal origin. Thus, in this study we wanted to determine the profile of AMR genes found in the RTE fish foods sold on retail market in India. A good understanding of the AMR profile in RTE- fish foods would help determine the risks consumers face by eating fish foods that has not been subjected to AMR testing on retails markets.

3. Objectives

The overall objective of the study was to determine whether the bacterial indicators found in terrestrial meat food animals were present in RTE fish food sold in retail markets in India and to determine the AMR profile found in the bacteria indicators of contamination.

Sub-goals; -

(a) Determine the profile of bacteria indicators of contamination found in RTE fish foods sold in retail markets in India

(b) Determine the antimicrobial resistance profile found in the indicator bacteria of contamination found in the RTE fish foods in India

4.0 Materials and Methods

4.1 Bacteria isolation and characterization

All samples were collected from different parts of India under the Indo-Norway collaborative project by Saurabh Dubey during his M.Sc. and Ph.D. studies. All bacterial isolates were obtained at the College of Fisheries, Mangalore, and Nitte University. Bacteriological characterization and disk diffusion tests were conducted at Nitte University. Bacterial DNA was isolated and sent to Nord University for the detection of AMR genes by PCR, whole-genome sequencing (WGS), and bioinformatics analysis.

Tabell 2. Bacteria isolation and characterization

Serial No	**Sample Name**	**Bacteria Name**	**Fish species – source of samples**
1	HL1	*Acinetobacter sp.*	*Labeo rohita*
2	HL2	*Acinetobacter lwoffii*	*Labeo rohita*
3	HL3	*Klebsiella sp.*	*Pangasius pangasius*
4	HL4	*Klebsiella oxytoca*	*Pangasius pangasius*
5	HL7	*Klebsiella pneumoniae*	*Pangasius pangasius*
6	HL8	*Aerococcus viridans*	*Clarias batrachus*
7	HL9	*Streptococcus dysgalactiae*	*Oreochromis niloticus*
8	HL10	*Citrobacter sp.*	*Pangasius pangasius*
9	HL11	*Escherichia coli*	*Cirrhinus mrigala*
10	HL12	*Citrobacter freundii*	*Pangasius pangasius*
11	HL13	*Staphylococcus aureus*	*Pangasius pangasius*
12	HL14	*Escherichia coli*	*Cirrhinus mrigala*
13	HL15	*Aerococcus urinaeequi*	*Clarias batrachus*
14	HL16	*Klebsiella pneumoniae*	*Pangasius pangasius*
15	HL17	*Klebsiella variicola*	*Pangasius pangasius*
16	HL18	*Staphylococcus pseudintermedius*	*Labeo rohita*
17	HL19	*Escherichia coli*	*Cirrhinus mrigala*
18	HL20	*Klebsiella pneumoniae*	*Pangasius pangasius*
19	HL21	*Citrobacter sp.*	*Pangasius pangasius*
20	HL22	*Escherichia coli*	*Cirrhinus mrigala*
21	HL23	*Escherichia coli*	*Cirrhinus mrigala*

4.2 Growth media preparation and bacterial culture

Tryptic Soy Broth (TSB) powder acquired from a commercial supplier (Sigma-Aldrich, Merk, Dorset, UK). A stock solution of TSB solution was prepared by dissolving 15 g of TSB powder in 600 ml of distilled water. The mixture was thoroughly mixed using a magnetic stirrer and sterilized by autoclaving at 121°C for 15 minutes. The sterile TSB solution was aliquoted into 15 mL Falcon tubes. Each bacterial isolate was inoculated into a falcon tube containing 5 mL of TSB. For bacteria inoculation, 100 µL of the bacterial isolate was added to each tube. The tubes were incubated in an incubator at 35°C for 24 hours. After 21-hour incubation, bacterial growth was visually assessed, noting variations in growth rates among the isolates.

4.3 Preparation of agar plates

Agar plates were prepared by dissolving 6 grams of Muller Hinton agar (Sigma-Aldrich, Merk, Dorset, UK) and 12 grams of TSB powder (Sigma-Aldrich, Merk, Dorset, UK) in 400 ml sterile distilled water in Erlenmeyer flasks. The solution was stirred thoroughly using a magnetic stirrer on a hotplate. The solution was sterilized by autoclaving at 121°C for 15 minutes, poured into Petri dishes and left to solidify at room temperature. Once solidified, the petri dishes were stored at 4°C for future use.

4.4 Disc diffusion methods

The antibiotic resistance (ABR) of all bacterial isolates was assessed using the Kirby-Bauer disk diffusion assay [144], following the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI) [145]. Commercially sourced Neo-Sensitabs™ antibiotic discs from Rosco (Neo-SensitabsTM, Rosco Diagnostica, Albertslund, Denmark) were used and the list of antibiotics and concentrations used are shown in Table 3. Antibiotic susceptibility/resistance was determined following the manufacturer's instructions (Neo-SensitabsTM, Rosco Diagnostica, Albertslund, Denmark). The disc diffusion test involved the use of two agar plates for each bacteria isolate, with each plate being used for six different antibiotic discs to allow for adequate spacing between the antibiotic discs. Altogether, each isolate was tested against a total of 12 antibiotics as shown in (Table 3).

A sterile cotton swab was used to evenly streak the bacterial suspension onto the Muller-Hinton agar plate, with the swab rotated at 60° between each streak. Subsequently, antibiotic discs of varying concentrations based on the manufacturer's guidance (Neo-SensitabsTM, Rosco Diagnostica, Albertslund, Denmark) (Table 3) were placed on the agar surface using sterile forceps, ensuring appropriate spacing to prevent overlap. The inoculated agar plates were then incubated at 25°C for 18-24 hours in a Memmert incubator to allow bacterial growth and colony formation. After 25 hours, the plates were removed from the incubator, and the zones of inhibition around each antibiotic disc were measured using a ruler. The diameter of each zone of inhibition was recorded in millimeters.

Tabell 3. List of antibiotics used in the disc diffusion test

Antibiotic family/class	**Antibiotics**	**Abbr.**	**Conc (µg)**	**Supplier**
Dihydrofolate reductase inhibitors	Trimethoprim	TRIM	5	Rosco Diagnostica A/ S
Amphenicol	Chloramphenicol	CLR	30	Rosco Diagnostica A/ S
Glycopeptide	Vancomycin	VAN	30	Rosco Diagnostica A/ S
Carbapenem	Imipenem	IMI	10	Rosco Diagnostica A/ S
Cephalosporin	Ceftriaxone	CTR	30	Rosco Diagnostica A/ S
Cephalosporin	Cefotaxime	CTX	30	Rosco Diagnostica A/ S
Tetracycline	Tetracyclines	TET	30	Rosco Diagnostica A/ S
Rifamcyn	Rifampicin	RIF	5	Rosco Diagnostica A/ S
Aminoglycoside	Gentamicin	GEN	30	Rosco Diagnostica A/ S
Macrolide	Erythromycin	ERY	15	Rosco Diagnostica A/ S
Beta-lactam	Ampicillin	AMP	10	Rosco Diagnostica A/ S
Beta-lactam	Penicillin	PEN	10	Rosco Diagnostica A/ S

4.5 Genomic DNA extraction

Genomic DNA (gDNA) extraction was performed following a previously published method [146] using the MagAttract® HMW DNA kit as per manufacturer's instructions (Qiagen GmbH, Hilden, Germany). A total of 21 bacterial isolates shown in Table 2 were obtained from pure cultures as described in section 4.1 above. Briefly, 20µl of bacteria solution was collected in 2ml Eppendorf tubes and mixed with 200µl of tissue lysis buffer (ATL) (Qiagen GmbH, Hilden, Germany) followed by vortexing. Subsequently, 50µl of Proteinase K solution (QIAGEN, Hilden, Germany) was added, and the samples were incubated at 56°C for 30 minutes in Labnet International's AccuBlock™ Digital Dry Baths. After incubation, 200µl of buffer AL (lysis buffer, QIAGEN) and 200µl of ethanol (96-100%) were added to each sample and thoroughly mixed. The mixture was then transferred to a DNeasy Mini Spin Column (QIAGEN, Hilden, Germany) placed in a 2ml collection tube and centrifuged at 8000 rpm for 1 minute at 20°C.

The flow-through vials were discarded, and each DNeasy Mini Spin Column was placed in a new 2ml collection tube. Subsequent washing steps were performed by adding 500µl of buffer AW1 (Wash buffer, QIAGEN, Hilden, Germany) followed by centrifugation at 8000 rpm for 1 minute. This was repeated with 500µl of buffer AW2 (wash buffer, QIAGEN, Hilden, Germany) and centrifuged at 14000 rpm for 3 minutes to dry the DNeasy membrane. The flow-through was discarded, and each DNeasy Mini Spin Column was transferred to a new 1.5ml microcentrifuge tube. Finally, 50µl of DNase-free water was added directly onto the DNeasy membrane, incubated for 1 minute at room temperature, and centrifuged at 8000 rpm for 1 minute. Afterward, the DNeasy Mini Spin columns were discarded while the 1.5ml tube that contained the DNA extract was retained. Purity and quality of the gDNA were evaluated using the nanodrop spectrophotometer (Thermo-Scientific, USA), and the quantity of the PCR product was measured using nanodrop followed by gel electrophoresis (Thermo-Scientific, USA) with a 1.2% agarose gel.

4.6 NanoDrop

The quantification of all samples was done using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop Spectrophotometers) to evaluate the quality and purity of genomic material. Buffer AE (elution buffer, QIAGEN, Hilden, Germany) was used for blanking, followed by adding 2 µl of each sample onto the lower pedestal for measurement. After each reading, any remaining reagent on the pedestal was meticulously wiped off with a clean paper towel.

Tabell 4. Composition of the PCR reaction mix for 16S rDNA

Component	Volume per Reaction	Supplier
Forward primer	1 µL	Thermo Fisher Scientific, USA
Reverse primer	1 µL	Thermo Fisher Scientific, USA
Template DNA	2.5 µL	Thermo Fisher Scientific, USA
DNA taq polymerase	10 µL	Thermo Fisher Scientific, USA
Nuclease free water	5.5 µL	Thermo Fisher Scientific, USA
Total volume	20 µL	

4.7 PCR amplification and sequencing of the 16S rDNA gene

The amplification by PCR was done using the universal primers of the 16S rRNA gene 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) to generate an amplicon of 1100bp for bacterial species identification [147]. The PCR reaction mix was prepared as shown in (Table 4). The PCR reactions were prepared in total reaction volumes of 20 µL per reaction. A negative control, consisting of distilled water instead of DNA template, was included in each PCR reaction. The specific amplification cycle conditions used are shown in the (Table 5). The visualization of PCR products was done using a 1.2% agarose gel prepared by dissolving 1.2 grams of agarose (Invitrogen - Life Technologies, ThermoScientific) in 100 ml of 10X Tris Borate (TBE) Buffer (Invitrogen, Life Technologies, ThermoScientific) followed by heating to 100 ºC. After cooling the gel solution to approximately 60 °C, 3 µL of SYBR Safe (Thermo Fisher Scientific, USA) was added to enhance the visualization of the bands. The gel was then poured into a gel tray with the appropriate comb that was inserted to create wells for sample loading. Subsequently, 2 µL of gel loading buffer was added to 20 µL of each PCR reaction to increase sample density and aid in sinking into the wells. A 10 µL DNA ladder (1 kb Plus DNA Ladder, ThermoFisher Scientific) was loaded in the first well of each cassette as a molecular size marker. The gel was subjected to electrophoresis at 100 volts until a dye line had migrated approximately 75% down the gel, indicating proper band separation. Visualization of the separated bands was done under UV light, and images were captured using the GeneSys software (SYNGENE G, Cambridge, UK). After completing the PCR run, the resulting bands were observed and extracted for sequencing commercially by EuroFin (EuroFin Engelsberg, Germany). To ensure the purity of the PCR products and eliminate any potential contaminants, purification was carried out using a PCR purification kit from GeneJET (ThermoFisher-Scientific, UK), following the manufacturer's guidelines. The samples underwent commercial sequencing by Eurofins Genomics in Germany (Engelsberg, Germany), and the resulting sequences were utilized in phylogenetic analysis for bacterial species identification.

Tabell 5. PCR amplification cycle condition

PCR step	Temperature (°C)	Duration(min/s)	Number of cycle
Initial denaturation	95 °C	5 mins	35 cycles
Denaturation	95 °C	30 Secs	35 cycles
Anneling	55 °C	30 Secs	35 cycles
External	72 °C	1 min	35 cycles
Final extension	72°C	5min	

4.8 Identify the presence of antibiotic-resistance genes using PCR analysis

The PCR reaction mix was prepared as outlined in Table 6, with each reaction containing a total volume of 20 µL. Each reaction included 1 µL of the forward primer,1 µL of the reverse primer, 2.5 µL of template DNA, 5.5 µL of distilled water, and 10 µL of Master Mix (Thermo Scientific). A negative control was included in each PCR run that consisted of distilled water in place of the DNA template, along with the (F) forward and reverse (R) primers altogether constituting the Master Mix. The PCR amplifications were conducted using the Bio-Rad thermocycler machine (Bio-Rad Laboratories, CA, United States, C1000 thermal cycler), which was programmed for the PCR conditions shown in Table 5. The specific amplification cycle conditions used are shown in Table 5, while the primers used are listed in Table 7.

Tabell 6. Composition of PCR reaction mix for detection of AMR genes

Component	Volume	Supplier
Forward primer	1 µL	Thermo Fisher Scientific, USA
Reverse primer	1 µL	Thermo Fisher Scientific, USA
Template DNA	2.5 µL	Thermo Fisher Scientific, USA
Master mix(DNA taq polymerase)	10 µL	Thermo Fisher Scientific, USA
Nuclease free water	5.5 µL	Thermo Fisher Scientific, USA
Total volume	20 µL	-

4.9 16S rRNA Phylogenetic Analysis

Phylogenetic analysis was conducted using the Molecular Evolutionary Genetic Analysis version 7 (MEGA-7) software [148] using the 16S rRNA gene sequences obtained from Eurofins (Engelsberg, Germany). Construction of phylogenetic trees was performed using the Neighbor-joining and BioNJ algorithms, with pairwise matrices estimated using the JTT model and quantified as the number of base substitutions per site [149]. This approach allowed for the accurate determination of evolutionary relationships among the sequences analyzed.

Tabell 7. Primer sequences, sizes, and annealing temperatures

Oligo name	Sequences (5′-3′)		Antibiotic	T°C	Pb size	Ref
QnrA	F	GGATGCCAGTTTCGAGGA	fluoroquinolone	60	492	[150]
	R	TGCCAGGCACAGATCTTG				
Sul 2	F	GCGCTCAAGGCAGATGGCATT	Sulfonamides	69	793	[151]
	R	GCGTTTGATACCGGCACCCGT				
bla$_{NDM}$	F	GGTTTGGCGATCTGGTTTTC	carbapenems	55	621	[150]
	R	CGGAATGGCTCATCACGATC				
ermA	F	AAGCGGTAAAACCCCTCTGAG	Erythromycin	55	442	[152]
	R	TCAAAGCCTGTCGGAATTGG				
CAT1	F	CGCCTGATGAATGCTCATCCG	Chloramphenicol	60	457	[153]
	R	CCTGCCACTCATCGCAGTAC				
bla$_{CTX-MA}$	F	SCSATGTGCAGYACCAGTAA	Cefotaxime	56	543	[154]
	R	CCGCRATATGRTTGGTGGTG				
rpoB	F	CGTCGTATCCGTTCCGTTGG	Rifampicin	61	885	[155]
	R	TTCACCCGGATAACATCTCGTC				
blaTEM	F	AGGAAGAGTATGATTCAACA	Penicillin Ampicillin	55	535	[156]
	R	CTCGTCGTTTGGTATGGC				
Ame3ant 2	F	GGGCGCGTCATGGAGGAGTT	Gentamycin	62	740	[157]
	R	TATCGCGACCTGAAAGCGGC				
tetE	F	TGATGATGGCACTGGTCA	Tetracyclines	56	262	[158]
	R	GCTGGCTGTTGCCATTA				
IMP	F	GGAATAGAGTGGCTTAAYTCTC	Imipenem	56	232	[159]
	R	GGTTTAAYAAAACAACCACC				
bla$_{SHV}$	F	TTATCTCCCTGTTAGCCACC	Penem, Carbapenem	58	860	[154]
	R	GATTTGCTGATTTCGCTCGG				
bla$_{KPC}$	F	CGTCTAGTTCTGCTGTCTTG	carbapenem	55	798	[150]
	R	CTTGTCATCCTTGTTAGGCG				

4.10 Whole genome sequencing, library preparation, and bioinformatics

Two bacteria isolates were randomly selected from the most dominant genera of the isolates shown in Table 2 characterized based on the 16S rDNA analysis above and were used for whole genome sequencing (WGS). The DNA used for whole genome sequencing was extracted using the method described in Section 4.5 above. Preparation of the paired-end DNA libraries was carried out using the Nextera DNA Flex Tagmentation method (Illumina Inc., San Diego, CA, United States; [160] while quantification of the Illumina library was done using the Qubit® DNA HS Assay Kit on a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, United States). Evaluation of the library fragments and size distribution was done using the Agilent HS DNA Kit on the Agilent 2,100 Bioanalyzer System (Agilent Technologies, CA, United States). The Illumina MiSeq platform (Illumina Inc., United States) was used for sequencing using V3 reagent kits, with paired end reads of 2 × 300 bp, following previously described methods [161]. The Galaxy platform (https://usegalaxy.no/) version 21.05 was used for bioinformatic analysis while quality assessment of both forward and reverse raw reads was done using the FastQC Version 0.11.9 software [162], followed by adapter removal and removal of low-quality reads from paired-end sequences using Trimmomatic version 0.38.1 [163]. After that, the paired-end sequence reads were assembled into contigs using the A5-miseq assembler [164] via *de novo* assembly. Quality read sequence contigs with k-mers ranging from 33 to 91 were also assembled using SPAdes v. 3.12.0 [165]. Genome annotation was done using the prokaryotic genome annotation pipeline [166] provided by the National Centre for Biotechnology information (NCBI), along with annotation performed using Prokka [167].

4.11 Prediction of antimicrobial resistance genes

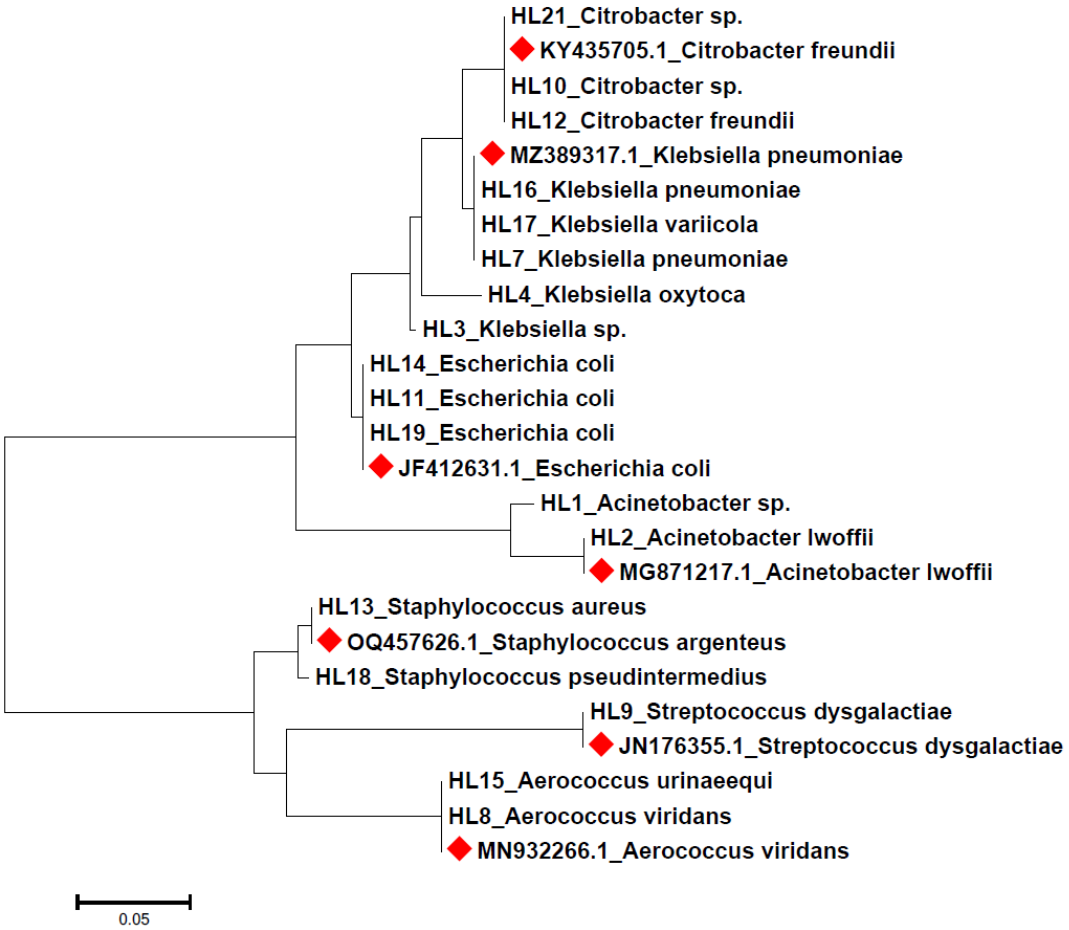
Identification of AMR genes, plasmids, and transposons was done for two isolates selected for WGS namely *Acinetobacter pittii* and *E. coli S100123*. Staramr version 0.7.2 [168] and ABRicate version 1.0.1 [167] software tools were used for AMR gene identification using the Comprehensive Antimicrobial Resistance Database (CARD) [169] with the threshold for gene identification set at 80%. Identification of the plasmids in bacterial genomes was done using Plasmidfinder v 2.0 [170] with the threshold for plasmid identification set at 80% . Circular maps of the *Acinetobacter pittii* and *E. coli S100123* genomes and plasmids were generated online using the Proksee software (https://proksee.ca/).

5.0 Results

5.1 Identification of Bacteria Isolates Based on the 16S rDNA

The bacterial isolates obtained in this study were characterized using 16S rDNA analysis, with a total of 18 out of 21 isolates included in the Phylogenetic Analysis. The analysis revealed the presence of seven genera among the 18 isolates as shown in Figure 5. The seven genera consisted of species namely *Citrobacter sp., Klebsiella sp., Escherichia coli, Acinetobacter sp, Staphylococcus sp, Streptococcus sp, and Aerococcus sp.*

Based on the Phylogenetic analysis (Figure 5), the isolates obtained were placed in two broad categories as Gram-positive and Gram-negative bacteria. The Gram-negative bacteria accounted for the largest proportion of 72.22% (13/18) while the Gram-positive bacteria only accounted for 27.78% (5/18). The Gram-negative bacteria belonged to four genera namely *Citrobacter*, *Klebsiella*, *Escherichia*, and *Acinetobacter* with each having 3, 5, 3, and 2 isolates, respectively.

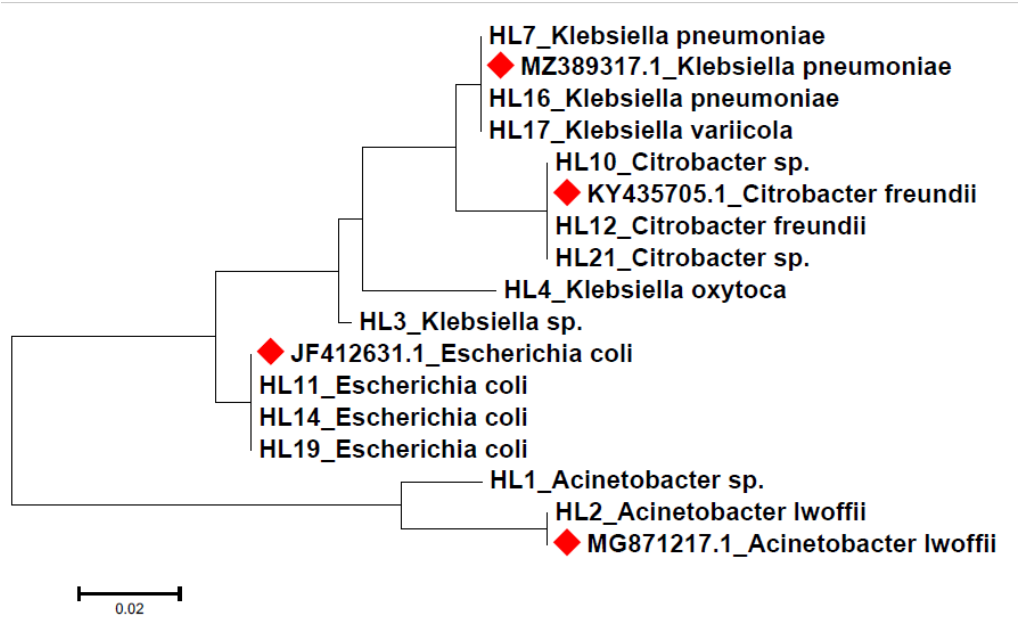


Figur 5. Phylogenetic tree of Indicator bacteria spp. isolates based on 16 S rDNA

Figure 5: Phylogenetic tree of indicator bacteria species isolates from fish used in this study based on 16 S rDNA sequencing. Sequence denoted by diamond shapes represent the 16S reference sequence from NCBI database compared with other bacteria used in this study.

5.1.1 Gram-Negative Bacteria

Among the Gram-negative bacteria, isolates numbers HL21 and HL10 were characterized as *Citrobacter sp*., while number HL12 was classified as *Citrobacter freundii* based on the phylogenetic analysis (Figure 6) and they all showed a high similarity (100%) with the reference strain KY435705.1_*Citrobacter freundii*. Members of the genus Citrobacter were grouped next to members of the genus Klebsiella. Altogether, five isolates were classified to be members of the genus Klebsiella namely isolates HL16 and HL7 which were characterized as *Klebsiella pneumoniae*, HL17 as *Klebsiella variicola*, HL4 as *Klebsiella oxytoca*, and HL3 as *Klebsiella sp* (Figure 6). All *Klebsiella* isolates were grouped close to the reference strain MZ389317.1_*Klebsiella pneumoniae*. Only isolate numbers HL14, HL11, and HL19 were classified as *Escherichia coli,* and all showed a high degree of homology (100%) with the reference strain JF412631.1_*Escherichia coli.* Isolate HL1 was identified as *Acinetobacter sp*., while HL2 was classified as *Acinetobacter lwoffii* showing 100% homology with the reference strain MG871217.1_*Acinetobacter lwoffii.*



Figur 6. Phylogenetic analysis of Gram-negative bacteria isolates based on 16 S rDNA

5.1.1.1 *Citrobacter* sp.

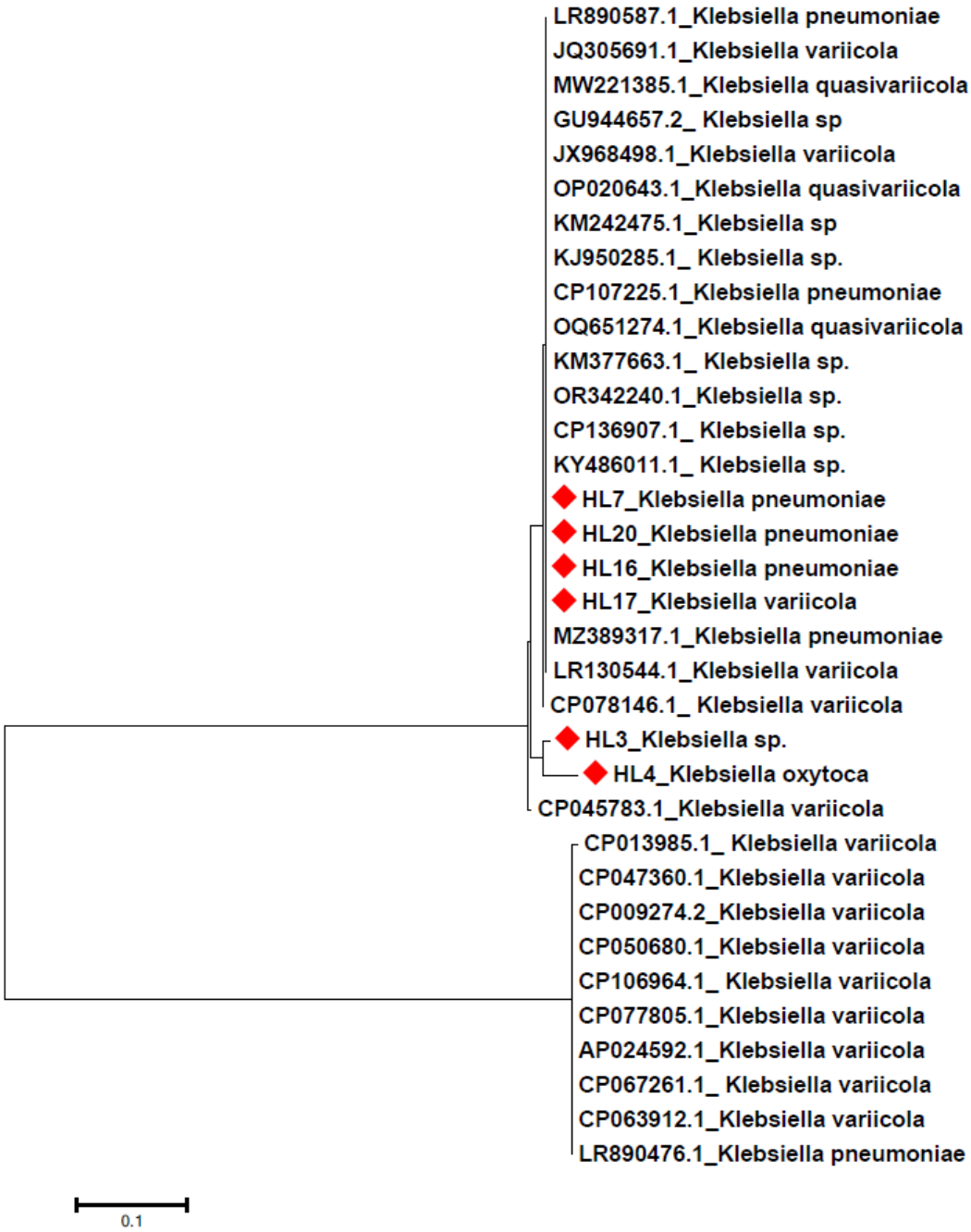
Comparison of 16S rDNA sequences of isolates HL12, HL21, and HL10 with several other sequences that were characterized as *Citrobacter* in (Figure 5) showed 100% similarity with other *Citrobacter freundii* deposited in the NCBI database (Figure 7). The characterization of the 18 isolates through 16S rDNA analysis revealed that isolate HL12 and HL21 was identified as *Citrobacter freundii*, exhibiting high homology with the reference strain KY435705.1. Additionally, isolate HL10 was classified as *Citrobacter sp*. and showed homology with strain KY435705.1 (Figure 7). Further phylogenetic analysis using the NCBI database confirmed that these isolates (HL21, HL10, HL12) displayed 100% homology with multiple *Citrobacter freundii* and *Citrobacter* sp. isolates (Figure 7).

Figur 7. Phylogenetic analysis of *Citrobacter freundii* isolates based on 16S rDNA

Figure 7 Phylogenetic analysis of *Citrobacter freundii* isolates in this study based on16S rDNA. The diamond-shaped markers denote the sequences *Citrobacter freundii* HL10, HL12, and HL21, which are the specific 16S rDNA sequences used in this study. These sequences are compared to the *Citrobacter freundii* 16S gene sequences available in the NCBI database.

5.1.1.2 *Klebsiella* species

Phylogenetic analysis of 18 out of 21 isolates using 16S rDNA sequencing revealed that two isolates (HL16, HL7 and HL20) were identified as *K. pneumoniae*, while one isolate (HL17) was classified as *Klebsiella variicola*, exhibiting high sequence homology to the Klebsiella pneumoniae reference strain MZ389317.1. (Figure 8). Further investigation showed that isolates HL7, HL20, and HL16 were also identified as *K. pneumoniae*, while isolate HL17 was characterized as *K. variicola*, all exhibiting 100% homology with sequences of other *Klebsiella* species deposited in the NCBI database. Additionally, isolates HL3 and HL4 were classified as *Klebsiella* sp. and *Klebsiella oxytoca*, respectively, showing high sequence similarity with other *Klebsiella* isolates in the NCBI database (Figure 8).



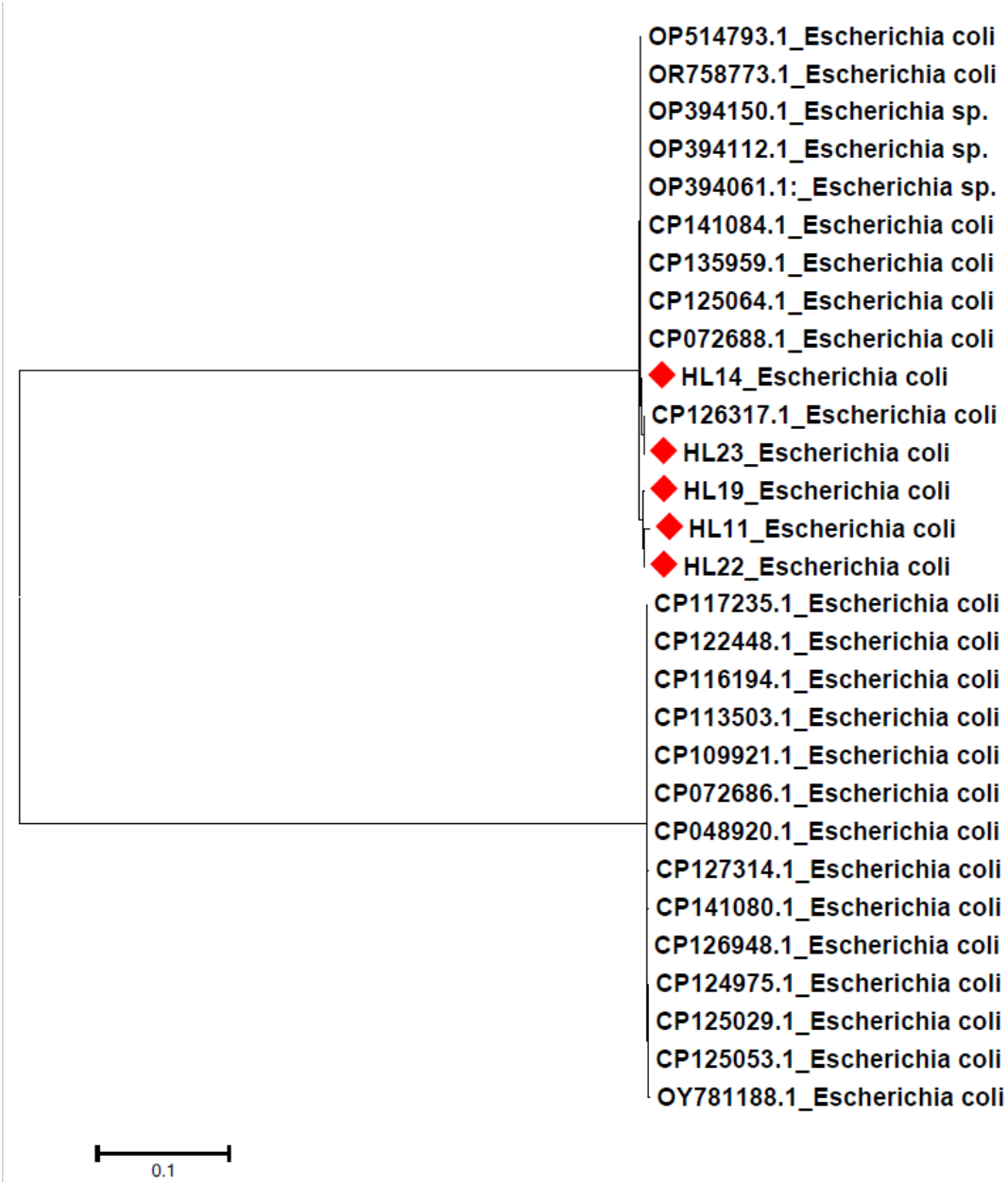
Figur 8. Phylogenetic analysis of *Klebsiella pneumoniae* isolates based on 16S rDNA.

Figure 8: Phylogenetic analysis of *K. pneumoniae* isolated in this study based on 16S rDNA. The sequences marked with diamond shapes are Klebsiella spp. HL7, HL20, HL16, HL17, HL3, and HL4 represent the 16S rDNA sequences analyzed in this research. These are compared against other 16S gene sequences of *Klebsiella spp.* sourced from the NCBI

5.1.1.3 *Escherichia coli*

Phylogenetic analysis of 16S rDNA sequences revealed that 3 isolates (HL14, HL11, HL19) were identified as *E. coli* species, demonstrating a high degree of similarity to the reference strain JF412631.1 (figure 5). Further examination using the NCBI database confirmed that these

isolates (HL14, HL23, HL19, HL11, HL22) classified as *E. coli* and shared 100% homology with multiple *E. coli* isolates (Figure 9).

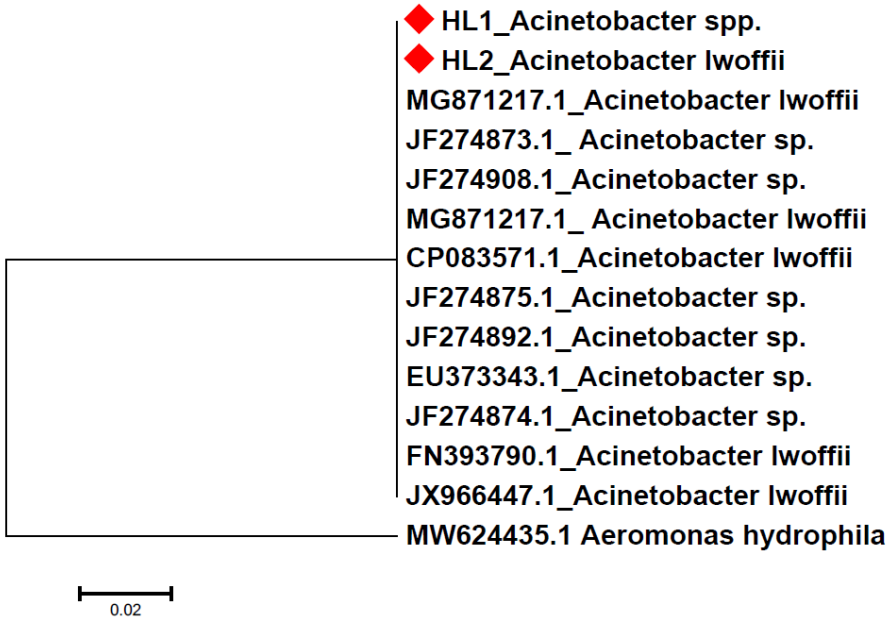


Figur 9. Phylogenetic analysis of *Escherichia coli* isolates based on 16S rDNA

Figure 9: Phylogenetic analysis of Escherichia coli isolated in this study based on 16S rDNA. with sequences denoted by diamond shapes are HL14, HL23, HL19, HL11 and HL22 representing the 16S sequences utilized in this study, are compared with additional E. coli 16S genes from the NCBI database.

5.1.1.4 *Acinetobacter* spp.

Phylogenetic analysis showed that isolates HL1 and HL2 were classified as *Acenitobacter* sp. and *Acenitobacter Iwoffii*, respectively. Both isolates showed a high similarity (100%) with other reference Acinetobacter strains deposited in the NCBI database as shown in Figure 10.

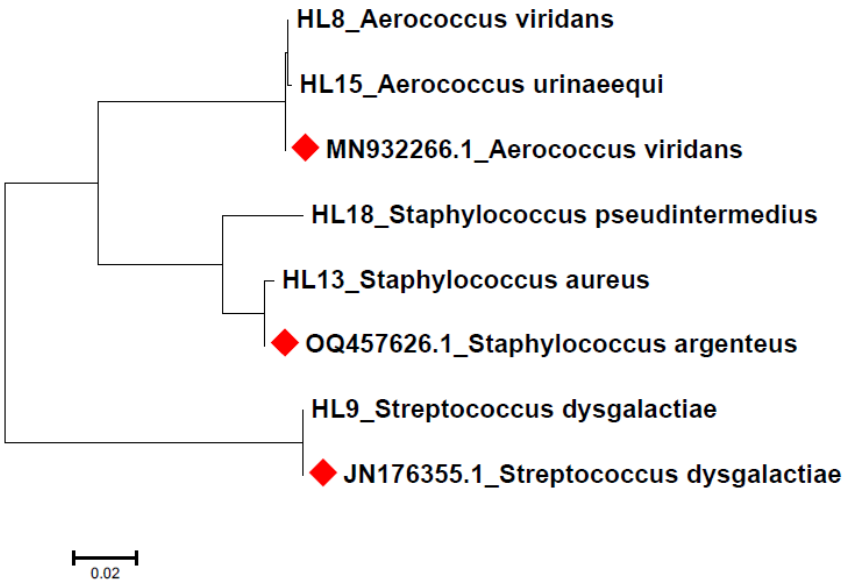


Figur 10. Phylogenetic tree of Indicator *Acinetobacter* spp. on 16 S rDNA

Figure 10: Phylogenetic tree of Indicator bacteria species isolates in this study based on 16 S rDNA. Sequence denoted by diamond shapes represent the 16S reference sequence from NCBI database compared with other bacteria used in this study.

5.1.2 Gram-Positive Bacteria

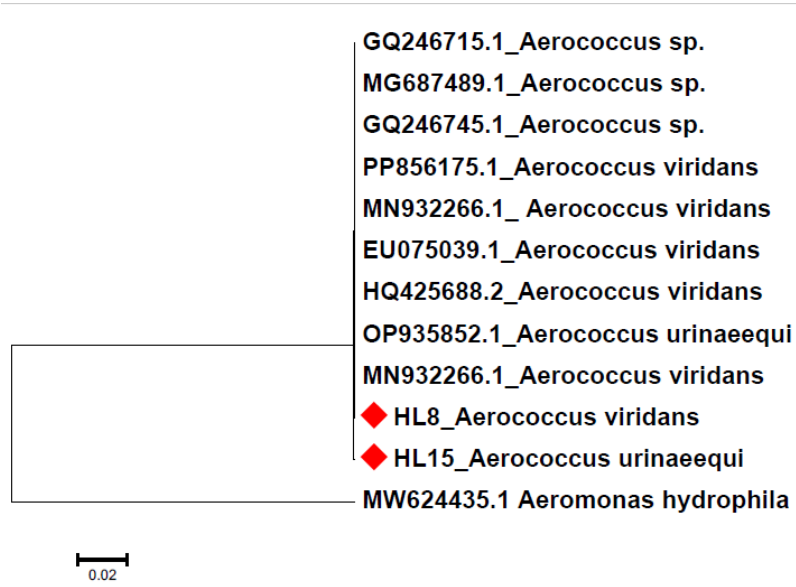
There five isolates out the 18 sequences generated by 16S rRNA analysis that were classified as Gram-Positive bacteria. Based on phylogenetic analysis of the 16S rRNA sequences generated the five isolates we classifed into three genera namely staphylococcus, Streptococcus and Aerococcus. Isolates numbers HL13 and HL18 were characterized as *Staphylococcus aureus* and *Staphylococcus pseudintermedius*, respectively, both showing high homology with the reference strain OQ457626.1_*Staphylococcus argenteus* (Figure 11). On the other hand, isolate HL9 was classified as *Streptococcus dysgalactiae* showing 100% similarity with the reference strain JN176355.1_*Streptococcus dysgalactiae*. Finally, isolate numbers HL15 and HL8 were identified as *Aerococcus urinaeequi* and *Aerococcus viridans*, respectively, both showing 100% homology with the reference strain MN932266.1_*Aerococcus viridans.*



Figur 11. Phylogenetic analysis of Garm-Positive bacteria based on 16S rRNA sequencing

5.1.2.1 *Aerococcus* species

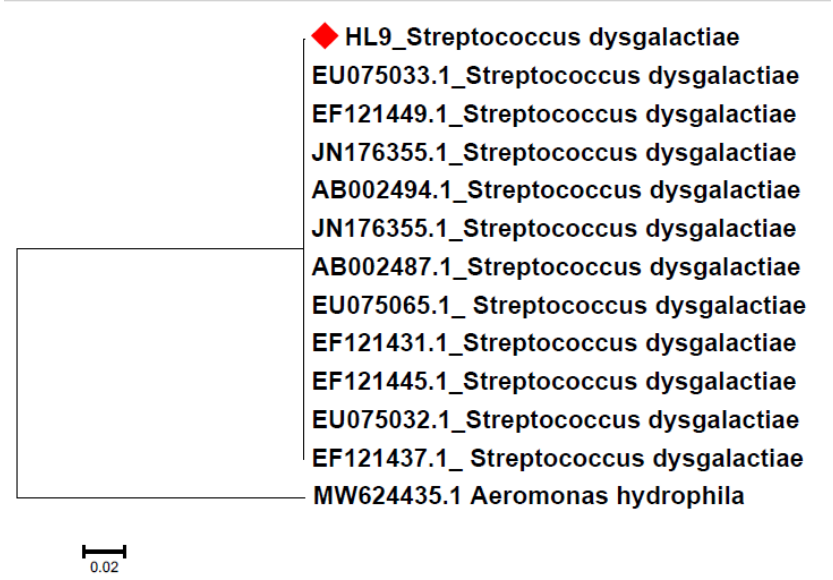
Phylogenetic analysis of the two isolates HL15 and HL18 characterized *Aerococcus* species and classified the two isolates as *Aerococcus urinaeequi* and *Aerococcus viridans* , respectively. Both isolates showed high 100% homology with 16S rDNA sequences of *other Aerococcus* species deposited in the NCBI database (Figure 12).



Figur 12. Phylogenetic analysis of *Aerococcus* isolates based on 16S rRNA sequences

5.1.2.2 *Streptococcus* species

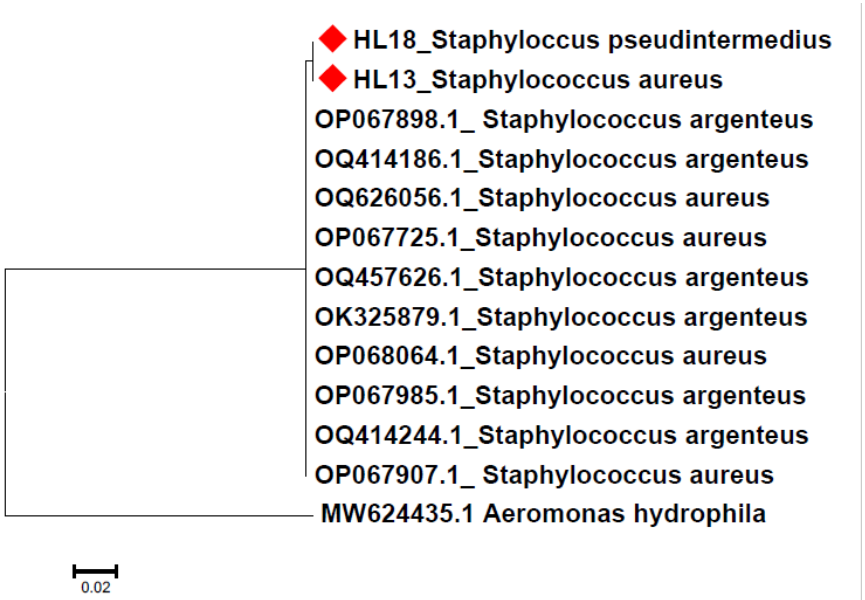
There was only one isolate (HL9) classified as *Streptococcus*, namely *S. dygalactiae* and it showed high homology with other 16S rDNA sequence of *S. dygalactiae* deposited in the NCBI (Figure 13).



Figur 13. Phylogenetic analysis of *Streptococcus dysgalactiae* based on the 16S rDNA

5.1.2.3 *Staphylococcus* species

There were only two isolates classified as *Staphylococcus* species namely *S. aureus* and *S. pseudintermedius* designated as HL13 and HL18, respectively. Phylogenetic analysis showed that both isolates exhibited high homology with 16S rDNA sequences of other Staphylococcus species depoisted in the NCBI database (Figure 14).



Figur 14. Phylogenetic analysis of the *Staphylococcus* species based on 16S rDNA

5.2 Characterizing antimicrobial resistance by disc diffusion test

5.2.1 Prevalence of antimicrobial resistance profile of the examined bacteria species

Tabell 8. Antibiotic resistance based on the disc diffusion test results

Sample ID	Bacteria species	Amoxycillin	Cefoxitin	Cephalothin	Ciprofloxacin	Erythryomicin	Gentamicin	Nitrofurantion	Rifampicin	Colisitn	Sulfonamide	Tetracycline	Trimethoprim	Sensitive Total	Resistance Total	Intermediate Total
HL1	*Acinetobacter sp.*	R	R	R	S	R	R	R	R	I	R	S	R	2	9	1
HL2	*Acinetobacter lwoffii*	R	R	R	S	S	S	R	S	S	R	S	R	6	6	0
HL3	*Klebsiella sp.*	R	S	R	S	R	S	S	R	I	R	R	R	4	7	1
HL4	*Klebsiella oxytoca*	R	R	R	R	S	S	R	S	S	R	S	R	5	7	0
HL7	*Klebsiella pneumoniae*	S	S	S	I	I	S	S	S	R	S	S	S	9	1	2
HL8	*Aerococcus viridans*	S	S	S	S	S	S	S	S	R	S	S	S	11	1	0
HL9	*Streptococcus dysgalactiae*	S	S	S	I	S	S	S	S	S	S	S	S	11	0	1
HL10	*Citrobacter sp.*	R	R	R	I	R	S	S	R	S	R	S	S	6	5	1
HL11	*Escherichia coli*	S	S	S	I	S	S	S	S	S	R	R	S	9	2	1
HL12	*Citrobacter freundii*	I	R	R	I	R	S	S	S	R	I	I	R	3	5	4
HL13	*Staphylococcus aureus*	S	S	S	S	S	S	S	S	S	S	S	S	12	0	0
HL14	*Escherichia coli*	S	S	S	S	S	S	S	I	I	S	S	S	10	0	2
HL15	*Aerococcus urinaeequi*	S	S	S	S	I	S	S	I	R	S	S	S	9	1	2
HL16	*Klebsiella pneumoniae*	S	S	S	I	I	S	S	R	S	S	S	S	9	1	2
HL17	*Klebsiella variicola*	S	S	S	I	S	S	R	S	I	S	S	S	9	1	2
HL18	*Staphylococcus pseudintermedius*	R	R	S	S	R	S	S	S	R	S	R	R	6	6	0
HL19	*Escherichia coli*	S	S	S	I	S	S	R	S	R	S	S	S	9	2	1
HL20	*Klebsiella pneumoniae*	S	S	S	I	R	S	S	R	R	S	S	S	8	3	1
HL21	*Citrobacter sp.*	R	R	R	I	R	S	S	R	S	S	S	S	6	5	1
HL22	*Escherichia coli*	S	S	S	I	S	S	S	R	S	S	R	S	9	2	1
HL23	*Escherichia coli*	S	S	S	I	S	S	S	S	S	S	S	S	11	0	1
	Intermediate Tota	1	0	0	12	3	0	0	2	4	1	1	0			
	Sensitive Total	13	14	14	8	11	20	16	12	10	14	16	15			
	Resistance Total	7	7	7	1	7	1	5	7	7	6	4	6			

Table 8 shows the results of the disc diffusion test based on the examined antibiotics. Of the 21 isolates examined only one had no AMR detected by the disc diffusion test (Tables 8 and 9). Table 9 shows that *Acinetobacter sp*. had the highest resistance of 83%, followed by *Citrobacter freundii* 75% and *Klebsiella sp*. 67%. The prevalence resistance for the rest of the isolates is

shown in Tables 8 and 9. Only *Staphylococcus aureus* had no resistance detected. Notably, *Acinetobacter sp*. isolate HL1 displayed the highest level of resistance, showing resistance to 10 different antibiotics. This was followed by *Citrobacter freundii* isolate HL12 with resistance to 9 antibiotics. *Klebsiella sp.* isolate HL3 exhibited resistance to 8 antibiotics, while *Klebsiella oxytoca* isolate HL4 was resistant to 7 antibiotics. Additionally, *Acinetobacter lwoffii* isolate HL2, *Citrobacter sp*. isolates HL10 and HL21, as well as *Staphylococcus pseudintermedius* isolate HL18 showed resistance to 6 antibiotics each. *Klebsiella pneumoniae* isolate HL20 demonstrated resistance to 4 antibiotics. Furthermore, *Klebsiella pneumoniae* HL7 and HL16, *Escherichia coli* HL11, HL19, and HL22, *Aerococcus urinaeequi* isolate HL15, and *Klebsiella variicola* isolate HL17 exhibited resistance to 3 antibiotics each. Lastly, *Escherichia coli* HL14 showed resistance to 2 antibiotics. Altogether, these findings show that most isolates had resistance against more than antibiotic indicating that most isolates showed a property of being multidrug resistance (MDR) (Table 8). Overall, amongthe 21 isolates exhibiting antibiotic resistance, 17 demonstrated (MDR), indicating resistance to multiple antibiotics. Conversely, only three isolates were only resistant to a single antibiotic each (Table 9).

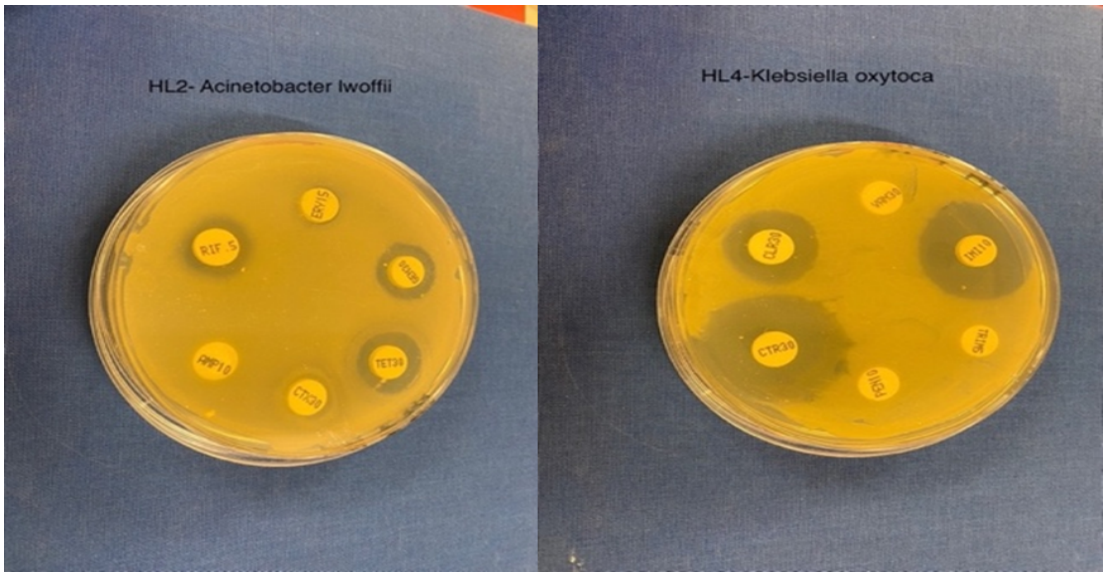
Tabell 9. Prevalence of antibiotic resistance in different bacteria species

Sample ID	Bacteria name	Sensitive total	Resistance total	Intermediated total	Total resistance	%
HL1	*Acinetobacter sp.*	2	9	1	10	83 %
HL12	*Citrobacter freundii*	3	5	4	9	75 %
HL3	*Klebsiella sp.*	4	7	1	8	67 %
HL4	*Klebsiella oxytoca*	5	7	0	7	58 %
HL2	*Acinetobacter lwoffii*	6	6	0	6	50 %
HL10	*Citrobacter sp.*	6	5	1	6	50 %
HL18	*Staphylococcus pseudintermedius*	6	6	0	6	50 %
HL21	*Citrobacter sp.*	6	5	1	6	50 %
HL20	*Klebsiella pneumoniae*	8	3	1	4	33 %
HL7	*Klebsiella pneumoniae*	9	1	2	3	25 %
HL11	*Escherichia coli*	9	2	1	3	25 %
HL15	*Aerococcus urinaeequi*	9	1	2	3	25 %
HL16	*Klebsiella pneumoniae*	9	1	2	3	25 %
HL17	*Klebsiella variicola*	9	1	2	3	25 %
HL19	*Escherichia coli*	9	2	1	3	25 %
HL22	*Escherichia coli*	9	2	1	3	25 %
HL14	*Escherichia coli*	10	0	2	2	17 %
HL8	*Aerococcus viridans*	11	1	0	1	8 %
HL9	*Streptococcus dysgalactiae*	11	0	1	1	8 %
HL23	*Escherichia coli*	11	0	1	1	8 %
HL13	*Staphylococcus aureus*	12	0	0	0	0 %

Table 10 shows the prevalence of antibiotic resistance based on disc diffusion tests for the various antibiotics tested (Table 10). Ciprofloxacin had the highest resistance of 62%, followed by colistin 52% erythromycin 48%, rifampicin 43%, and amoxicillin 38%. Cefoxitin, cephalothin and sulphonamide had a prevalence of 33%. Trimethoprim had a prevalence of 29%, while tetracycline and nitrofurantoin had a prevalence of 24%. On the other hand, gentamicin exhibited the lowest resistance of 5%. Overall, Table 10 shows that all antibiotics tested showed resistance to some of the bacteria tested.

Tabell 10. Prevalence of Antibiotic Resistance based on Disc Diffusion Test

Antibiotic	Amount	Sensitive	Resistance	Intermediate	Total R	%
Ciprofloxacin	5	8	1	12	13	62 %
Colisitn	10	10	7	4	11	52 %
Erythryomicin	15	11	7	3	10	48 %
Rifampicin	5	12	7	2	9	43 %
Amoxycillin	**30**	**13**	7	1	8	38 %
Cefoxitin	30	14	7	0	7	33 %
Cephalothin	30	14	7	0	7	33 %
Sulfa	240	14	6	1	7	33 %
Trims	5	15	6	0	6	29 %
Nitrofurantion	300	16	5	0	5	24 %
Tetra	30	16	4	1	5	24 %
Gentamicin	250	20	1	0	1	5 %



Figur 15. Example of the results of disc diffusion test

Figure 15: Example of the results of disc diffusion testing exhibiting *Acinetobacter lwoffii* (HL2) showing intermediate zones of inhibition around rifampicin (5 µg), gentamicin (30 µg), erythromycin (15 µg), tetracycline (30 µg), and cefotaxime (30 µg) while a high resistance observed to around ampicillin (10 µg). For *Klebsiella oxytoca* (HL4), an intermediate zone of inhibition was observed around chloramphenicol (30 µg), while susceptibility was seen with ceftriaxone (30 µg) and imipenem (10 µg). Resistance was observed against vancomycin (30 µg), trimethoprim (5 µg), and penicillin (10 µg).

5.2.2 Resistance prevalence of the Gram-negative bacteria based on the disc diffusion test

Altogether there were four genera belonging to the Gram-negative bacteria accounting for 13 of the 18 isolates examined using the disc diffusion test. The four genera were represented by

Escherichia, *Acinetobacter*, *Klebsiella* and *Citrobacter*. The prevalence of antibiotic resistance for the four genera based on the disc diffusion test is summarized below: -

(a) *Escherichia coli* isolates HL11, HL19, and HL22 displayed resistance against 25% of the tested antibiotics (3/12), while *E. coli* isolate HL14 exhibited resistance against 17% of the tested antibiotics (2/12).

(b) *Acinetobacter sp.* HL1 showed the highest resistance, with resistance against 83% of the tested antibiotics (10/12), and *Acinetobacter lwoffii* (HL2) demonstrated resistance against 50% of the tested antibiotics (6/12).

(c) Klebsiella sp. HL3 exhibited resistance against 67% of the tested antibiotics (8/12), and *Klebsiella oxytoca* HL4 showed resistance against 58% of the tested antibiotics (7 out of 12). *Klebsiella pneumoniae* HL20 displayed resistance to 33% of the tested antibiotics (4/12), while *Klebsiella pneumoniae* isolate (HL7, HL16), and *klebsiella variicola* HL17 demonstrated resistance against 25% of the tested antibiotics (3/12).

(d) *Citrobacter freundii* (HL12) exhibited resistance against 75% of the tested antibiotics (9/12), and *Citrobacter sp.* (HL10, HL21) showed resistance against 50% of the tested antibiotics (6/12).

5.2.3 Resistance profile of the Gram-positive bacteria based on the disc diffusion test

There were three genera belonging to the Gram-positive bacteria accounting for 27.78% of the total 18 isolates examined based the disc diffusion test results. The three genera were represented Staphylococcus, Streptococcus and Aerococcus. The prevalence of antibiotic resistance for each genera are summarized below; -

(a) Staphylococcus pseudintermedius (HL18) exhibited the highest resistance against 50% of the tested antibiotics (6/12), while *Staphylococcus aureus* (HL13) showed no resistance against any of the antibiotics tested, with a total resistance of 0%.

(b) Streptococcus dysgalactiae (HL9) displayed resistance against 8% of the tested antibiotics (1/12).

(c) Aerococcus urinaeequi (HL15) exhibited resistance against 25% of the tested antibiotics (3/12), while *Aerococcus viridans* (HL8) exhibited resistance against 8% of the tested antibiotics (1/12).

5.3 Identification of Antimicrobial Resistance Genes by PCR

5.3.1 Prevalence of antimicrobial resistance genes based on PCR analysis

The PCR results are shown in Table 11 (and Figure 18, appendix) for the AMR genes examined.

Tabell 11. Antimicrobial resistance profiles based on PCR analysis

ID	Bacteria species	QnrA	Sul2	NDM	ermA	CAT1	*bla*CTX-	rpoB	*bla*TEM	AME3A	tetE	IMP	*bla*SHV	KPC
HL1	*Acinetobacter sp.*	-	+	-	-	-	-	-	-	-	-	-	-	-
HL2	*Acinetobacter lwoffii*	-	-	-	-	-	-	-	-	-	-	-	-	-
HL3	*Klebsiella sp.*	-	+	-	-	-	+	-	+	-	-	-	-	-
HL4	*Klebsiella oxytoca*	-	+	-	-	-	+	-	+	-	-	-	-	-
HL7	*Klebsiella pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	+	-
HL8	*Aerococcus viridans*	-	+	-	-	-	-	-	-	-	-	-	-	-
HL9	*Streptococcus dysgalactiae*	-	+	-	-	-	-	-	-	-	-	-	-	-
HL10	*Citrobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-	-
HL11	*Escherichia coli*	-	-	-	-	-	-	+	-	-	-	-	-	-
HL12	*Citrobacter freundii*	-	-	-	-	-	+	+	-	-	-	-	-	-
HL13	*Staphylococcus aureus*	-	+	-	-	-	+	-	-	-	-	-	-	-
HL14	*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-	-	-
HL15	*Aerococcus urinaeequi*	-	-	-	-	-	+	+	-	-	-	-	-	-
HL16	*Klebsiella pneumoniae*	-	+	-	-	-	+	+	-	-	-	-	+	-
HL17	*Klebsiella variicola*	-	-	-	-	-	+	+	-	-	-	-	+	-
HL18	*Staphylococcus pseudintermedius*	-	-	-	-	-	-	-	-	-	-	-	-	-
HL19	*Escherichia coli*	-		-	-	-	+	+	-	-	-	-	+	-
HL20	*Klebsiella pneumoniae*	-	-	-	-	-	-	+	-	-	-	-	+	-
HL21	*Citrobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-	-
HL22	*Escherichia coli*	-	-	-	-	-	-	+	-	-	-	-	-	-
HL23	*Escherichia coli*	-	-	-	-	-	+	+	-	-	-	-	+	-
	Total resistance	0	7	0	0	0	9	9	2	0	0	0	6	0
	Percentage (%)	0	33	0	0	0	43	43	10	0	0	0	29	0

The PCR results presented in Tables 11 and 12 show that only three isolates did not have AMR genes while the other 18 isolated had AMR genes detected by PCR. The highest number of AMR genes was in *Klebsiella pneumoniae* isolate HL16 among the bacterial isolates tested, with 4 out of the 13 genes showing resistance, accounting for 31% of the tested genes. This was followed by *Klebsiella sp.* (HL3), *klebsiella oxytoca* (HL4), *Klebsiella variicola* (HL17), *Escherichia coli* (HL19 and HL23) each showing resistance to 23% of the tested genes. Other

bacterial species like *Citrobacter freundii* (HL12), *Staphylococcus aureus* (HL13), *Aerococcus urinaeequi* (HL15), and *Klebsiella pneumoniae* (HL20) exhibited resistance against 15% of the tested genes each. In contrast, *Acinetobacter lwoffii* (HL2), *Citrobacter sp.* (HL10), *Escherichia coli* (HL14), *Staphylococcus pseudintermedius* (HL18), and *Citrobacter sp.* (HL21) showed no resistance to any of the tested genes, with a resistance percentage of 0%.

Tabell 12. Ranking of antimicrobial resistance bacteria profile based on PCR analysis

ID	**Bacteria species**	**Number of the reistance out of 13 genes**	**Percentage (%)**
HL16	*Klebsiella pneumoniae*	4	31 %
HL3	*Klebsiella sp.*	3	23 %
HL4	*Klebsiella oxytoca*	3	23 %
HL17	*Klebsiella variicola*	3	23 %
HL19	*Escherichia coli*	3	23 %
HL23	*Escherichia coli*	3	23 %
HL12	*Citrobacter freundii*	2	15 %
HL13	*Staphylococcus aureus*	2	15 %
HL15	*Aerococcus urinaeequi*	2	15 %
HL20	*Klebsiella pneumoniae*	2	15 %
HL1	*Acinetobacter sp.*	1	8 %
HL7	*Klebsiella pneumoniae*	1	8 %
HL8	*Aerococcus viridans*	1	8 %
HL9	*Streptococcus dysgalactiae*	1	8 %
HL11	*Escherichia coli*	1	8 %
HL22	*Escherichia coli*	1	8 %
HL2	*Acinetobacter lwoffii*	0	0 %
HL10	*Citrobacter sp.*	0	0 %
HL14	*Escherichia coli*	0	0 %
HL18	*Staphylococcus pseudintermedius*	0	0 %
HL21	*Citrobacter sp.*	0	0 %

The ranking of the AMR genes examined by PCR showed that the highest resistance was observed for the *bla*$_{CTX}$ gene, associated with cefotaxime resistance (43%), and the *rpoB* gene associated with resistance for Rifampicin resistance (43%) (Table 13). This was followed by the *Sul-2* gene for the sulfonamides resistance (33%), *bla*$_{SHV}$ gene for penem resistance (29%), *bla*$_{TEM}$ gene for Penicillin and Ampicillin resistance (10%). On the other hand, no bands were detected for the *tetE* gene for tetracyclines resistance, *QnrA* for fluoroquinolones resistance, *bla*$_{NDM}$ gene for carbapenem resistance, *ermA* gene for erythromycin resistance, and *CAT1* gene for Chloramphenicol resistance, *IMP* gene for Imipenem resistance, *AME3ANT2* gene for gentamycin and *bla*$_{KPC}$ gene for carbapenem resistance, indicating a 0% detection of resistance for these genes.

Tabell 13. Ranking of AMR gene profile based on PCR analysis

Antibiotic	Gene	Number positive out of 21 bacteria examined	Percentage (%)
Cefotaxime	CTX-MA	9	43%
Rifampicin	rpoB	9	43%
Sulfonamides	Sul 2	7	33%
Penem,carbapenem	blaSHV	6	29%
Penicillin Ampicillin	blaTEM	2	10%
Tetracyclines	tetE	0	0%
Gentamycin	AME3ANT2	0	0%
fluoroquinolones	QnrA	0	0%
carbapenems	NDM	0	0%
Erythromycin	ermA	0	0%
Chloramphenicol	CAT1	0	0%
Imipenem	IMP	0	0%
carbapenem	KPC	0	0%

5.3.2 Resistance prevalence of the Gram-negative bacteria based on PCR analysis

All four genera belonging to the Gram-negative bacteria accounting for 13 of the 18 isolates examined using the disc diffusion test in this study had AMR genes detected by PCR. The four genera were represented by *Escherichia*, *Acinetobacter*, *Klebsiella* and *Citrobacter*. The prevalence of antibiotic resistance for the four genera detected by is summarized below: -

(a) *Klebsiella pneumoniae* isolate HL16 exhibited the highest level of resistance to 4 out of 13 genes indicating MDR, HL20 showed resistance to 2 out of 13 AMR genes and HL7 exhibited one out of 13 AMR genes. While *Klebsiella sp.* (HL3*), Klebsiella oxytoca* (HL4), and *Klebsiella variicola* (HL17) showed resistance to 3 genes had a prevalence of 23%. Also,

(b) Citrobacter freundii isolate (HL12) showed resistance against two genes leading to a prevalence of 15% (2/13).

(c) *Escherichia coli* isolates (HL19, HL23) exhibited resistance against three of 13 genes leading to a prevalence of 23%.

(d) *Acinetobacter sp* isolate (HL1) showed resistance against just 1 out of 13 genes giving a prevalence of 8%.

5.3.3 Resistance profile of the Gram-positive bacteria based on PCR analysis

All three Gram-positive genera represented Staphylococcus, Streptococcus and Aerococcus had AMR genes detected by PCR. The prevalence of the AMR genes for each genera are summarized below; -

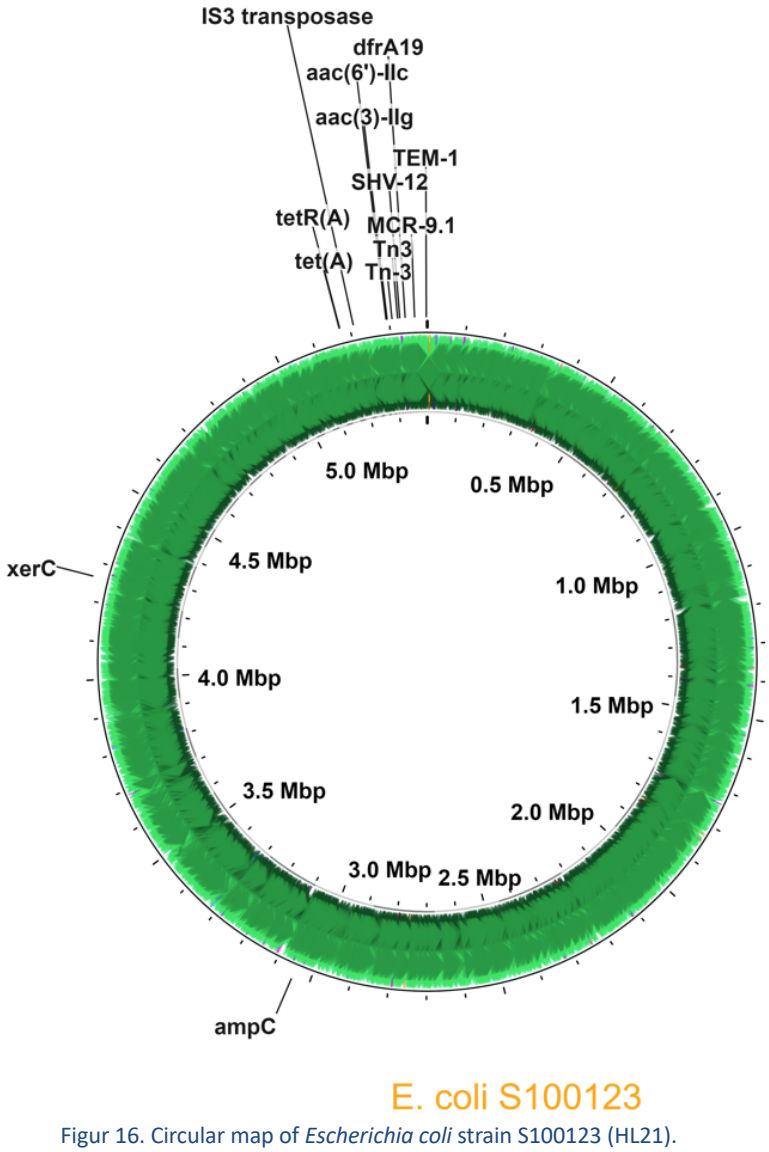
(a) Staphylococcus aureus (HL13), and *Aerococcus urinaeequi* (HL15) had resistance against two out of 13 genes leading to a prevalence 15%

(b) Streptococcus dysgalactiae (HL9) and *Aerococcus viridans* (HL8) had resistance against one out of 13 genes leading to a prevalence of 8%.

5.4 Whole Genome Sequencing

Whole genome sequence analysis (WGS) was carried out for two isolates randomly selected from the two most abundance genera namely Escherichia and Acinetobacter out of the 18 isolates sequenced using 16S rDNA primers. The randomly selected isolates were *E. coli* strain S100123 (isolate number HL22) and *A. pittii* strain HL1 which belonged to the most prevalent genera in this study. Data generated after identifying the AMR genes based on the CARD analysis is shown and discussed below.

5.4 .1 Escherichia coli strains S100123



Figur 16. Circular map of *Escherichia coli* strain S100123 (HL21).

Figure 16: Circular map of *E. coli* S100123 (HL22). Gene distribution and specific antimicrobial resistance (AMR) genes. The position of AMR genes like '*ampC*', *'MCR-9.1'*, and *'tet(A)*', among others, are marked on the outer edge of the circle of the genome. Each feature is positioned according to its location within the 5.0 megabase pair (Mbp) genome, with the scale provided in megabase pairs."

The circular map of *E. coli S100123* HL22 was generated to visualize the distribution of genes across the genome, with a specific focus on AMR genes and mobile genetic elements (MGEs). The circular map shows presence of the *ampC* gene, located at 3.1 (Mbp) on the genome, encoding a β-lactam enzyme that provides resistance to cephalosporins and penems (Figure 16). This gene shows 100% coverage, and 97.97% identity based on the CARD database analysis. The *xerC* gene is located at 4.2 Mbp and has been shown to plays a role in site-specific recombination, which can contribute to the stability and maintenance of plasmids that carry AMR genes. The *tetR* and *tet* genes found at 5.0 Mbp are associated with resistance to tetracycline. The *tet* gene encodes a protein that actively pumps tetracycline out of the cell, while *tetR* regulates this process. An IS3 transposase was identified near the *tet* genes. Transposases like *IS3* enable the movement of transposable elements within the genome. Thus, the proximity of *IS3* to *tetR* and *tet* suggests that these AMR genes might be part of an MGE, potentially facilitating their spread among bacteria. A cluster containing multiple resistance genes, including *MCR-9.1*, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-1}}$, *aac(6')-llc*, *aac(3)-llg*, as well as *Tn3* transposons, was observed in the 5.3 Mbp region of the genome. The genome shows presence of *MCR-9*, which provides resistance to colistin, an antibiotic used as a last resort for multidrug resistance (MDR) infection, with 100% coverage and identity. In addition, the genome also shows presence of the beta lactam genes like $bla_{\text{SHV-12}}$ that confer resistance to carbapenems and cephalosporins, showing 100% coverage and 99.88% identity. Further, the genome shows the $bla_{\text{TEM-1}}$ gene, which is also a beta lactamase, that also provide resistance to cephalosporins and penems, with 100% coverage and 99.88% identity (Figure 16).

Table 14 shows some of the AMR genes detected in the genome of *E. coli* strain S100123 HL22 that include *Aac(6')-llc* and *aac(3)-llg* genes that encode the aminoglycoside modifying enzymes that confer to resistance to aminoglycosides, both showing 100% coverage and identity. Transposons like Tn3 can move between different DNA molecules, like between plasmids and the chromosome, thereby spreading resistance genes.

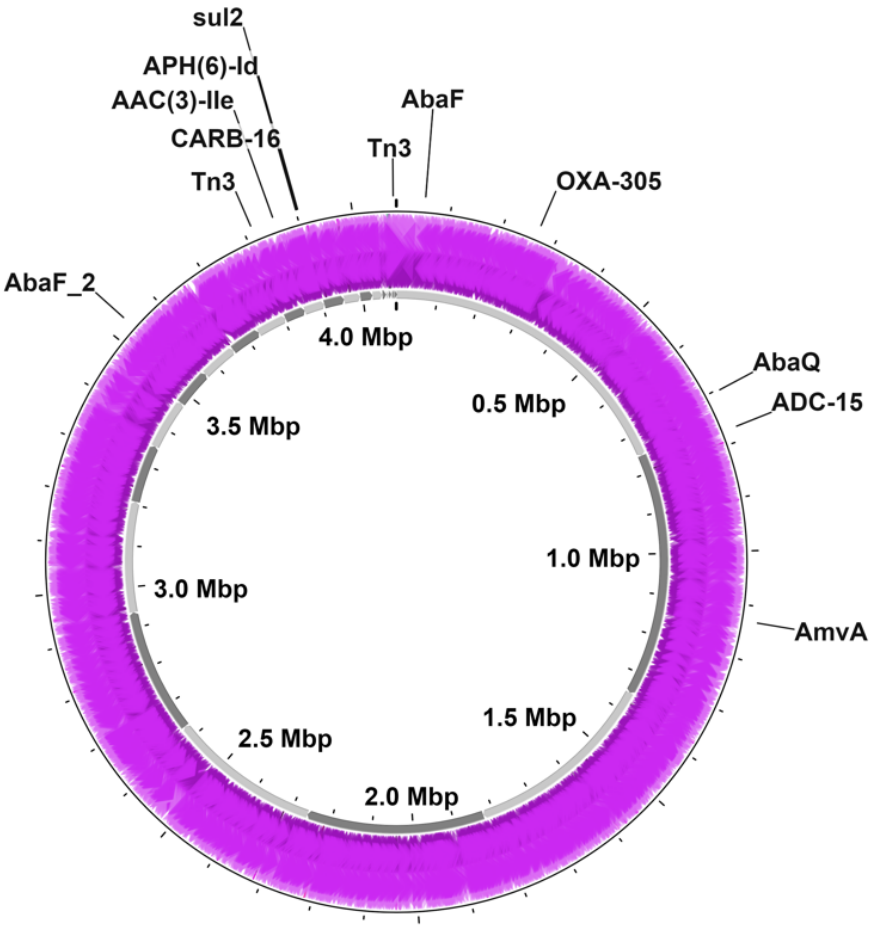
Tabell 14. Antibiotic genes detected in Escherichia coli strain S100123

GENE	%Coverage	%Identity	Database	Resistance
ampC	100.00	97.97	card	Cephalosporin, Penam
tet(A)	97.80	100.00	card	tetracycline
AAC(6')-IIc	100.00	100.00	card	aminoglycoside
SHV-134	100.00	99.88	card	Carbapenem, cephalosporin,
dfrA19	100.00	99.83	card	diaminopyrimidine
MCR-9	100.00	100.00	card	peptide
APH(6)-Id	100.00	99.88	card	aminoglycoside
APH(3")-Ib	100.00	99.88	card	aminoglycoside
TEM-1	100.00	99.88	card	Cephalosporin, Penam

Table 14: This table enumerates the antimicrobial resistance (AMR) genes identified in *E. coli* strain S100123 (HL21). Each gene is accompanied by its percentage coverage and identity, verifying the extent of sequence alignment and similarity respectively.

5.4.2 *Acinetobacter pittii* strain HL1

Whole genome sequence (WGS) analysis shows that *Acinetobacter pittii* strain HL1 has a size greater than 4.0 Mbp. The genome of *Acinetobacter pittii* strain showed the presence of the beta-lactam *bla*$_{OXA-306}$, sulfonamide *sul2*, aminoglycoside *AAC(3)* and *APH(6)-id*, macrolide A*mvA*, fluoroquinolone *AbaQ*, fluoroquinolone *adeG* and Fosfomycin *AbaF* genes (Figure 17). In addition, the genome also encoded the *Tn3* transposase. Table 15 shows the genes detected in the genome based on the CARD analysis with more than 80% identity similarity and a coverage of more than 99.9%.



Figur 17. This circular map illustrates the genome of *Acinetobacter pittii*

Figure 17: This circular map illustrates the genome of *Acinetobacter pittii*, highlighting its gene distribution, particularly the AMR genes. Notable the position of AMR genes such as *bla*$_{OXA-305}$, *ADC-15*, and *CARB-16* are shown around the circumference of the circle, corresponding to their positions in the genome. The scale in megabase pairs (Mbp) is provided to show the size and location of these genes within the genome.

Tabell 15. Antimicrobial resistance genes identified in Acinetobacter pittii strain HL1

Gene	% Coverage	% Identity	Database	Resistance
AbaF	99.92	94.10	card	fosfomycin
OXA-305	100.00	98.42	card	Cephalosporin, penam
AbaQ	100.00	90.19	card	fluoroquinolone
AAC(3)-IIe	100.00	99.53	card	aminoglycoside
sul2	100.00	100.00	card	sulfonamide
APH(6)-Id	100.00	99.88	card	aminoglycoside
APH(3")-Ib	100.00	99.88	card	aminoglycoside
CARB-16	100.00	100.00	card	penam
ADC-15	100.00	92.45	card	cephalosporin
adeG	100.00	86.57	card	Fluoroquinolone, tetracycline
AmvA	99.73	89.80	card	macrolide

Table 15. This table enumerates the AMR genes identified in *Acinetobacter pittii* strain HL1. Each gene is accompanied by its percentage coverage and identity, verifying the extent of sequence alignment and similarity, respectively.

6. Discussion

6.1 Detection of indicator bacteria in RTE fish foods

The present study detected different bacteria indicators of contamination in the RTE fish food samples collected from different places in India. The detected indicator bacteria belonged to seven genera comprising of four Gram-negative and three Gram-positive. The detection of *E. coli*, *Klebsiella*, and *Citrobacter* points to the presence of coliforms and Enterobacteriaceae. This finding is also indicative of contamination by fecal material. As pointed out by other scientists [74-76] that *Klebsiella* spp. like *K. pneumoniae* might be indicative of contamination from industrial waste while the presence of *Citrobacter freundii* could be indicative of contamination from vegetation and other sources [77, 78]. Thus, contamination from fecal materials, environmental sources, and the water used for cleaning the fish were likely sources of contamination of retail fish in this study. Like our finding, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Klebsiella* spp., have been isolated from different retail fish and seafood markets in India and other countries [171-177] . Equally, *Citrobacter freundii* and other *Citrobacter* species have been isolated together with other Enterobacteriaceae like *Klebsiella* spp., *E coli*, and *Enterococci* spp. from fish at retail markets in India and other countries [172, 178]. The *Enterococci* spp. and *Acinetobacter* spp. have also been detected alongside other indicator bacteria species in retail markets in India [108, 113, 174]. For example, Naik et al [174] found *Staphylococcus* spp., *Klebsiella pneumoniae*, *Enterobacter* spp., and *Acinetobacter* spp. in marine fish were procured from different markets in Mumbai. Thus, our findings are in line with several other studies that have reported the presence of indicator bacteria from RTE fish sold at different markets in India.

Visnuvinayagamet al [179] pointed out that most Indian consumers prefer to buy fish in retail markets. They carried out a multi-year environmental study using FIBs to determine the hygienic status in retail markets in the Navi region in Mumbai India. The FIBs found in fish from retail markets included *E. coli*, and fecal *Streptococci.* In another study, Visnuvinayagam et al [180] examined the presence of FIBs in the water used in the retail fish markets in Navi in Mumbai and found a high presence of *E. coli*, faecal streptococci, and sulfite-reducing clostridia. They pointed out that the repeated use of the same water without replacement was a major source of higher FIB levels in fish sold at retail markets. They recommended the use of a running water facility to clean the fish to reduce the contamination levels unlike the repeated use of the same water without replenishment. Dutta et al [181] found a high prevalence of *E.coli* in fish and shrimps sold in retail markets in Kolkata, India. They observed that *E. coli* increased remarkably in the summer months than in winter. Amin et al [182] found a significant difference ($p < 0.005$) in the prevalence of *E. coli* in fish sold in the wet markets compared to fish sold in supermarkets in retail markets across Dhaka in Bangladesh. They found that the mean concentration of *E. coli* on fish from the wet market (3.0 ± 0.9 $\log_{10}$ CFU/g) was twice higher than that of supermarkets (1.6 ± 0.9 $\log_{10}$ CFU/g). Thus, there is a need for further investigations to identify the factors influencing the occurrence of FIBs in fish foods sold at retail markets as an overture to developing effective food safety control measures against bacteria contamination in the production, processing, and supply chain of fish foods in India.

The detection of *Staphylococcus* species like *S. aureus* in this study is suggestive that fish might have been contaminated by human handlers given that the *Staphylococcus* spp. are mostly found on the skin surfaces and in the upper respiratory tract of humans and some terrestrial animals. Our findings corroborate with Roy et al [183] who found *S. aureus* in fish collected from the Baghajatin wholesale fish market and Garia retail fish market in Kolkata, India. Sivaraman et

al [136] found biofilm-forming methicillin-resistant *S. aureus* (MSRA) clones circulating in retail markets in Assam India. Similarly, the MSRA *S. aureus* has been reported in seafood in other places in India [184, 185]. Put together, these studies attributed the presence of *S. aureus* to possible contamination from fish handlers in retail fish markets or wastewater contamination of fish foods. Thus, there is a need for further investigation to determine the role of fish handlers in the production and supply chain on the contamination of fish products and RTE fish foods.

6.2 Prevalence of antimicrobial resistance in the indicator bacteria in RTE fish foods

In general, the AMR prevalence detected by both the disc diffusion test and PCR was higher in the Gram-negative bacteria than the Gram-positive bacteria. The highest prevalence detected by the disc diffusion was in *Acinetobacter* sp. HL1 (83%) followed by *Citrobacter freundii* HL12 (75%), *Klebsiella sp.* HL3 (67%), and *Klebsiella oxytoca* HL4 (58%) while *Citrobacter* sp. HI10 and H21 together with *Staphylococcus pseudintermedius* (HL18) had a prevalence of 50%. Thus, our observation shows that the top eight isolates were Gram-negative bacteria belonging to the family Enterobacteriaceae and the coliform group. The prevalence of antibiotic resistance in *E. coli* isolates varied between 8 to 25%. Except for *S. pseudintermedius* HL18, the rest of the Gram-positive bacteria had a low prevalence of antibiotic resistance (ABR) detected by the disc diffusion test as shown that *A. urinaequi* HL15, *A. viridans* HL8, *S. dysgalactiae* HL9, and *S. aureus* HL13 had a prevalence of 25%, 8%, 8%, and 0%, respectively. Overall, our findings show that the Gram-negative bacteria had a higher prevalence of antibiotic resistance than the Gram-positive bacteria. The high prevalence of coliform bacteria in this study could be attributed to the fact that they are the most common contaminants associated with food safety because they can be from various sources that include fecal material, water used for fish processing, and the aquatic environment used for fish culture.

Much as the prevalence of AMR genes detected by PCR was lower than the prevalence of ABR detected by the disc diffusion test, our observations show that the top seven isolates having the highest prevalence of AMR genes detected by PCR were members of the coliform bacteria. As shown in the PCR results, *K. pneumoniae* HL16 had the highest prevalence of 31% followed by *Klebsiella* sp. HL3, *K. oxytoca* HL4, *K. variicola* HL17, *E. coli* HL19, and *E. coli* HL23 all with a prevalence of 23%. This was followed by 10 isolates with a low prevalence of AMR genes varying between 8% and 15%. Finally, five isolates had no AMR genes detected by PCR, which included *Citrobacter* spp. (HL10 and HL21), *E. coli* (HL14), *Acinetobacter lwoffii* (HL2) and *S. pseudintermedius* (HL18). Altogether our observations show that the coliform bacteria mainly comprising the *Klebsiella* species and *E. coli* accounted for the top species that had the highest prevalence of AMR genes detected by PCR. These findings suggest that coliforms are the major indicator of bacteria contaminants in fish food in retail markets in India.

6.3 Antimicrobial resistance profile detected in the indicator bacteria species

The profile of antibiotic resistance detected using the disc diffusion test showed a wider prevalence range of 5% to 62% inclusive of all 12 antibiotics tested showing that each of the 18 isolates examined was resistant to at least one antibiotic. This is contrary to the PCR results that had a narrow prevalence range of 0 to 43% with only five of the 13 AMR genes being positive. Like our findings, several other scientists have reported different antibiotic resistance profiles in indicator bacteria isolated from retail fish in India. For example, Naik et al [174] found resistance to multiple antibiotics such as erythromycin, kanamycin, neomycin,

streptomycin, penicillin, cefotaxime, bacitracin, rifampicin, trimethoprim, ciprofloxacin, and doxycycline in marine fish procured from different fish markets in Mumbai that had different bacteria species inclusive of *Staphylococcus spp.*, *K. pneumoniae*, *Enterobacter spp.*, and *Acinetobacter spp*. Singh et al [175] found a high prevalence of *E. coli* isolates that were resistant to cephalosporins, cefotaxime, cefpodoxime, ceftazidime, imipenem, cefoxitin and meropenem in fresh seafood in retail markets in India while investigating for the antibiotic susceptibility patterns of the fecal indicator *E. coli* and distribution of important β-lactamase genes. These studies show that the antibiotic resistance profile detected by the disc diffusion test in the indicator bacteria isolated from retail fish in India in this study is comparable to previous findings obtained by other scientists.

The resistance profile of AMR genes detected by PCR in this study showed that only five of the 13 genes tested had resistance to some of the bacteria examined. The resistance by PCR was only seen in the ESBL and carbapenem genes like *bla*$_{\text{CTX-MA}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ together with the *rpoB* gene for rifampicin and *sul2* gene for sulphonamide. Resistance against these genes has been detected in indicator bacteria in RTE fish sold in retail markets by different scientists. For example, Sivaraman et al [171] found 11 AMR genes comprising of the *bla*$_{\text{TEM}}$*, bla*$_{\text{SHV}}$*, bla*$_{\text{OXA-1-like}}$*, tetA, strA, strB, dfrA1, sul1, sul2, qnrB, qnrS, aac(6')-Ib-cr, oqxA, oqxB* in *K. pneumoniae* and *E. coli* from fish food in Assam in India. They also observed that all *E. coli* and *K. pneumoniae* isolates had the *bla*$_{\text{CTX-M}}$ gene in almost all *E. coli* isolates. Similarly, Singh et al [175] found a high prevalence of ESBL genes like *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ together with the β-lactamase genes in the fecal indicator *E. coli* isolated from fresh fish and shellfish sold in the retail markets in India. Dwivedi et al [108] found different carbapenem and ESBL genes that included *bla*$_{\text{IMI}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{EBC}}$, *bla*$_{\text{CIT}}$, *bla*$_{\text{ACC}}$, and *tet(E)*, *bla*$_{\text{NDM-5}}$ *bla*$_{\text{OXA-48}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M-15}}$, *tet(A), sul1*, and *qnrB* in different *Enterococus* spp. and *E. coli* isolated from freshwater fish sold in retail markets in India. Thus, the findings of *bla*$_{\text{CTX-MA}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ genes in the indicator bacteria isolated from RTE fish sold in retail markets in this study corroborate with previous findings suggesting that indicator bacteria found in RTE fish foods might be carriers of different ESBL and carbapenem genes.

In the present study, the *tetE*, *AME3ANT2*, *QnrA*, *bla*$_{\text{NDM}}$, *ermA*, *CAT1*, *IMP*, and *bla*$_{\text{KPC}}$ genes for resistance against tetracycline, gentamycin, fluoroquinolones, carbapenems, erythromycin, chloramphenicol, impenem, and carbapenem, respectively, were not detected by PCR. But other studies have detected the presence of these genes in retail fish in India and other countries. For example, Singh et al [175] and Dwivedi et al [108] found the *bla*$_{\text{NDM}}$ in different indicator bacteria species isolated from fish sold in retail markets in India while Dwivedi et al [108] also found the *tetE* and *bla*$_{\text{KPC}}$ genes in indicator bacteria isolated from farmed freshwater fish in India. Sivaraman et al [171] found the *qnrB,* and *qnrS* genes in *K. pneumoniae* isolated from fish foods in retail markets in Assam, India.

6.4 Comparison of the disc diffusion test, PCR, and whole genome sequencing

The highest prevalent among the antibiotics tested using the disc diffusion test was against ciprofloxacin (62%) and colistin (52%) followed by erythromycin (48%), rifampicin (43%), Amoxycillin (38%), cefoxitin (33%), cephalothin (33%), sulphonamide (33%), trimethoprim (29%), nitrofurantoin (24%), tetracycline (24%), and gentamicin (5%). Thus, all the antibiotics tested using the disc diffusion test showed resistance with varying prevalence of 5% to 62%. On the contrary, the PCR analysis only showed resistance to five genes of the 13 AMR genes examined with the *bla*$_{\text{CTX-MA}}$ gene for cefotaxime and *rpoB* for rifampicin being highest with a prevalence of 43% each followed by the *sul-2* gene for sulfonamide (33%), *bla*$_{\text{SHV}}$ gene for

penem and carbapenem (29%), and the *bla*$_{TEM}$ gene for ampicillin and penicillin (10%). Thus, although the selection of AMR genes used in the PCR analysis was matched with the antibiotics tested in the disc diffusion test, the total number of resistance genes detected by PCR was less than half of the total examined (38.5%, 5/13) unlike the disc diffusion test where all antibiotics tested showed resistance to at least one of the bacteria tests (100%, 12/12).

The difference between the detection of AMR using the disc diffusion test and PCR is influenced by different factors. The disc diffusion test can detect AMR based on the extrusion of the antibiotics by efflux pumps. This is particularly true when there is a mutation in the efflux pumps leading to excessive extrusion of antibiotics. These elements cannot be easily detected by PCR, which in most cases is focused on detecting mutational changes in specific target genes that mediate antimicrobial sensitivity. Changes in the target sites often lead to resistance against antibiotic treatment because the ability of the antibiotic to execute its bactericidal or bacteriostatic effect has been altered. Thus, these factors could account for reasons why there was more resistance to several antibiotics in the disc diffusion test than PCR. However, the disc diffusion test has the restriction of only depending on the selected antibiotics used in the assay and, therefore, risks omitting other antibiotics not included in the assay. Similarly, PCR is also limited to the selected primers that target the chosen resistance genes but also risks omitting other AMR genes not included in the assay. Moreover, the selected primers in the PCR assays have the potential to fail to detect mutation in the target AMR genes. Thus, both assays cannot detect other causes of resistance outside the commercial antibiotics or primers used in the assay. Also, neither the disc diffusion nor PCR can determine whether the resistance is intrinsically encoded in the chromosomes, or they are extrinsically encoded in the MGEs. As shown in this study, WGS is a better tool for determining all AMR genes present in a bacteria genome. For example, the disc diffusion test showed resistance against tetracycline in some of the isolates while PCR showed no resistance against the *tetE* gene for tetracycline. However, WGS showed the presence of the *tetA* gene in *E. coli* HL22, which could not be detected by the *tetE* primers used in the PCR. Thus, WGS provides a comprehensive insight into all AMR genes present in the genome and the presence of the MGEs. As shown in this study, there were more genes detected by WGS including those not included in the PCR assay. In addition, WGS provided additional information such as the location of the AMR genes, and the presence of transposon. Nonetheless, both the disc diffusion test and PCR-based assay are the most widely used for the detection of AMR because they are less expensive than WGS, which requires advanced bioinformatics analysis and the use of specialized software to determine the presence and location of the AMR genes in microbial genomes.

7. Conclusion

This study reports the detection of different indicator bacteria species associated with the contamination of RTE fish sold in retail markets in India. The indicator bacteria identified belonged to seven genera consisting of four Gram-negative and three Gram-positive genera. The Gram-positive bacteria accounted for five isolates while the Gram-negative accounted for 13 of the 18 isolates examined. The Gram-negative species detected included *K. pneumoniae*, *K. variicola*, *K. oxytoca*, *Citrobacter freundii*, *Citrobacter* sp., *E. coli*, *Acinetobacter lwoffii* and *Acinetobacter* sp. On the other hand, the Gram-positive bacteria detected comprised *S. aureus*, *S. pseudintermedius*, *S. dysgalactiae*, *A. viridans*, and *A. urinaequi*. Overall, members of the Enterobacteriaceae dominated by the coliform group accounted for the largest proportion of the indicator bacteria species identified. The detection of the *Staphylococcus* species points to possible contamination by fish handlers while the high prevalence of coliforms points to fecal contamination.

The disc diffusion test showed a variable prevalence of resistance against the 12 antibiotics that included ciprofloxacin (62%), colistin (52%), erythromycin (48%), rifampicin (43%), amoxicillin (38%), cefoxitin (33%), cephalothin (33%), sulphonamide (33%), trimethoprim (29%), nitrofurantoin (24%), tetracycline (24%), and gentamicin (5%). Thus, all 13 antibiotics were found resistant in one or more bacteria isolates tested using the disc diffusion test. On the contrary, the detection of AMR genes using PCR showed that only five of the 13 AMR genes were found resistant in some of the bacteria isolates examined. This implied that although the selection of AMR genes used in the PCR was matched with antibiotics used in the disc diffusion test, the result of the PCR test showed a lower resistance prevalence than the disc diffusion test that varied between 0 and 43%. The AMR genes detected by PCR consisted of the $bla_{\text{CTX-MA}}$ gene for cefotaxime and *rpoB* for rifampicin being the highest with a prevalence of 43% each followed by the *sul-2* gene for sulfonamide (33%), bla_{SHV} gene for penem and carbapenem (29%), and the bla_{TEM} gene for ampicillin and penicillin (10%). The detection of $bla_{\text{CTX-MA}}$, bla_{SHV}, and bla_{TEM} points presence of the ESBL and carbapenems in the indicator bacteria examined. Comparative analysis of PCR and WGS showed that WGS was a better tool for the detection of all AMR genes together with MGEs encoded in bacteria genomes. This study has shown that WGS provided extra information on AMR genes not included in the primers used in the PCR assay. In addition, it provided extra information on the location of the AMR genes and MGEs in bacteria genomes and helped to determine whether the AMR genes are intrinsically located in the chromosomes or extrinsically located in the MGEs.

Overall, this study has shown the presence of bacteria indicators of contamination in fish foods sold in retail markets in India, and that the identified indicator bacteria contain AMR genes inclusive of the ESBLs and carbapenems. Thus, there is a need to develop fish food safety control measures aimed at preventing bacteria contamination and the spread of AMR from fish to humans.

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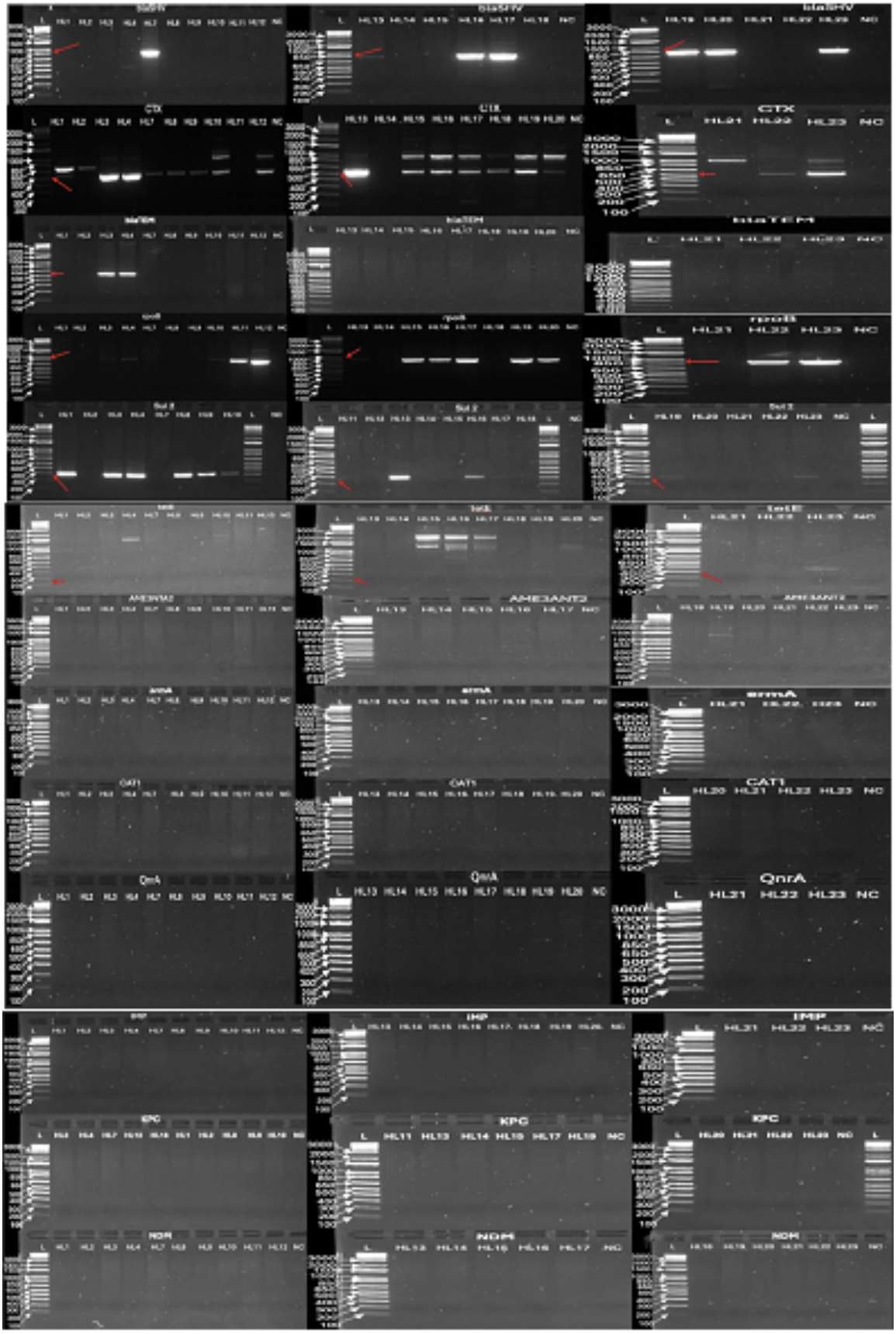
8. Appendix

Tabell 16. Nanodrop results for the DNA extraction

Sample ID	Concentration (ng/µl)	A260/A280	A260/A230
HL1	61.1	2.00	1.34
HL2	64.6	1.94	1.19
HL3	97.6	2.03	1.59
HL4	84.6	2.07	1.74
HL7	141.3	1.81	0.90
HL8	22.3	2.02	0.97
HL9	14.7	1.92	0.63
HL10	65.2	2.07	1.45
HL11	216.4	1.98	1.26
HL12	112.1	1.99	1.45
HL13	66.1	1.88	1.15
HL14	204.5	2.01	1.81
HL15	52.5	1.86	1.01
HL16	61.1	1.99	1.35
HL17	139.8	2.02	1.56
HL18	27.3	1.81	0.85
HL19	118.9	2.05	1.57
HL20	62.5	2.03	1.32
HL21	70.4	1.77	0.79
HL22	122.7	1.99	1.18
HL23	126.0	1.91	0.97



Figur 18. PCR electrophoresis gel results