Doctoral theses at NTNU, 2023:279

Hyejeong Lee

Mesophilic *Aeromonas* spp. isolated from ready-to-eat seafood

Insight into antimicrobial resistance, virulence factors, and growth kinetics in food

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
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Trondheim, September 2023

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Summary

In the last few years, more attention has been given to the role of *Aeromonas* as a foodborne pathogen as well as a spoilage organism. This is due to their widespread occurrence in different types of water and food, their ability to grow at a wide range of temperatures, especially at low temperatures, and lastly, their pathogenic potential by the production of virulence-associated toxins as well as other virulence factors. With the recent trend of consuming more natural and less processed food, there is growing concern over *Aeromonas* as microbiological hazards when consuming raw ready-to-eat (RTE) seafood such as retail sushi or sashimi. However, there is limited information on virulence factors, growth kinetics, and the spoilage potentials of *Aeromonas* if present in RTE seafood. Moreover, the emergence of antimicrobial resistance (AMR) in the food chain is a complex and cross-sectional challenge, and the consumption of contaminated food with antimicrobial resistant bacteria (ARB) or antimicrobial resistance genes (ARGs) is a potential transmission route of AMR to consumers. Due to the ubiquitous nature of *Aeromonas* in aquatic environments and their ability to acquire and transfer genetic materials within and among other species, a better understanding of AMR among *Aeromonas* spp. present in food and their potential role as a vector in the food chain is necessary. Thus, the aim of this work was to establish more knowledge on mesophilic *Aeromonas* isolated from RTE seafood, with a special focus on antimicrobial resistance, virulence factors, and growth kinetic parameters.

Available information on the prevalence of *Aeromonas* in RTE seafood in Norway is rather limited and only covers retail sushi products. In **Paper I**, the occurrence of *Aeromonas* was examined in different types of RTE seafood, and the highest prevalence was found in retail sushi products (17 %), followed by oysters (10 %), fresh salmon loins (10 %) and scallops (4 %). Among 43 *Aeromonas* identified based on *gyrB* gene sequences, *A. media* (75 %) was the most prevalent, followed by *A. salmonicida* (23 %), and *A. bestiarum* (2 %). Most of the isolates contained several virulence-associated toxin genes encoding hemolysin (*hylA),* aerolysin (*aerA),* cytotoxic enterotoxin (*act),* and heat-labile cytotonic enterotoxin (*alt*). Moreover, all isolates were resistant to ampicillin and erythromycin, and most of them (98 %) were considered multidrug resistant. Our study demonstrated the presence of potentially virulent and multidrug resistant *Aeromonas* in RTE seafood. To our knowledge, our study reported for the first time, the occurrence of multidrug resistant *Aeromonas* associated with Norwegian RTE seafood.

Our knowledge of the virulence factors and AMR of *Aeromonas* present in RTE seafood and a salmon processing environment has been extended in the further study (**Paper II**)**,** where whole genome sequences of the selected *Aeromonas* strains were examined for the presence of virulence genes, ARGs and mobile genetic elements (MGEs) using different databases. Of 22 *Aeromonas* whole

genomes that have been analysed for species identification, eight different species, including *A. caviae, A. dhakensis, A. hydrophila, A. media, A. rivipollensis, A. salmonicida, A. bestiarum,* and *A. piscicola* were identified based on a multilocus phylogenetic analysis (MLPA) and average nucleotide identity (ANI) analysis. In addition, the genes responsible for adherence and motility (Msh type IV pili, tap type IV pili, polar flagella), type II secretion system (T2SS) and hemolysin were present in all strains, while the genes encoding type VI secretion system (T6SS) including major effectors were found in 68 % of the strains. Moreover, all strains contained multiple ARGs encoding β-lactamases such as *cphA* and bla_{OXA}. One strain *A. rivipollensis* A539 had a IncQ type plasmid carrying the quinolone resistance gene, *qnrS2*, and another strain *A. caviae* SU4-2 contained a class I integron (*intI1*) with two ARGs (*sul1* and *aadA1*), and a Tn521 transposon carrying a mercury operon. These findings confirmed that *Aeromonas* strains in RTE seafood were potentially pathogenic by carrying several virulence-related genes, and in particular, those strains carrying some ARGs located in MGEs could potentially be involved in the dissemination of ARGs to other bacterial species residing in the food chain and possibly to humans.

Several factors influencing the growth kinetics of *Aeromonas* in food and understanding the growth behaviours of *Aeromonas* strains under different growth conditions is critical to prevent and control the growth of potentially pathogenic and multidrug resistant *Aeromonas* in RTE seafood. In **Paper III**, the effects of food processing factors often applied in RTE seafood such as low temperatures (4 and 8 °C), salting (0.5–6.5 %), and two types of purified condensate smokes (PCS) at different concentrations, were tested on *Aeromonas* growth in liquid cultures. The combined effect of low temperatures and 3.5 % NaCl was insufficient to inhibit the growth of *A. media, A. bestiarum, A. piscicola,* and *A. salmonicida*, while the antimicrobial potential of using PCS at the maximal allowed concentration (0.26 %) was observed. In addition, the growth kinetics of three *Aeromonas* strains were studied on vacuum-packed fresh and cold-smoked salmon stored at 4 °C for 14 and 21 days, respectively. Vacuum packaging combined with cold storage at 4 °C was insufficient to inhibit the growth of *Aeromonas* in fresh salmon, while the growth was inhibited in a minimally salted cold-smoked salmon (salt content of 1.8 %). Our study demonstrated that mild processing factors used in producing RTE seafood might not guarantee the total inhibition of *Aeromonas*. Nevertheless, the cold-smoking process including the application of PCS could be a promising approach to control the growth of *Aeromonas*.

In conclusion, this work demonstrated the occurrence of mesophilic *Aeromonas* in Norwegian RTE seafood, and most of them could grow under different food processing conditions that are often applied in the production of RTE seafood. They are potentially pathogenic, carrying multiple virulence factors, and thus, consumption of RTE seafood contaminated with a high concentration of potentially pathogenic *Aeromonas* may be a risk factor to the frequent consumers of RTE seafood. Moreover, our

study confirmed the occurrence of multidrug resistant *Aeromonas* strains in RTE seafood, and some strains carrying ARGs in their MGEs potentially act as a vector of ARGs to other bacterial species. Our finding suggests that consumption of contaminated RTE seafood with multidrug resistant *Aeromonas* can be a potential transmission route of AMR (or ARGs) from aquatic animals or environments to humans. Furthermore, *Aeromonas* residing in the food chain could be potential reservoirs of ARGs and virulence genes, and they might play an important role in the emergence and spread of multidrug resistant pathogens in the food chain.

Thesis outline and list of papers

This thesis consists of two parts.

Part I is an introduction to the research topic of my PhD work with a short review of current scientific knowledge of this field and discussion against my own findings. My contributions to the field are indicated by reference to Paper I-III included in Part II.

Part II includes three peer-reviewed papers published in scientific journals as follows. Paper I is published in the *Journal of Applied Microbiology*, and Paper II is published in the *Frontiers in Microbiology*. Lastly, paper III is published in the *International Journal of Food Microbiology*.

(I) **Lee, H. -J**., Hoel, S., Lunestad, B. -T., Lerfall, J. and Jakobsen, A. N. (2021). *Aeromonas* spp. isolated from ready-to-eat seafood on the Norwegian market: prevalence, putative virulence factors and antimicrobial resistance. *J. Appl. Microbiol*. 130, 1380-1393. doi: 10.1111/jam.14865

(II) **Lee, H. -J**., Storesund, J. E., Lunestad, B. -T., Hoel, S., Lerfall, J. and Jakobsen, A. N. (2023). Whole genome sequence analysis of *Aeromonas* spp. isolated from ready-to-eat seafood: antimicrobial resistance and virulence factors. *Front. Microbiol*. 14, 1175304. doi: 10.3389/fmicb.2023.1175304

(III) **Lee, H. -J.**, Tokle, I. F., Lunestad, B. -T., Lerfall, J., Hoel, S. and Jakobsen, A.N. (2023). The effect of food processing factors on the growth kinetics of *Aeromonas* strains isolated from ready-to-eat seafood. *Int. J. Food Microbiol*. 384, 09985. doi: 10.1016/j.ijfoodmicro.2022.109985

List of abbreviation

16S rRNA gene: 16S ribosomal RNA gene

AMR: Antimicrobial Resistance

ARB: Antimicrobial Resistant Bacteria

ARGs: Antimicrobial Resistance Genes

AST: Antimicrobial Susceptibility Testing

CLSI: Clinical and Laboratory Standards Institute

DDH: DNA-DNA Hybridization

EFSA: European Food Safety Authority

EU: European Union

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FDA: Food and Drug Administration

HG: Hybridization Groups

ICNP: International Code of Nomenclature of Prokaryotes

LPSN: List of Prokaryotic names with Standing in Nomenclature

MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry

MGEs: Mobile Genetic Elements

MIC: Minimal Inhibitory Concentration

MLPA: Multilocus Phylogenetic Analysis

MLSA: Multilocus Sequence Analysis

NGS: Next-Generation Sequencing

PCS: Purified condensate smokes

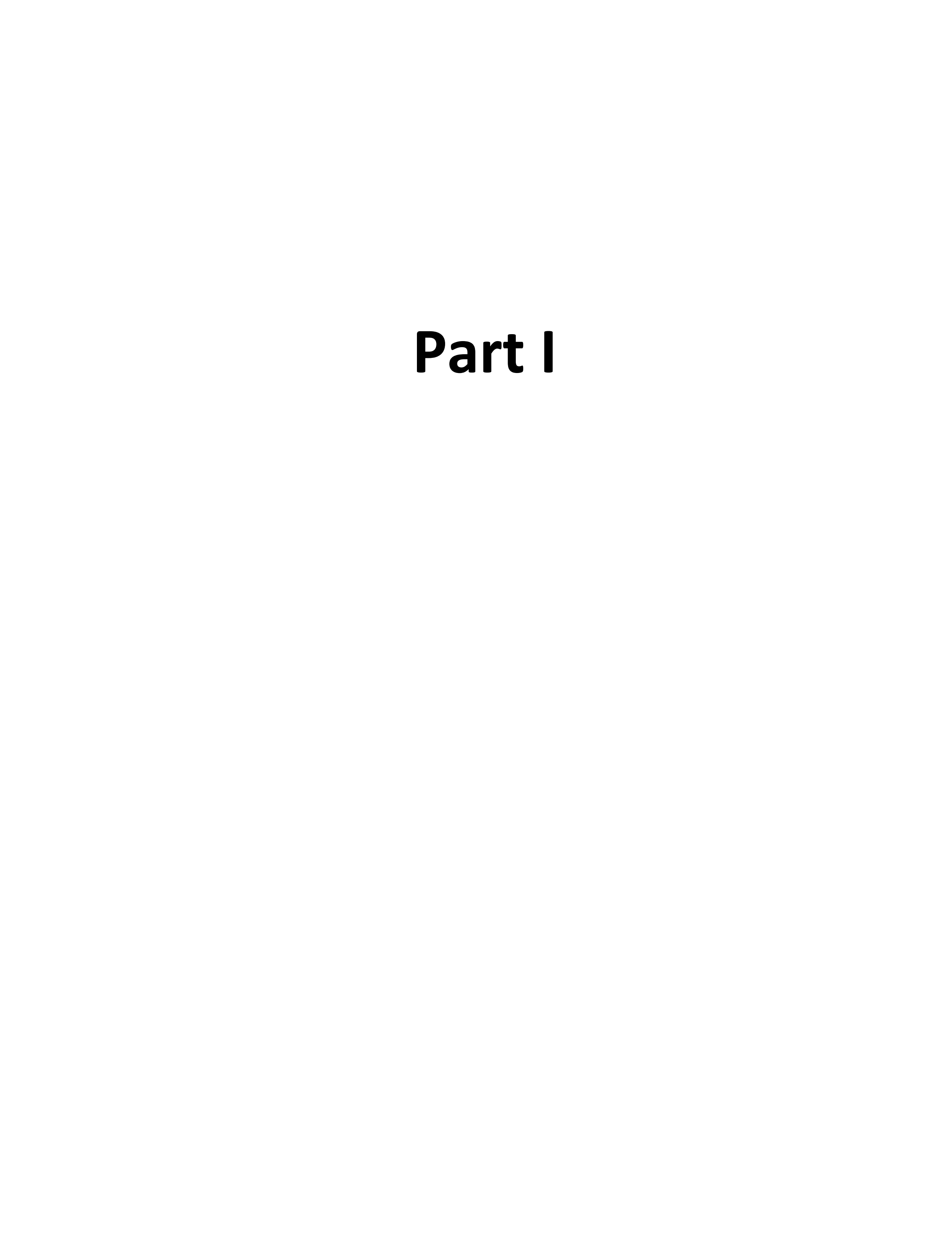
RFLP: Restriction Fragment Length Polymorphism

RTE: Ready-To-Eat

WGS: Whole Genome Sequencing

WHO: World Health Organization

Part I



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1. Introduction

Members of the genus *Aeromonas* are ubiquitous in aquatic environments such as fresh, brackish, estuarian and marine waters (Martin-Carnahan and Joseph, 2005). Psychrophilic *A. salmonicida* and some mesophilic *Aeromonas* spp. like *A. hydrophila* are known as fish pathogens causing furunculosis and motile *Aeromonas* septicemia (MAS), while several species of mesophilic *Aeromonas* can cause a wide spectrum of human infections, such as gastroenteritis, wound infections, or bacteremia (Janda and Abbott, 2010; Beaz-Hidalgo and Figueras, 2013). The taxonomy of the genus *Aeromonas* is complex and still controversial. Since the first establishment of the genus in the International Committee of Systematic of Prokaryotes (ICSP) in 1943, at least 36 species have been described in the genus *Aeromonas* as of June 2023. In a recent study, *A. caviae. A. dhakensis, A. veronii, and A. hydrophila* were the four species responsible for 95 % of the human infections that have been reported so far (Fernández-Bravo and Figueras, 2020). In a current theory, the pathogenicity of *Aeromonas* is multifactorial, where several virulence factors related to adhesin, secretion systems, and toxins are involved (Tomás, 2012, Fernández-Bravo and Figueras, 2020).

The role of *Aeromonas* as a true foodborne pathogen has been controversial, due to the limited epidemiological data on acute gastroenteritis and outbreaks. Only few cases of foodborne outbreaks were previously linked to the consumption of food or water contaminated by *Aeromonas* (Zhang et al., 2012; Ventura et al., 2015). Despite so, mesophilic *Aeromonas* species are considered emerging foodborne pathogens as well as spoilage organisms (Teunis and Figueras, 2016; Wu et al., 2018; Hoel et al., 2019; Jakobsen et al., 2020). The main routes of *Aeromonas* infection are via the consumption of contaminated food and water (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020). *Aeromonas* are widespread in different types of food and water, and they can grow at a wide range of temperatures and produce cytotoxic enterotoxins (Pablos et al., 2011; Stratev et al., 2012). Particularly, a high prevalence of potentially pathogenic *Aeromonas* in ready-to-eat (RTE) seafood has been reported in previous studies (Di Pinto et al., 2012; Hoel et al., 2015;2017; Park et al., 2021; Teodoro et al., 2022). In addition, the ability of mesophilic *Aeromonas* strain to grow at cold temperatures has been demonstrated in a nigiri sushi model (Hoel et al., 2018). Thus, there is a growing concern over *Aeromonas* as microbiological hazards; however, there is limited knowledge on growth kinetics in food and virulence factors of *Aeromonas* present in RTE seafood and seafood processing environments.

Antimicrobial resistance (AMR) is a global public health problem affecting humans, animals, and the environments (WHO, 2014; 2021). Current challenges in AMR with multiple and interconnected drivers must be addressed within the *One Health* approach (Mcewen and Collignon, 2018; Velazquez-

Meza et al., 2022). Especially, antimicrobial resistant bacteria (ARB) or antimicrobial resistance genes (ARGs) residing in the food chain can spread to humans via the foodborne route, and consumption of contaminated food is a potential transmission route of AMR from animals to humans (Founou et al., 2016; EFSA Panel on Biological Hazards (BIOHAZ), 2021). *Aeromonas* are at the interface of all *One Health* components due to their widespread occurrence in environments, animals, and humans (Lamy et al., 2022). While aquatic environments are considered primary reservoirs of *Aeromonas,* they can rapidly adapt to environmental changes, form a biofilm, and colonize a wide range of niches and hosts (Fernández-Bravo and Figueras, 2020; Lamy et al., 2022). The prevalence of multidrug resistant *Aeromonas* has frequently been reported from various sources, including fresh- or wastewater (Esteve et al., 2015; Piotrowska et al., 2017), fish (Dhanapala et al., 2021), marine bivalves (Albini et al., 2022), seafood (Yano et al., 2015; Hossain et al., 2020), dairy products and vegetables (Stratev and Odeyemo, 2016). Additionally, the potential capacity of *Aeromonas* to transfer AMR to other bacterial species has been shown by carrying multiple ARGs and mobile genetic elements (MGEs) in their genomes (Piotrowska and Popowska, 2014; 2015; Dubey et al., 2022a, b). Therefore, *Aeromonas* are considered a key amplifier of genetic material, particularly ARGs (Lamy et al., 2022). However, current knowledge is rather limited to the occurrence of AMR among *Aeromonas* spp. in the clinical setting or aquatic environments. Considering the high occurrence of *Aeromonas* in RTE seafood and other types of food, more information on AMR of *Aeromonas* present in RTE seafood is needed to understand their potential role as a vector of AMR in the food chain.

Therefore, the main aim of this research work is to gain more knowledge of AMR, virulence factors, and growth kinetics of *Aeromonas* isolated from RTE seafood, and the specific objectives of the research works are summarised in Chapter 2. Moreover, Chapter 3 briefly introduces the genus *Aeromonas* regarding their taxonomy, identification methods, and human infections. Chapter 4 discusses the significance of *Aeromonas* as a potential foodborne pathogen and spoilage organism, particularly with the growing consumption of RTE seafood. Chapter 5 discusses the potential role of *Aeromonas* as a vector of AMR in the food chain. Finally, Chapter 6 includes the conclusion of this research work and further perspectives.

2. Aims and specific research objectives

The aim of the present Ph.D. work was to establish more knowledge on the mesophilic *Aeromonas* spp. isolated from RTE seafood, with the focus on AMR, virulence factors and growth kinetics, and to investigate the significance of *Aeromonas* as potentially pathogenic and multidrug resistant bacteria in the food chain.

Specific research objectives were:

1) Examine the prevalence of *Aeromonas* spp. in RTE seafood on the Norwegian market and characterize the AMR patterns and virulence-associated toxin genes of the *Aeromonas* isolated from the RTE seafood (**Paper I**).

2) Evaluate overall virulence and ARGs profile based on whole genome sequencing (WGS) analysis of *Aeromonas* isolated from RTE seafood (**Paper II**).

3) Investigate the growth kinetics of selected strains of *Aeromonas* (representing seven species) isolated from RTE seafood, under the effects of different food processing factors such as temperatures, salt contents, and smoke condensates (**Paper III**).

3. The genus *Aeromonas*

3.1. *Aeromonas* species: past to present

The genus *Aeromonas* belongs to the class of Gammaproteobacteria, order Aeromonadales, and the family *Aeromonadaceae* (Colwell et al., 1986). *Aeromonas* (*aer*- is gas, and -*monas* is units, in Greek, meaning gas-producing units) is the name given to the group of Gram-negative rods that resembled pseudomonads in being motile but differed by producing gas when carbohydrates were fermented (Kluyver and van Niel, 1936). Bacteria in the genus *Aeromonas* are Gram-negative, rod-shaped, facultatively anaerobic, oxidase positive, and catalase positive (Martin-Carnahan and Joseph, 2005). The genus *Aeromonas* was established by the ICSP in 1943. The genus was initially included in the family *Vibrionaceae* in 1965, but later research revealed a unique phylogenetic branch of this genus belonging to the new family *Aeromonadaceae* in 1986 (Colwell et al., 1986). From the beginning of the mid-1970, the species in the genus *Aeromonas* have traditionally been classified into two major groups: one comprising mesophilic and motile species, typified by *Aeromonas hydrophilia* which are mainly associated with human infections, and the other is psychrophilic and non-motile species, represented by *Aeromonas salmoncida* which cause diseases in fish (Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010). The occurrence of atypical *A. salmonicida* strains (being mesophilic and motile) has been described from other studies (Pavan et al., 2000; Vincent et al., 2017), showing a great diversity in the strains belonging into *A. salmonicida*. Taxonomic classification of *Aeromonas* is a challenging task like other bacteria, and the taxonomy of the genus *Aeromonas* has undergone numerous changes (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020). One of the main reasons is due to the diversity of *Aeromonas* species and their erratic behaviours, causing difficulties in species identification when using biochemical tests (Beaz-Hidalgo et al., 2010; Fernández-Bravo and Figueras, 2020). The development of molecular-based methods has enabled the identification of several new species and the verification of their taxonomic position (see more detail in **Chapter 3.2**).

At the time of the most recent publication of the Bergey's Maunal in 2005, the genus *Aeromonas* comprised 14 species and the so-called Enteric Group 501 (currently known as *A. diversa*) (Martin-Carnahan and Joseph, 2005). Based on DNA-DNA hybridization (DDH), the species were classified into different DNA hybridization groups (HG); *A. hydrophila* (HG1), *A. bestiarum* (HG2), *A. salmonicida* (HG3), *A. caviae* (HG4), *A. media* (HG5), *A. eucrenophila* (HG6), *A. sobria* (HG7), *A. veronii (biovar sobria and biovar veronii)* (HG8/10), *A. jandaei* (HG9), *A. encheleia* (HG11, HG16), *A. schubertii* (HG12), *A. diversa* (HG13), *A. trota* (HG14), *A. allosaccharophila* (HG15), and *A. popoffii* (HG17). With the development of novel markers including 16S ribosomal RNA (16S rRNA) gene or housekeeping genes, and subsequent development of a multilocus phylogenetic analysis (MLPA) using concatenated

sequences of several housekeeping genes, the number of new species or subspecies has continued to rise and some of the species has been reclassified as a new species (Fernández-Bravo and Figueras, 2020). As of June 2023, at least 36 species have been recognized, including new species: *A. simiae, A. molluscorum, A. bivalvium, A. tecta, A. piscicola, A. diversa, A. fluvialis, A. sanarellii, A. taiwanensis, A. rivuli, A. australiensis, A. cavernicola, A. dhakensis, A. aquatica, A. finlandiensis, A. lacus, A. rivipollensis, A. lustitana, A. aquatilis, A. crassostreae, A. enterica* and *A. intestinalis* (**Table 1**). *Aeromonas* species have been isolated from various sources including freshwater, drinking water, sewage, fish, marine bivalves, as well as clinical samples, and the source of isolation and identification methods used for 36 species are summarised in **Table 1**. According to the List of Prokaryotic names with Standing in Nomenclature (LPSN) database, 31 species have been validly published under the International Code of Nomenclature of Prokaryotes (ICNP), which requires the deposit of pure cultures of the type strain in collections in at least two distinct countries (Parte et al., 2020).

Table 1. 36 species belonging to the genus *Aeromonas* in chronological order (year of isolation), including the source of isolation and identification methods used.

Year	Species name	Source of isolation	Methods used for species identification
1943	*Aeromonas hydrophila*	Canned milk	Phenotypic characterisation (Stanier, 1943)
1953	*Aeromonas salmonicida*	Fish	Phenotypic characterisation (Griffin et al., 1953)
1981	*Aeromonas sobria*	Clinical samples, fish, frog, and fresh water	Phenotypic characterisation and DNA base compositions (Popoff and Véron, 1976)
1983	*Aeromonas media*	River water	Phenotypic characterisation and DNA base compositions (Allen et al., 1983)
1984	*Aeromonas caviae*[a]	Guinea pig	Phenotypic characterisation (Popoff, 1984)
1987	*Aeromonas veronii*	Clinical samples (Human faeces, blood, and wounds)	Phenotypic characterisation and DDH (Hickman-Brenner et al., 1987)
1988	*Aeromonas schubertii*	Clinical samples (Blood, wounds)	Phenotypic characterisation and DDH (Hickman-Brenner et al., 1988)
1988	*Aeromonas eucrenophila*	Carp and fresh water	Phenotypic characterisation (Schubert and Hegazi, 1988) and DDH (Huys et al., 1997)
1991	*Aeromonas trota*	Human faeces	Phenotypic characterisation and DDH (Carnahan et al., 1991a)
1991	*Aeromonas jandaei*	Clinical samples (Human faeces, blood, and wounds)	Phenotypic characterisation and DDH (Carnahan et al., 1991b)
1992	*Aeromonas allosaccharophila*	European eel and human faeces	Phenotypic characterisation, 16S rRNA gene sequencing (Martinez-Murcia et al., 1992), and DDH (Esteve et al., 1995)
1995	*Aeromonas encheleia*	European eel	Phenotypic characterisation and DDH (Esteve et al., 1995)
1996	*Aeromonas bestiarum*	Diseased fish, water, and human faeces	Phenotypic characterisation and DDH (Ali et al., 1996)
1997	*Aeromonas popoffii*	Drinking water	Phenotypic characterisation and DDH (Huys et al., 1997)
2004	*Aeromonas simiae*	Monkey faeces	Phenotypic characterisation, DDH, and 16S rRNA gene sequencing (Harf-Monteil et al., 2004)
2004	*Aeromonas molluscorum*	Bivalve molluscs	Phenotypic characterisation, DDH, 16S rRNA gene sequencing, and RFLP analysis (Miñana-Galbis et al., 2004)

<table>
<tr><td>2007</td><td>Aeromonas bivalvium</td><td>Bivalve molluscs</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, and RFLP analysis (Miñana-Galbis et al., 2007)</td></tr>
<tr><td>2008</td><td>Aeromonas tecta</td><td>Human faeces, fish, and tap water</td><td>Phenotypic characterisation, DDH, 16S rRNA, gyrB, and rpoD gene sequencing (Demarta et al., 2008)</td></tr>
<tr><td>2009</td><td>Aeromonas piscicola</td><td>Diseased fish</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, MLPA using 5 housekeeping genes (16S rRNA, rpoD, gyrB, recA, and dnaJ), and MALDI-TOF MS analysis (Beaz-Hidalgo et al., 2009)</td></tr>
<tr><td>2010</td><td>Aeromonas diversa</td><td>Clinical samples (Wound infection)</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, MLPA using 4 housekeeping genes (cpn60, dnaJ, gyrB, and rpoD), and RFLP analysis (Miñana-Galbis et al., 2010)</td></tr>
<tr><td>2010</td><td>Aeromonas fluvialis</td><td>River water</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, and MLPA using 5 housekeeping genes (gyrB, rpoD, recA, dnaJ, and gyrA) (Alperi et al., 2010b)</td></tr>
<tr><td>2010</td><td>Aeromonas sanarellii</td><td>Clinical samples (Wound infection)</td><td rowspan="2">Phenotypic characterisation, DDH, 16S rRNA and rpoD gene sequencing, and MPLA using 5 housekeeping genes (gyrB, rpoD, recA, dnaJ, and gyrA) (Alperi et al., 2010a)</td></tr>
<tr><td>2010</td><td>Aeromonas taiwanensis</td><td>Clinical samples (Wound infection)</td></tr>
<tr><td>2011</td><td>Aeromonas rivuli</td><td>Water rivulet</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, and MLPA using 5 housekeeping genes (gyrB, rpoD, recA, dnaJ, and gyrA) (Figueras et al., 2011)</td></tr>
<tr><td>2013</td><td>Aeromonas australiensis</td><td>Irrigation water</td><td>Phenotypic characterisation, DDH, rpoD and gyrB gene sequencing, and MLPA using 6 housekeeping genes (gyrB, rpoD, recA, dnaJ, gyrA, and dnaX) (Aravena-Román et al., 2013)</td></tr>
<tr><td>2013</td><td>Aeromonas cavernicola[c]</td><td>Fresh water</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, and MLPA of 7 housekeeping genes (gyrB, rpoD, recA, dnaJ, gyrA, dnaX, and atpD) (Martínez-Murcia et al., 2013)</td></tr>
<tr><td>2013</td><td>Aeromonas dhakensis[b]</td><td>Aquarium water, ornamental fish, and human faeces</td><td>Phenotypic characterisation, DDH, 16S rRNA, rpoD and gyrB gene sequencing, and MLPA using 5 housekeeping genes (gyrB, rpoD, recA, dnaJ, and gyrA) (Beaz-Hidalgo et al., 2013)</td></tr>
<tr><td>2015</td><td>Aeromonas aquatica</td><td>Lake water</td><td rowspan="3">Phenotypic characterisation, 16S rRNA and rpoD gene sequencing, MLPA using 15 housekeeping genes (gyrB, rpoD, recA, dnaJ, gyrA, dnaX, atpD, cpn60, dnaK, gltA, mdh, radA, rpoB, tsf, and zipA), ANI analysis, and in silico DDH (Beaz-Hidalgo et al., 2015b)</td></tr>
<tr><td>2015</td><td>Aeromonas finlandiensis</td><td>Lake water</td></tr>
<tr><td>2015</td><td>Aeromonas lacus</td><td>Lake water</td></tr>
<tr><td>2015</td><td>Aeromonas rivipollensis</td><td>River water</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, and MLPA using 5 housekeeping genes (gyrA, gyrB, rpoD, recA, and dnaJ) (Marti and Balcázar, 2016)</td></tr>
<tr><td>2016</td><td>Aeromonas lusitana[c]</td><td>Untreated water and vegetables</td><td>Phenotypic characterisation, DDH, 16S rRNA gene sequencing, and MLPA using 7 housekeeping genes (gyrB, rpoD, recA, dnaJ, gyrA, dnaX, and atpD) (Martinez-Murcia et al., 2016)</td></tr>
<tr><td>2017</td><td>Aeromonas aquatilis[d]</td><td>Lake water</td><td rowspan="4">Phenotypic characterisation, 16S rRNA and rpoD gene sequencing, MLPA using 6 housekeeping genes (rpoD, gyrB, gyrA, recA, dnaJ, and dnaX), and in silico DDH (Figueras et al., 2017)</td></tr>
<tr><td>2017</td><td>Aeromonas crassostreae[c]</td><td>Oyster</td></tr>
<tr><td>2017</td><td>Aeromonas enterica[d]</td><td>Human faeces</td></tr>
<tr><td>2017</td><td>Aeromonas intestinalis[d]</td><td>Human faeces</td></tr>
</table>

[a] Synonym with *A. puntata* (Snieszko, 1957)
[b] Synonym with *A. hydrophila subsp. dhakensis* (Huys et al., 2002) and *A. aquariorum* (Martínez-Murcia et al., 2008)
[c] not validly published under the ICNP
[d] not registered in the LPSN database nor published under the ICNP

3.2. Taxonomic classification and identification methods

Isolation and detection of *Aeromonas* are complex and labour-intensive (Perales, 2003). The choice of culture medium will depend on the type of test sample and influence the recovery and prevalence of *Aeromonas* (Latif-Eugenín et al., 2016). A numbers of culture media have been proposed for the isolation of *Aeromonas* and most of the media are selective for *Aeromonas* isolation to contain antibiotics (ampicillin), bile salts, or other selective agents. While no single medium has officially been recommended for *Aeromonas* detection, ampicilin dextrin agar (ADA) is the most used medium for water samples (Havelaar et al., 1987; Borrell et al., 1998) and starch ampicillin agar (SAA) seems to be the best medium for testing food samples, including fish and shellfish (Palumblo et al., 1987; NMKL, 2004; **Paper I**). Other selective media designed for testing food samples includes MacConkey xylose agar (Okrend et al., 1987), MacConkey mannitol agar (Okrend et al., 1987), and bile salts irgasan brilliant green modified (BIBG-m, Neyts et al., 2000b). Nevertheless, selective media does not solely isolate *Aeromonas* but also *Vibrio* spp. or possibly other species, thus, further biochemical tests are recommended to confirm typical characteristics of *Aeromonas*. For instance, unlike *Vibrio* spp., *Aeromonas* are generally resistant against vibriostaticum O/129 and show no growth at 6 % sodium chloride (NMKL, 2004). Despite so, correct identification at the species level using biochemical tests remains challenging due to the erratic behaviours of the *Aeromonas* strains (Beaz-Hidalgo et al., 2010).

Several molecular-based methods have been developed and used for the species identification and taxonomic classification of *Aeromonas.* The advantages and remaining challenges in identification methods are further discussed. Traditionally, the DDH method has been a gold standard for classifying bacterial species based on DNA relatedness (Wayne et al., 1987). The principle of this method is based on thermal stability of hybridized DNA of two closely related organisms, and strains are considered to belong to the same species when ≥ 70 % of the DNA between the strains reassociates within a 5-degree difference in melting temperatures (Brenner, 1973). Generally, several strains sharing DDH values with ≥ 70% similarity are classified into one genomic group (or HG) (Wayne et al., 1987). The taxonomic classification of *Aeromonas* had previously been based on different HGs (*e.g. A. hydrophila* = HG1), as described in Chapter 3.1. Even though the DDH method has widely been used for species delineation, it has been criticised as being laborious and inaccurate, difficult to reproduce and for the challenges in the production of cumulative databases (Stackebrandt, 2002; Gevers et al., 2005; Rosselló-Mora, 2006). The conventional DDH method has now largely been replaced by *in silico* DDH (Meier-Kolthoff et al., 2013) or average nucleotide identity (ANI) analysis (Richter and Rosselló-Móra, 2009), and more detailed information of these methods will be discussed below.

With the development of DNA sequencing technology, the 16S rRNA gene sequencing has been considered a stable evolutionary marker, and it has widely been used for the identification of many bacterial species together with DDH analysis (Stackebrandt and Goebel, 1994; Stackebrandt et al., 2002). For the first time, Martinez-Murcia et al. (1992) used the 16S rRNA gene to identify a new *Aeromonas* species (*A. allosaccharophila*). However, the 16S rRNA genes of the genus *Aeromonas* share high homology, and the interspecies similarity of the gene sequences is known as 96.7–100 % (Martinez-Murcia et al., 1992), indicating the limitation of using this gene for species delineation. Particularly, the high interspecies similarity (99.6–100 %) of this gene has been reported for tightly closed species such as *A. bestiarum*, *A. salmonicida*, and *A. piscicola* (Martinez-Murcia et al., 2005; Beaz-Hidalgo et al., 2009). Alternatively, protein-coding housekeeping genes have been suggested as new markers for phylogenetic analysis, and several housekeeping genes such as *gyrB* (DNA gyrase subunit B) (Yanez et al., 2003; **Paper I**), *rpoD* (RNA polymerase sigma factor) (Soler et al., 2004), *rpoB* (RNA polymerase subunit beta) (Küpfer et al., 2006), *dnaJ* (chaperone protein) (Nhung et al., 2007), or *recA* (DNA recombination/repair protein) (Sepe et al., 2008) have been used for *Aeromonas* identification. These markers have shown better discriminatory powers than the 16S rRNA gene in differentiating the species in genus *Aeromonas*, with relatively lower interspecies similarities (89–93 %) than the 16S rRNA gene. Particularly, partial *gyrB* gene sequences could successfully discriminate the tightly closed group of *A. salmonicida, A. bestiarum,* and *A. piscicola* (Hoel et al., 2017; **Paper I**). Nevertheless, some limitations of using single housekeeping gene to infer the phylogeny of the *Aeromonas* species have been found due to the different phylogenetic resolution of the housekeeping genes or possible horizontal gene transfer or recombination (Navarro and Martinez-Murcia, 2018).

The approach using several housekeeping genes to assign species identity was first named multilocus sequence analysis (MLSA) by Gevers et al. (2005), which is defined as a method for the genotypic characterisation of a more diverse group of prokaryotes (including entire genera) using the concatenated sequences of multiple housekeeping genes. The MLSA has been recommended by *ad hoc* committee for delineation of the bacterial species (Stackebrandt, 2002). Later, multilocus phylogenetic analysis (MLPA) has been suggested as a more appropriate term since the main purpose is on the data analysis of phylogeny. The study by Martinez-Murcia et al. (2011) was the first to apply the MLPA to investigate the inter- and intra- species relationship of 115 *Aeromonas* strains, and they used the concatenated sequences of seven housekeeping genes (*gyrB, rpoD, recA, dnaJ, gyrA, dnaX*, and *atpD*). They emphasized the importance of using several "*bona fide*" strains (reference strains) representing all described species for reliable phylogenetic species delineation for the MLPA. Since then, several studies have used the MLPA for the phylogenetic analysis of *Aeromonas* species for the verification of taxonomic classification (Beaz-Hidalgo et al., 2013; 2015b; Martinez-Murcia et al., 2016;

Talagrand-Reboul et al., 2017b; **Paper II**). In the study by Beaz-Hidalgo et al. (2013), the MLPA using five housekeeping genes (*gyrB, rpoD, recA, dnaJ,* and *gyrA*) has confirmed that formerly recognized two unique species of *A. hydrophila subsp. dhakensis* (Huys et al., 2002) and *A. aquariorum* (Martínez-Murcia et al., 2008) belonged to a unique taxon, and thus *Aeromonas dhakensis* sp. nov. comb nov. has been proposed as a new name. Moreover, the MLPA using sixteen housekeeping genes could delineate the species inside *A. media* species complex into three different clades representing *A. media, A. rivipollensis,* and *A. paramedia* (which is suggested as a new candidate species) (Talagrand-Reboul et al., 2017b). The reference sequences of these three species inside *A. media* species complex have been included in the MLPA in **Paper II**, and this study revealed that many of the isolates were incorrectly assigned to *A. media* based on *gyrB* gene sequence in **Paper I**, and they were more closely related to *A. rivipollensis*.

In recent years**,** WGS technology has become more common, and the first complete genome sequence of *A. hydrophila* subsp*. hydrophila* ATCC 7966 became available in 2006 (Seshadri et al., 2006). In 2012, only 6 *Aeromonas* genomes were available in the National Center for Biotechnology Information (NCBI) Genome database, and after a few years, the number of available genomes became 410 in 2019 (Fernández-Bravo and Figueras, 2020). As of June 2023, about 1518 *Aeromonas* genomes, including 345 complete genomes are available in the NCBI Genome database. The number of available *Aeromonas* genome sequences has increased almost four-times in the last five years. At the same time, new bioinformatic tools are developed to make use of the WGS data for species identification based on the calculation of traditional DDH value. One is *in silico* DDH (*is*DDH) which has been developed to determine the genetic similarity of two bacterial genomes based on genome-to-genome comparisons (GGDC), to overcome the limitation of the experimental DDH method (Meier-Kolthoff et al., 2013). The other is ANI analysis, which is also based on the comparison of average nucleotide identity among conserved and shared genes, with organisms belonging to the same species showing ≥ 95–96 % ANI value (Konstantinidis and Tiedje, 2005; Richter and Rosselló-Móra, 2009). An ANI value of 95–96 % corresponded to the traditional DDH value of 70 % used for species identification (Goris et al., 2007). Currently, ANI is the gold standard for the substitution of DDH and the cut-off value of 96 % is generally recommended for the species boundaries of prokaryotic genomes (Richter and Rosselló-Móra, 2009). The whole-genome ANI analysis has been used to assess species boundaries and confirm identification (Jain et al., 2018; **Paper II**). An ANI value of > 96 % is the suggested cut-off value for species delineation of *Aeromonas* spp. (Colston et al., 2014). Genomic comparison based on both ANI value > 96 % and *is*DDH value < 70 %, together with MLPA have been used to compare the genomic relatedness of *Aeromonas* strains for the taxonomy classification of a new species (Figueras et al., 2014; Figueras et al., 2017) or for the identification the taxonomic misidentification (Beaz-Hidalgo et al., 2015a).

Besides, other databases such as Kraken 2 and KmerFinder have been developed for the rapid identification and taxonomic classification of the WGS data using fast *k-mer* algorithm (Hasmann et al., 2014; Wood and Salzberg, 2014). In principle, each *k-mer* within a query sequence is matched against the *k-mer* present in the genomes of all known organisms, and then the query sequence is classified based on the *k-mer* assignment. KmerFinder has been designed for the rapid diagnostic purpose of clinical samples (Hasmann et al., 2014; Larsen et al., 2014), while Kraken 2 is a taxonomic classifier particularly designed for metagenomic data (Wood and Salzberg, 2014; Wood et al., 2019). Despite so, both were initially used to check the purity and taxonomy of the whole genome sequence of *Aeromonas* strains in **Paper II** (data not included). These tools seemed to be valuable tools for checking the purity of the genome sequences, since some of the isolates contaminated with other bacterial species have been identified and discarded for further analysis. Except for the contaminated isolates, all others were correctly identified as the genus *Aeromonas*. However, it was inappropriate to identify *Aeromonas* at the species level since the identification for some isolates was incorrectly made at species level. This is mainly due to the limited number of *Aeromonas* reference genomes in the database. For the species such as *A. piscicola* and *A. bestiarum*, only two and three reference sequences are currently available in the NCBI database, respectively; however, none of these were updated in the KmerFinder or Kraken 2 database, thus impossible to make a correct identification for these species.

3.3. Clinical infection and epidemiology

Aeromonas are considered emerging opportunistic pathogens in humans and animals. In aquaculture, psychrophilic *A. salmonicida* and some mesophilic *Aeromonas* are responsible for the fish diseases such as furunculosis and motile *Aeromonas* septicemia (MAS) (Beaz-Hidalgo and Figueras, 2013). On the other hand, mesophilic *Aeromonas* are mainly associated with gastrointestinal and extraintestinal infections with various clinical manifestations in humans (Janda and Abbott, 2010; Tomás, 2012). Of 36 species that have been recognized, at least 19 are considered clinically significant (Fernández-Bravo and Figueras, 2020). Of the clinical cases that have been reported so far, *A. caviae* (37.26 %), *A. dhakensis* (23.49 %), *A. veronii* (21.54 %), and *A. hydrophila* (13.07 %) are the four major pathogens (Figueras and Beaz-Hidalgo, 2015; Fernández-Bravo and Figueras, 2020).

Aeromonas infections include gastroenteritis, wound infections, and bacteremia, affecting both healthy and immunocompromised people (Fernández-Bravo and Figueras, 2020). The most common clinical manifestation is gastroenteritis, and the main source of infection is known as the consumption of contaminated water and food (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020). *Aeromonas* gastroenteritis is generally accompanied by a mild and self-limiting watery diarrhea, but

occasionally a bloody diarrhea or chronic diarrhea are seen (Agger et al., 1985; George et al., 1985; von Graevenitz, 2007). The symptoms are most severe in people at risk groups such as young children and elderly immunocompromised patients (San Joaquin and Pickett, 1988; Fernández-Bravo et al., 2020; Greiner et al., 2021). *Aeromonas* have also been linked to the Travel's diarrhea (Yamada et al., 1997; Vila et al., 2003), as well as the rare complication of haemolytic uremic syndrome (HUS) (Figueras et al., 2007a). Wound infection is the second manifestation, and direct exposure to contaminated water is considered the main route of infections (Janda and Abbott, 2010). Reported cases of *Aeromonas* wound infections have been linked to traumatic injuries or exposure to aquatic environments (Vally et al., 2004; Rutteman et al., 2017) or natural disasters like Tsunamis or Hurricanes (Hiransuthikul et al., 2005; Presley et al., 2006; Lamy et al, 2009). In addition, several cases of *Aeromonas* bacteremia have been reported (Tang et al., 2014; Wu et al., 2015; Rhee et al., 2016). Other infections include respiratory and urinary tract infections (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020).

The overall incidence of *Aeromonas* infections worldwide is unknown or most likely not-reportable due to the self-limiting course of the disease. In addition, there is no reporting requirement for these bacteria nor any routine testing in patient or food. Nevertheless, epidemiological data on *Aeromonas* infections has occasionally been reported in different geographical locations, and the incidence of *Aeromonas* infections seems to be more prevalent in developing countries than in developed countries. The epidemiological data on *Aeromonas* infection was first published in California in 1988, where the overall incidence of *Aeromonas* infections was about 10.5 cases per million people (King et al., 1992). In addition, the estimated incidence of *Aeromonas* bacteremia was 1.5 cases per million people in England in 2004 (Janda and Abbott, 2010), and approximately 1.6 cases per million in France in 2006 (Lamy et al., 2009). In Taiwan, the incidence of bacteremia by *Aeromonas* was about 76 cases per one million individuals between 2008 and 2010 (Wu et al., 2014). Moreover, in Japan, the incidence of traveler's diarrhea caused by *Aeromonas* was about 5.5 % of the patients (1265 out of 23215) who travelled to developing countries in 1997 (Yamada et al., 1997), and 2 % of the patients (18 of 863 patients) travelled to Africa, Asia and Latin America in Spain in 2003 (Vila et al., 2003). In Hong Kong, the incidence of *Aeromonas* infection was about 6.9 % among 130 outpatients with acute gastroenteritis in 2003 (Chan et al., 2003).

However, the role of *Aeromonas* in gastroenteritis has been controversial, mainly due to the non-fulfilment of Koch's postulates, the lack of an animal model, the low number of reported acute illness or outbreaks compared to other enteropathogens (Chu et al., 2006; Figueras et al., 2007b; Janda and Abbott, 2010). Critics have pointed out a lack of epidemiological data and evidence showing the specific linkage that *Aeromonas* cause human gastroenteritis. In fact, until now, there is no adequate

animal model for mimicking *Aeromonas* infections causing gastroenteritis (Tomás, 2012). Additionally, one human-challenging study failed to prove the causation of *Aeromonas* in gastroenteritis, where only 2 out of 57 humans experienced diarrheas after ingesting a high concentration of *Aeromonas* (Morgan et al., 1985). On the other hand, *Aeromonas* have frequently been isolated from the stool samples of patients, as well as from food and water for human consumption (Khajanchi et al., 2010; Pablos et al., 2011; Stratev et al., 2012; **Paper I**). *Aeromonas* have also been recognized as etiological agents in some cases of gastroenteritis or outbreaks (Ramalivhanae et al., 2010; Zhang et al., 2012; Ventura et al., 2015). These findings have proven the epidemiological linkage between the source of infection and clinical isolates. In addition, it is most probable that most cases of *Aeromonas* gastroenteritis with mild symptoms (or asymptomatic in some cases) have been unreported, or unidentified even with severe symptoms since the isolation and detection methods of *Aeromonas* are not well-established (Janda and Abbott, 2010). Furthermore, one study by Tenius and Figueras (2016) assessed the risk associated with the concentrations of *Aeromonas* found in different water and food matrix using dose-response experiments, suggesting that *Aeromonas* even with low concentration, is highly infectious to human like other enteropathogens such as *Campylobacter* or *Salmonella*.

4. *Aeromonas* as a foodborne pathogen and spoilage organisms

4.1. *Aeromonas* in ready-to-eat (RTE) seafood

In recent years, ready-to-eat (RTE) food has become a global trend with a growing demand for high-quality convenience food products among consumers with less time for cooking and meal preparation (Casini et al., 2015; Contini et al., 2016). Reflecting the current trend of consuming food perceived as healthier and more natural, consumption of raw or minimally processed RTE seafood such as sushi, sashimi, and cold-smoked fish products has been increasing around the world (Carlucci et al., 2015; Menozzi et al., 2020). According to the Commission Regulation (EC) No 2073/2005, RTE food or seafood is defined as "food or seafood intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level microorganism of concern". Fresh fish and seafood are highly perishable since they can be easily contaminated with a high level of microorganisms present in marine environments. As RTE seafood is consumed without a cooking process to kill potential microbial pathogens, it can become a potential vehicle of pathogens circulating in marine environments (Herrera et al., 2016). To overcome such microbiological challenges, various novel processing technologies have been developed for mild processing of seafood (Abel et al., 2022). Nevertheless, mild processing technologies combined with cold storage have been shown not to completely inhibit the growth of foodborne pathogens, such as *Listeria monocytogenes*, or *Aeromonas* spp. (EFSA Panel on Biological Hazards (BIOHAZ), 2018; Hoel et al., 2019; **Paper III**). Consequently, consumption of contaminated RTE seafood poses a potential food safety risk to consumers, and it is often considered the main source of foodborne illnesses (Lehel et al., 2020; EFSA and ECDC, 2022).

Aeromonas are natural inhabitants of aquatic environments such as fresh- and brackish water and marine, and thus, it is not uncommon that fish and seafood are contaminated with *Aeromonas* (Castro-Escarpulli et al., 2003, Di Pinto et al., 2012, Stratev et al., 2012; Yano et al., 2015). The occurrence of mesophilic *Aeromonas* spp. has also been observed in different types of water (Borrell et al., 1998; Pablos et al., 2011), and food, including meat (Neyts et al., 2000a), dairy products (Borrell et al., 1998; Tahoun et al., 2016), vegetables (McMahon and Wilson 2001; Xanthopoulos et al., 2010; Umutoni et al., 2020), and seafood (Ullmann et al., 2005; Di Pinto et al., 2012; Hoel et al., 2015; **Paper I**). In addition, their abilities to grow at refrigeration temperatures (4–10 °C) and produce enterotoxins have contributed to the concerns over *Aeromonas* as a foodborne pathogen (Pablos et al., 2011; Stratev et al., 2012; Hoel et al., 2018;2019). Moreover, *Aeromonas* have occasionally been identified as an etiological agent of foodborne illnesses associated with the consumption of oysters (Abeyta et

al., 1986), Swedish salad containing shrimp (Krovacek et al., 1995), frozen shrimp (Hänninen et al., 1997), salad ingredient (Zhang et al., 2012), and drinking water (Ventura et al., 2015).

Recently, more attention has been given to *Aeromonas* as a potential foodborne pathogen, particularly in connection to the consumption of raw and minimally processed RTE seafood (Hoel et al., 2019). The high prevalence of *Aeromonas* in RTE seafood has been reported in previous studies conducted in different geographical regions. In Germany, about 32 % (27/84) of the retail seafood, including raw seafood products, sushi, and smoked salmon were contaminated by *Aeromonas* spp., and *A. hydrophila* was the most frequently found species (Ullmann et al., 2005). A higher prevalence (70 %, 57/81) of *Aeromonas* in different types of RTE seafood including sushi, sea salad, surimi and peeled shrimp, was shown in a study conducted in Italy (Di Pinto et al., 2012). In addition, a high number (67 %, 20/30) of RTE seafood (temaki) was contaminated by *Aeromonas* in a Brazilian study (Teodoro et al., 2022). In South Korea, the highest prevalence (57 %, 47/91) of *A. hydrophila* was observed in raw oyster followed by sashimi, and RTE sushi (Park et al., 2021). In Norway, a high prevalence (70 %, 41/58) of *Aeromonas* was observed in retail sushi products, where mesophilic *A. salmonicida* was the most prevalent (Hoel et al., 2015;2017). In contrast to the previous results, a relatively low level (8 %, 12/144) of *Aeromonas* was observed in Norwegian RTE seafood (**Paper I**). It seems that the contamination level of *Aeromonas* in RTE seafood can vary dependent on the sampling sites and time, type and source of the products, isolation methods, seasonal and geographical variation. While the reported occurrence of *Aeromonas* in RTE seafood has generally been high, a combination of mild processing factors, such as low temperatures and high salt contents, commonly applied in the production of RTE seafood seemed not to guarantee the total inhibition of *Aeromonas* (**Paper III**, see chapter 4.2). Previously, the growth potential of the foodborne *Aeromonas* strain in a nigiri sushi model stored at cold temperatures has been demonstrated (Hoel et al., 2018). Furthermore, virulence-associated toxins genes of the *Aeromonas* isolated from RTE seafood have been characterised from several studies (Ullmann et al., 2005; Hoel et al., 2017; Park et al., 2021; Teodoro et al., 2022; **Paper I**), suggesting that they are potentially pathogenic. Some of the other major virulence factors detected in the *Aeromonas* genomes are likely to contribute to their pathogenicity (**Paper II**, see chapter 4.3).

4.2. The growth and spoilage potential of *Aeromonas* in RTE seafood

Food spoilage results from three mechanisms; autolysis, microbial activity, and chemical oxidation, rendering the product either unacceptable or undesirable for human consumption (Gram and Huss, 1996; Gram et al., 2002). Microbial growth and metabolism are by far the most common cause of food spoilage, and it is characterised by visible growth, textural changes, off-flavours, or off-odours (Gram

et al., 2002; Gram and Dalgaard, 2002). However, not all but specific spoilage microorganisms (SSOs) with spoilage potential and activity cause food spoilage (Gram and Huss, 1996, Gram and Dalgaard, 2002). The composition of spoilage microbiota depends on the initial microbiota of food, and several factors, *e.g*. processing conditions, packaging and storage condition, and microbial interactions, affect the growth of microorganisms in food (Boziaris and Parlapani, 2017).

In the food industry, hurdle technology combining a series of hurdles to challenge microbial growth, is primarily applied as a strategy to ensure food safety but also to prolong product's shelf life (Leistner, 2000). Mild processing technologies commonly used in the production of RTE seafood include chilling, salting, and smoking, and a combination of two or more mild processing technologies is applied as the hurdles to retain high nutritional and sensory value as well as to prevent the growth of spoilage organisms and potentially pathogenic microorganisms such as *Aeromonas* (Abel et al., 2022).

Like other bacteria, several intrinsic and extrinsic factors, such as temperature, water activity, and pH, influence the growth of *Aeromonas*. Clinical isolates of *Aeromonas* usually grow well at 20 to 35 °C, but most *Aeromonas* species are known to grow at a wide range of temperatures from 0 and 42 °C (Palumbo et al., 1985b). Some of the foodborne *Aeromonas* isolates from milk, chicken, meat, lettuce, and RTE seafood were able to grow under refrigeration (Palumbo et al., 1985a; Vivekanandhan et al., 2003; Hoel et al., 2018; Umutoni et al., 2020; **Paper III**), and thus refrigerated storage of the food product alone might not guarantee the total inhibition of some *Aeromonas* species. On the other hand, most heat treatments used during food processing could destroy the *Aeromonas* spp. (Palumbo et al., 1987).

In general, the higher concentration of sodium chloride (NaCl) is more effective in inhibiting the growth of *Aeromonas* spp., but the inhibitory effect of NaCl is temperature dependent (Delamare et al., 2000; **Paper III)**. Most foodborne *Aeromonas* isolates could tolerate 4 % NaCl at 28 °C, while only a few grew at 3 % NaCl at a lower temperature of 4 °C (Palumbo et al., 1985b). Likewise, some of the *Aeromonas* isolates from RTE seafood could tolerate the presence of 3.5 % NaCl at 4 °C, whereas their growth was suppressed with higher NaCl concentration (≥ 5.0 %) at 8 °C (**Paper III**). In another study, some *Aeromonas* could still grow well at 4.5 % of NaCl at 4 °C (Pin et al., 1996), and other *Aeromonas* can grow at the concentration of 4.0 % NaCl at 5 °C (Vivekanandhan et al., 2003). The combination of high salt contents and low temperature can limit *Aeromonas* growth (Gram, 1991; **Paper III**); however, the tolerance seems to be strain dependent. Moreover, *Aeromonas* is sensitive to low pH, and only a few *Aeromonas* strains could grow at pH 4.5 at 4 °C (Pin et al., 1996; Hoel et al., 2018). The effect of low pH is also dependent on the temperature (Hoel et al., 2018). The low pH was more effective in

inhibiting the growth of *Aeromonas* spp. when combined with low temperature and NaCl (Palumbo and Williams, 1992).

Cold-smoked salmon products are among the most popular RTE seafood, especially in Europe. Smoking is one of the oldest methods of preserving fish, and a traditional cold-smoking process includes salting, dehydration, and smoking, which establish combined hurdles (Leroi et al., 2000). Due to the potential health risk of polycyclic aromatic hydrocarbons (PAHs) from traditional wood smoking, the use of atomised purified condensate smokes (PCS) has been suggested as an alternative to producing healthier smoked fish products with increased sensory properties (Lingbeck et al., 2014; Valø et al.,2020; Waldenstrøm et al., 2021). PCS consist of several compounds, including phenols, organic acids, and carbonyls, and phenol concentration and pH of the smoke extract are considered important factors responsible for the antimicrobial effects in smoked products (Lingbeck et al., 2014). In previous studies, *Aeromonas hydrophila* was shown to be sensitive to smoke condensates derived from different types of wood (Sofos et al., 1988), and the antimicrobial effect of smoke condensate on *Aeromonas* spp. was demonstrated (Suñen et al., 2001; Suñen et al., 2003). Similarly, a recent study has demonstrated the antimicrobial potential of PCS against *Aeromonas* in liquid culture (**Paper III**); however, further studies are still needed in a real food matrix to confirm the antimicrobial effects of PCS on *Aeromonas.* In the same study, the growth of the same *Aeromonas* strains was inhibited when inoculated in a lightly salted cold-smoked salmon stored at low temperature but not in the fresh salmon stored at the same condition. It is likely that the combination of smoking, salting and low temperature might have established the effective hurdles to control the growth of the tested *Aeromonas* strains.

Packaging technology such as vacuum packaging (VP) or modified atmosphere packaging (MAP), in combination with refrigeration is often applied to extend the shelf life of food products including RTE seafood (Aberoumand and Baesi, 2020). Several studies have shown that VP in combination with refrigeration at 4 °C was insufficient to inhibit the growth of *Aeromonas* spp. in RTE salmon and trout products (Suñen et al., 2003; Jakobsen et al., 2020; Hoel et al., 2022; **Paper III**). On the other hand, MAP enriched with CO_2 has been shown to have a better antimicrobial effect on *Aeromonas* spp. (Devlieghere and Debevere, 2000; Jakobsen et al., 2020; Hoel et al., 2022). The study by Jakobsen et al. (2020) demonstrated significantly lower growth rates and maximum population density of *Aeromonas* in cold-stored RTE salmon packed in MA (60 % CO_2/40 % N_2) compared to VP. In addition, the growth of inoculated *A. salmonicida* in Atlantic cod was significantly inhibited when the cod was packed in an atmosphere consisting of 67 % CO_2/33 % O_2 or 33 % N_2 and stored at 4 °C, while VP was not sufficient to inhibit their growth (Hoel et al., 2022). Moreover, the study by Provincial et al. (2013)

has shown that the growth of *A. hydrophila* was only inhibited when sea bream fillets were packaged in 80 % CO_2/20 % N_2 at 4 °C, but not in 60 % CO_2/40 % N_2 or 70 % CO_2/30 % N_2.

Furthermore, *Aeromonas* have been identified as major spoilage bacteria in ice-stored sea salmon (*Pseudopercis semifasciata*) (Hozbor et al., 2006), ice-stored sea bream (Parlapani et al., 2013), MAP (50 % CO_2/50 % N_2) shrimp (*Penaeus vannamei*) (Macé et al., 2014), as well as spoiled grass carp (*Ctenopharyngodon idellus*) (Wang et al., 2014). In addition, *Aeromonas* were recognized as potential spoilage organisms in VP cold-smoked salmon (*Salmo salar* L.) stored at 8 °C (Leroi et al., 1998) and VP RTE salmon (*Salmo salar* L.) stored at 4 °C (Jakobsen et al., 2020). The spoilage potential of *Aeromonas* has been demonstrated by the production of different spoilage metabolites such as trimethylamine (TMA) in shrimp and salmon (Macé et al., 2014; Jakobsen et al., 2020), or biogenic amines in grass carp (Wang et al., 2014). Despite so, more knowledge is needed to explicate the actual role of *Aeromonas* as a spoilage organism in RTE seafood.

Predictive models of bacteria including *Aeromonas* have been developed to predict and control microbial growth in liquid media or food matrix, and these models are available in public database such as Combase (combase.cc). However, growth kinetic information regarding *Aeromonas* species is limited to certain species, such as *A. hydrophilia, A. cavaie,* and *A. sorbia*, in some of the food matrixes. A recent study has reported interspecies diversity in the growth pattern against different food processing factors in liquid cultures (**Paper III**), and such information is critical to predict their growth in certain processing conditions and develop the appropriate technology to avoid their growth in food. Considering the spoilage potential of *Aeromonas*, more information on the growth kinetic of *Aeromonas* species in the food matrixes is required for growth prediction of *Aeromonas* in RTE seafood and spoilage potential caused by *Aeromonas*.

4.3. Pathogenicity and virulence factors of *Aeromonas* in RTE seafood

Pathogenicity can be defined as the ability of bacteria to cause disease in a particular host (Casadevall and Pirofski, 1999). The term 'virulence' is often confused with the term pathogenicity. Virulence is a quantitative expression of pathogenicity, and it can be defined as the relative capacity of bacteria to cause damage to a host, or the degree of pathogenicity caused by the bacteria (Casadevall and Pirofski, 1999). The measurement of bacterial virulence has mostly been based on quantification outcomes on the host, such as the lethal dose required to kill 50 % of infected hosts, referred to as LD_{50}. (Casadevall, 2017). With the development of molecular microbiology, virulence can be assessed by identifying the genes encoding virulence factors such as toxins, secretion systems, or adhesins (Wassenaar and Gaastra, 2001). A subset of the genes that play important roles in bacterial pathogenicity are called virulence genes. While every gene involved in the pathogenicity might be considered a virulence gene,

some of the genes might be needed for the survival of the bacteria but might be not critical for the pathogenicity. Genes uniquely present in pathogenic strains can be defined as major virulence genes or virulence determinants causing pathogenicity (Wassenaar and Gaastra, 2001). Thus, information on the major virulence gene or virulence determinants in significant pathogens can help a better understanding of the virulence mechanisms of pathogens and the development of preventative and control measures for pathogens. Such information could be a basis to differentiate pathogenic and non-pathogenic strains in epidemiological studies, such as source tracking of foodborne outbreaks.

Considering the broad spectrum of disease caused by *Aeromonas* and various clinical manifestation that have been reported so far, *Aeromonas* pathogenicity is considered a multifactorial process, where a complex network of multiple virulence factors forms to fight the defense mechanisms of the host's immune system (Tomás, 2012; Fernández-Bravo and Figueras, 2020). Major virulence factors associated with *Aeromonas* pathogenicity have been reviewed by Tomás (2012) and Lowry et al. (2014), which can be divided into seven categories: (1) structural components: capsules, lipopolysaccharide (LPS), and S-layers, (2) motility: polar and lateral flagella, (3) adherence: Type I and IV pili, (4) secretion systems: type II secretion system (T2SS), type III secretion system (T3SS) and type VI secretion system (T6SS), (5) toxins: enterotoxins and exotoxins, (6) iron-binding systems, and (7) quorum sensing, and a schematic diagram of the major virulence factors is shown in **Figure 1**.

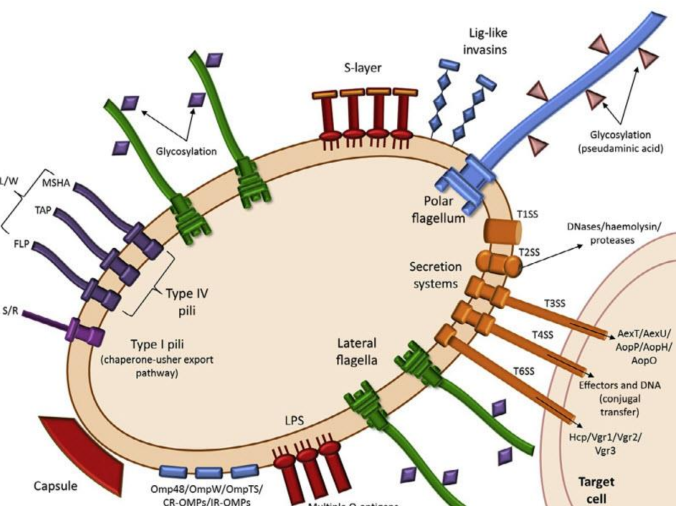

Figure 1. Schematic diagram showing the numerous structures associated or implicated in the aeromonad colonisation process. These include the motility organelles: the polar flagellum glycosylated with pseudaminic acid for swimming motility and lateral flagella for swarming motility over surfaces. For adherence to biotic and abiotic surfaces, type I and type IV pili (Flp, MSHA, Tap), outer-membrane proteins have been implicated. For protection from the host immune response, lipopolysaccharide (LPS), capsule and S-layers have been shown to be important. For the delivery of effectors that target host or bacterial cells, multiple types of secretion systems (T1SS, T2SS, T3SS, T4SS, and T6SS) are present. Adapted from Lowry et al. (2014).

In the last two decades, the pathogenicity and virulence of *Aeromonas* have experimentally been studied using *in vitro* cell lines and different animal models to identify the major virulence factors (Fernández-Bravo and Figueras, 2020). Available information on the major virulence factors of *Aeromonas* is primarily based on the molecular characterisation of virulent *A. hydrophila* strains, particularly SSU and AH-3, which have been reclassified as *A. dhakensis* SSU and *A. piscicola* AH-3 (Rasmussen-Ivey et al., 2016). It is worth noting that this reclassification has raised the question of the applicability of previous research on *A. hydrophila* virulence mechanisms, and the virulence factors of *A. hydrophila* has been clarified by Rasmussen-Ivey et al. (2016). Some of the virulence factors such as toxin genes (*aerA*, *act*), secretion system (T3SS, T6SS), and enzymes have been considered as the virulence markers of *Aeromonas*; however, there are no ultimate virulence markers that separate pathogenic strains from the non-pathogenic strains (Tomás, 2012; Rasmussen-Ivey et al., 2016).

Among several virulence factors described so far, the production of toxins such as hemolysin, aerolysin, or cytotoxic enterotoxin is probably the most well-studied virulence factors. The presence of virulence-associated toxin genes in the *Aeromonas* genome does not directly imply that they are pathogenic to cause clinical symptoms of an infection; however, there is some evidence showing the correlation between the isolates harbouring toxin genes and their virulence traits (Albert et al., 2000; Sha et al., 2002; Ottaviani et al., 2011). Screening specific toxin genes has been suggested as an effective and practical way of characterising the virulence of *Aeromonas* strains (Wang et al., 2003), rather than animal-based studies which are currently outdated. Several studies have evaluated the pathogenic potential of *Aeromonas* strains by PCR-based methods targeting the virulence-associated toxin genes such as *alt* and *ast* (cytotonic enterotoxin), *act* (cytotoxic enterotoxin), *aerA* (aerolysin), *hlyA* (hemolysin), or *stx-1* and *stx-2* (Shiga-like toxins) (Pablos et al., 2011; Hoel et al., 2017; Umutoni et al., 2020; **Paper I)**. Not only toxins, but some of the recent studies have also included the genes encoding other virulence factors such as T3SS (*aopB, ascV, aexT*), T6SS (*traJ*), polar flagella (*flaA, flaB*), lateral flagella (*lafA*), lipase (*lip*), serine protease (*ser*), DNase (*exu*), or elastase (*ahp*), as the virulence markers of *Aeromonas* (Ahangarzadeh et al., 2022; Qu et al., 2022; Xu et al., 2022; Youssef et al., 2022). Using a few target genes as markers can be a practical approach to assess the pathogenic potential, while such information is limited to the selected virulence factors.

More recently, the development of WGS technology and WGS databases such as the virulence factor database (VFDB), has enabled a more comprehensive analysis of virulence factors. By simply uploading bacterial genomes of interest to the VFDB, the overall virulence gene profile of the bacteria can be obtained to predict the presence of virulence determinants in the bacterial genomes (Liu et al., 2022). Currently, the VFDB covers 32 genera of well-studied bacterial pathogens, including *Aeromonas*. In this database, information on all known virulence factors of *Aeromonas* has been collected based on

the comparative pathogenomics of four reference genomes, including *A. hydrophila* ML09-119, *A. hydrophila* subsp*. hydrophila* ATCC 7966, *A. salmonicida* subsp*. salmonicida* A449, and *A. veronii* B565. Recently, VFDB has been used as a basis by several studies to examine the virulence gene profile of several *Aeromonas* species for the prediction of their pathogenic potential (Tekedar et al., 2019; Dubey et al., 2022b; Vasquez et al., 2022; Abdella et al., 2023; **Paper II**). For instance, the study by Tekedar et al. (2019) has determined the pathotype of *A. veronii* isolated from aquaculture. In the study by Dubey et al. (2022b), the virulence gene profile of *A. media* strain isolated from marine sediments was characterised in comparison with the profile of the reference strains in the VFDB. The study by Vasqueze et al. (2022) reported that the major difference between typical and atypical *A. salmoncida* strains isolated from fish was related to the virulence factors such as T3SS, effectors and toxins. In the study by Abdella et al. (2023), the pathogenic potential of several *A. hydrophila* strains from various sources were investigated based on comparative pathogenomics. To our knowledge, no comparative genome studies based on whole genomes have been dedicated to investigating the virulence factors of foodborne *Aeromonas* isolates.

In **Paper II,** the virulence gene profiles of seafood-borne *Aeromonas* strains representing eight different species were compared with the profiles of the reference strains. The potential role and function of each virulence factor in virulence mechanisms of the *Aeromonas* strains were discussed, and most of the *Aeromonas* isolated from RTE seafood were considered potentially pathogenic to contain several genes encoding the major virulence factors, particularly high prevalence of toxin genes, T6SS and its effectors. In general, *A. piscicola, A. bestiarum, A. salmonicida, A. hydrophila and A. dhakensis* strains contained more virulence factors than other strains representing *A. media, A. rivipollensis,* and *A. caviae*, implying higher chance of those strains being virulent. Some virulence genes such as *aerA* and *ahh1*, seemed less prevalent in the *A. media, A. rivipollensis,* and *A. caviae* strains*.* Among them, the *A. piscicola* strain had the most virulent profile containing T3SS (without effectors), T6SS, lateral flagella, and all kinds of examined toxins except for the repeat toxins (RTX). On the contrary, the *A. caviae* strain seems less virulent than others, containing the lowest number of virulence factors.

5. Antimicrobial resistance (AMR) of *Aeromonas*

5.1. The emergence of AMR in the food chain

Since the first antibiotic, penicillin, was discovered in 1928 by Alexander Fleming, antimicrobials including antibiotics, antifungals, and antivirals have been essential medicines to prevent and control microbial infections in humans and animals (Kumar et al., 2020). Through the process of Darwinian selection, microorganisms can adapt to new environments exposed to different antimicrobials, and the emergence of AMR in microorganisms is a natural phenomenon (Holmes et al., 2016; Mcewen and Collignon, 2018). Bacteria can be intrinsically resistant to certain antimicrobials by phenotypic expression of pre-existing ARGs, leading to intrinsic or natural resistance (Jian et al., 2021). On the other hand, bacteria can acquire new resistance mechanisms by obtaining new ARGs, called acquired resistance (Holmes et al., 2016; Jian et al., 2021). The acquisition of the ARGs occurs after horizontal gene transfer (HGT), and HGT is mainly driven by MGEs such as plasmids, transposons, and integrons (Piotrowska and Popowska, 2015; Holmes et al., 2016). A pool of genes located in MGEs are considered flexible and transferrable so those genes can be transmitted between and within environments, animals, and humans (Piotrowska and Popowska, 2015). Three main mechanisms of HGT include conjugation (transfer of DNA between two bacterial cells via direct contact), transformation (uptake of free DNA in competent bacteria), and transduction (a bacteriophage-mediated transfer of DNA) (Thomas and Nielsen, 2005).

According to the European Food Safety Authority (EFSA and ECDC, 2023), "AMR is defined as the inability or reduced ability of an antimicrobial agent to inhibit the growth of a bacterium, which, in the case of a pathogenic organism, can lead to therapy failure". AMR is a global public health threat facing humanity with multiple and interconnected drivers (WHO, 2014; 2021). Global action is needed within the *One Health* approach, a collaborative effort of multiple health science professionals to attain optimal health for people, animals, and environments (Mcewen and Collignon, 2018; Velazquez-Meza et al., 2022). One of the important drivers of AMR is the over- and misuse of antimicrobial drugs for humans, animals and environments, and modern selective pressure given by antimicrobial usages in multiple sectors has resulted in the spread of ARB and ARGs locally and globally (Mcewen and Collignon, 2018; WHO, 2021). In fact, antimicrobials have not only been used for therapeutic purposes in humans and animals, but also for prophylactic or metaphylactic purposes in livestock farming, aquaculture, and agricultural crop production (Founou et al., 2016; Mcewen and Collignon, 2018). In addition, antimicrobials have been used for growth-promoting purposes in livestock or aquaculture farming at an industrial scale (WHO, 2014; Mcewen and Collignon, 2018). The European Union (EU) banned the usage of antimicrobials as growth promotors in 2006, while substantial amounts of

antibiotics are still used in agricultural and food animal production in many Asian and African countries (Founou et al., 2016).

The emergence and transmission of AMR in the food chain is a complex pathway and a cross-sectional challenge, representing a severe risk to the exposed workers and consumers (Founou et al., 2016; 2021). In principle, frequent usage of antibiotics along the food chain can result in multiple reservoirs of ARB and ARGs at each stage of the food production chain. Food animals, fish, and vegetables are considered large reservoirs of ARB and ARGs (EFSA Panel on Biological Hazards (BIOHAZ), 2021). These reservoirs of resistance can be transmitted to humans via two major routes: (1) by direct contact with the contaminated food producing animals or human carriers, and (2) indirectly via post or cross-contamination with antibiotic residues in the food chain or through exposure to highly contaminated ecological niches in the wider environments (hospitals, agricultural run-off, or wastewater) (Economou and Gousia, 2015; Founou et al., 2016) (**Figure 2**). Consumption of contaminated food or food-derived products is a potential transmission route of ARB and ARGs, where food may act as a vector for transferring AMR to humans (Verraes et al., 2013; Economou and Gousia, 2015). Particularly, raw food products can hold a substantial risk for transferring AMR to humans, if these products are consumed without further processing that might kill ARB and ARGs (Verraes et al., 2013; Schar et al., 2021). For example, the prevalence of ARGs in RTE salad and their potential impact of transferring ARGs to consumer has been highlighted (Zhou et al., 2020).

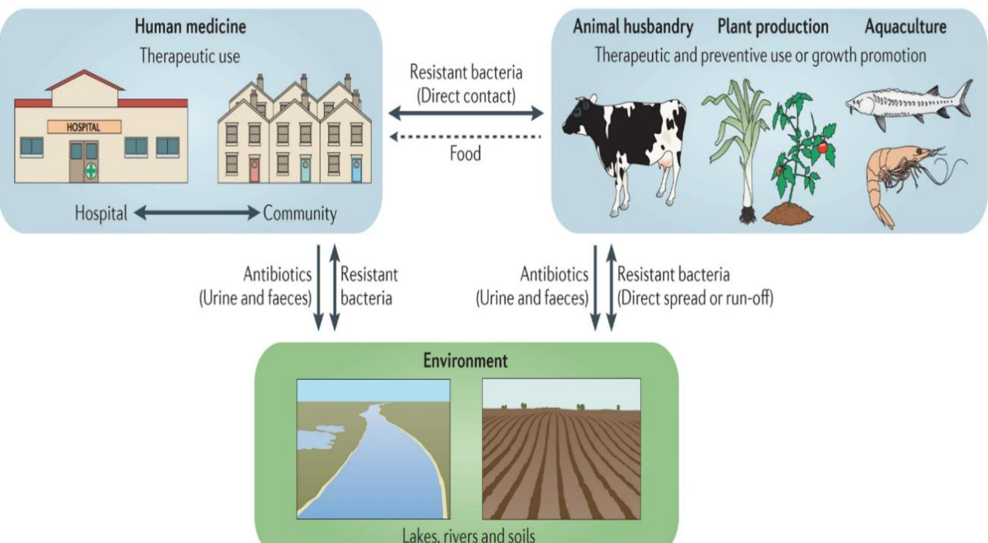

Figure 2. An overview of antibiotic use and transmission routes of AMR between different environments, such as medical environments, livestock and agricultural production, aquaculture, and the wider environments, showing the major transmission routes of AMR in the food chain. Adapted from Andersson and Hughes (2014).

The excessive usage of antibiotics in food animal production has been criticised as a major contributor to the emergence of AMR (Economou and Gousia, 2015). The occurrence of ARB and ARGs in food animal and derived food products has been reported as a potential threat to public health from several studies, including multidrug resistant or extended-spectrum cephalosporin-resistant *Salmonella* in poultry (Castro-Vargas et al., 2020), multidrug resistant *Campylobacter* spp. in poultry (Yang et al., 2019), extended-spectrum β-lactamase (ESβL) producing *Escherichia coli* in pig farm (Fischer et al., 2012) or in minced meat, raw milk (Geser et al., 2012), and methicillin-resistant *Staphylococcus aureus* (MRSA) in livestock (Vanderhaeghen et al., 2010; Cuny et al., 2015). Moreover, the prevalence of ARB and ARGs present in fresh produce, including vegetables, fruits, sprouts consumed raw, and RTE salad, has systematically been reviewed by Rahman et al. (2021), and the most frequently observed bacteria was *E. coli* (42.5 %), followed by *Klebsiella* spp. (22.5 %), and *Salmonella* (20 %). In the EU, monitoring and reporting of AMR is mandatory in *Salmonella*, *C. coli*, *C. jejuni* and indicator *E. coli*, in food animals and their derived meat since 2021, and further characterisation is required for *E. coli* and *Salmonella* isolates showing resistance to extended-spectrum cephalosporins and carbapenems (EFSA and ECDC, 2023).

The status of antimicrobial usage in aquaculture in different countries and their regulations has been summarised by Lulijwa et al. (2019). Particularly in Norway, a strict policy on antimicrobial usage in aquaculture together with effective vaccination, has significantly decreased the antimicrobial usage in aquaculture since the top in 1987 (Lulijwa et al., 2019; NORM/NORM-VET, 2021). However, a large proportion of world aquaculture production is carried out in developing countries, where there is a lack of regulation or system to monitor antimicrobial usage (Founou et al., 2016; Watts et al., 2017). The antimicrobial usage in aquaculture has more likely contributed to the emergence and transmission of ARB and ARGs in aquatic environments (Preena et al., 2020). Aquaculture system and fish farms are considered the hotspots for ARGs, and fish are considered large reservoirs of resistance in the food chain (Watts et al., 2017). Resistant pathogens or antibiotic residues in aquaculture could easily pass into the marine environment, and they have a negative impact on humans and animals by transferring ARGs (Buschmann et al., 2012). The occurrence of ARB such as *Aeromonas* spp., *Vibrio* spp., and Enterobacteriaceae as well as multiple ARGs has been observed in aquaculture, cultured fishes, and crustaceans (Preena et al., 2020; Schar et al., 2020). In addition, previous studies have shown that bacteria from marine and terrestrial environments can share antimicrobial resistant determinants for quinolone and tetracycline, and they are most likely of aquatic bacterial origin (Rhodes e al., 2000; Cattoir et al., 2008; Cabello et al., 2013).

Not only the antimicrobial usage in primary production, but selective pressure given by the wide use of biocides or heavy metal containing products in the food production chain or in the environment

has also contributed to the development of AMR and co-resistance (Romero et al., 2017; Donaghy et al., 2019). Biocides including sanitisers, disinfectants, and cleaning agents are commonly used in the food production chain for food hygiene assurance (Donaghy et al., 2019; Jones and Joshi, 2021). Inappropriate usage of biocides may give selective pressure on bacteria, contributing to the co-selection process of bacteria for antibiotic resistance (Jones and Joshi, 2021). In addition, heavy metals containing products such as feed additives, fertilizers, pesticides, and anti-fouling products are widely used in different settings in agricultural and aquaculture practices (Han et al., 2002; Burridge et al., 2010). The frequent exposure to both heavy metals and antimicrobials may increase the chance of selection and co-selection of AMR (Seiler and Berendonk, 2012).

Furthermore, multidrug resistant pathogens such as *Campylobacter, Salmonella*, and *Shigella*, transmitted in the food chain can be a serious threat in the food industry, as they could contribute to the emergence and transmission of more resistant, pathogenic, and mobile bacterial strains, which could not be treated with existing antibiotics (Doyle, 2015; Pérez-Rodríguez and Taban, 2019). The study by Pérez-Rodríguez and Taban (2019) has critically reviewed the current knowledge on multidrug resistant foodborne pathogen, and they pointed out that the food chain can act as a 'resistances' modulator' to reduce the incidence of ARB by controlling food process parameters. They suggested that risk factors and transmission routes in the food chain can be identified based on the combination of WGS technology and predictive microbiology.

5.2. *Aeromonas* as a vector of AMR in the food chain

The ubiquitous nature of *Aeromonas* in the environments and their ability to acquire and exchange genetic materials make *Aeromonas* the multifaceted middleman in the *One Health* world (Lamy et al., 2022). Antimicrobial resistant *Aeromonas* have been isolated from virtually any types of water sources, including freshwater (Esteve et al., 2015; Baron et al., 2017), estuarian environments (Chaix et al., 2017), drinking water (Kivanc et al., 2011; Pablos et al., 2011), wastewater (Figueira et al., 2011; Piotrowska et al., 2017), and aquaculture (Rhodes e al., 2000; Schmidt et al., 2001). The occurrence of ARGs among the *Aeromonas* species in aquatic environments has frequently been reported (Piotrowska and Popowska, 2014; Piotrowska et al., 2017), showing that *Aeromonas* are the important reservoirs of ARGs in various water sources. Obviously, they can acquire and exchange ARGs located in MGEs by HGT (Piotrowska and Popowsk, 2015), and the ability of *Aeromonas* to form biofilm may facilitate the transfer of genetic materials (Talagrand-Reboul et al., 2017a). Therefore, *Aeromonas* has been suggested as indicator bacteria for monitoring AMR in aquatic environments (Patil et al., 2016; Usui et al., 2016; Baron et al., 2017; Gomes et al., 2021).

In aquatic environments, generated resistance can be easily distributed locally as well as globally via different routes such as water discharge and fish consumption (Schar et al., 2021). Thus, it is not unusual to find the high prevalence of multidrug resistant *Aeromonas* in fish or seafood in various geographical regions (Yano et al., 2015; Dahanayake et al., 2020; Hossain et al., 2020; Dhanapala et al.,2021; **Paper I**). In a meta-systematic study of AMR in marine bivalve, the genus *Aeromonas* showed the highest prevalence of AMR followed by *Vibrio* spp. *Salmonella*, and *E. coli* (Albini et al., 2022). In addition, multidrug resistant *Aeromonas* has been found in other types of food, including meat, dairy products, and vegetables (Stratev and Odeyami, 2016). These findings have suggested that fish, seafood as well as various food products can be potential reservoirs of resistant *Aeromonas* in the food chain.

Moreover, several studies have reported the presence of MGEs in the *Aeromonas* isolated from aquatic environments, or fish and their association with resistant determinants (Cattoir et al., 2008; McIntosh et al., 2008; del Castillo et al., 2013; Dubey et al., 2022a; Song et al., 2023). In addition, a recent study has shown the potential role of foodborne *Aeromonas* as a vector of AMR in the food chain (**Paper II**). In **Paper I**, the high prevalence of multidrug resistant *Aeromonas* strains was reported for the first time from the RTE seafood, especially in retail sushi products, on the Norwegian market. The occurrence of multidrug resistant pathogen like *Aeromonas* in raw fish or seafood for direct consumption can represent a potential risk of pathogen transmission (Schar et al., 2021). A further study in **Paper II** has shown that most of multidrug resistant *Aeromonas* carried multiple ARGs (mostly β-lactams). In addition, one strain carried a quinolone resistance gene *qnrS2* in a plasmid, and another strain had a transposon carrying mercury and class I integron carrying two ARGs, implying their potential to disseminate their ARGs to other species in the food chain.

5.3. Antimicrobial susceptibility testing (AST) and susceptibility patterns of *Aeromonas*

Considering the AMR issue, AST of significant bacterial pathogens becomes an important task to determine the susceptibility of pathogens against antibiotics and to detect possible drug resistance in common pathogens, for effective treatments of infectious diseases (Jorgensen and Ferraro, 2009; Salam et al., 2023). Phenotypic AST focuses on a set of observable characteristics of microbes against the group of selected antimicrobial agents, while genotypic AST is based on the detection of resistance genes present in microbes' genomes as resistance determinants associated with resistant phenotypes (Salam et al., 2023). Conventional phenotypic AST methods include disc diffusion, broth dilution, and gradient diffusion, which usually require a few days for diagnostics of AMR (Jorgensen and Ferraro, 2009; Idelevich and Becker, 2019). For rapid diagnostics of AMR, automated AST or MALDI-TOF MS-based AST methods have recently been introduced (Idelevich and Becker, 2019; Benkova et al., 2020).

In addition, novel methods using microfluidics or microdroplets systems seem promising for rapid phenotypic AST (Benkova et al., 2020; Zhang et al., 2020). Besides, genotypic AST methods are based on PCR-based amplification of target genes, or sequence-based methods such as DNA microarray or DNA chips (Salam et al., 2023). More recently, WGS-based AST (see more information in **Chapter 5.5**), in combination with bioinformatic tools, has facilitated our ability to explore the AMR, and several studies have shown the potential of using WGS for accurate predication of the genetic determinants strongly associated with resistant phenotypes in the genome of microbes (Hendriksen et al., 2019; Maboni et al., 2022; **Paper II**).

Even with the development of modern diagnostics methods, disk diffusion tests and broth microdilution are still the most widely used methods for the determination of phenotypic resistance (Idelevich and Becker, 2019). The guidelines on the performance of AST using disk diffusion and broth microdilution methods have been published by the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and these guidelines are globally used as references for interpretative breakpoints zone diameters and minimal inhibitory concentration (MIC) values for different bacterial groups and antimicrobial agents (Idelevich and Becker, 2019; Salam et al., 2023). In the disk diffusion method, the zone of growth inhibition around each antibiotic disk is measured and interpreted as resistant, intermediate, and susceptible according to the interpretative breakpoints published by the CLSI or EUCAST (CLSI, 2016; EUCAST, 2023). This method has great advantages of simplicity in the performance and flexibility in the choice of drug to test with the availability of commercial antibiotic disks (used in **Paper I**), while there is a possibility of performance or interpretation error (Jorgensen and Ferraro, 2009; Idelevich and Becker, 2019). In broth microdilution, bacterial growth is tested against different concentrations of antibiotics, and the lowest concentration of antibiotic that prevented growth represents the MIC (CLSI, 2016; EUCAST, 2023). This method has the advantage of obtaining both quantitative and qualitative information on antimicrobial susceptibility; however, the preparation of antibiotic solutions at different concentrations can be tedious and time-consuming task for rapid diagnostics (Jorgensen and Ferraro, 2009; Idelevich and Becker, 2019). Despite of some limitations, both methods are cost-effective and reliable when performed according to the guidelines, and unlike genotypic methods, information on resistance and susceptibility can be predicted simultaneously, when using dilution methods (Jorgensen and Ferraro, 2009; Salam et al., 2023).

For mesophilic *Aeromonas* spp., current guidelines published by the CLSI or EUCAST are made mostly based on the most clinically relevant *Aeromonas* species such as *A. hydrophila* complex*, A. caviae* complex, and *A. veronii* complex, while there is one specific guideline published by CLSI for the susceptibility testing of psychrophilic *A. salmonicida* (CLSI, 2014;2016; EUCAST 2023). It is unclear

whether those profiles can be extrapolated to other less significant *Aeromonas* species (Janda and Abbott, 2010, Chen et al., 2012), and the interpretative breakpoints for some antimicrobial agents are not provided in these guidelines. Nevertheless, these guidelines have widely been used as a reference to perform AST and examine the susceptibility patterns of other *Aeromonas* species originating from different sources such as water and seafood (Dahanayake et al., 2020; Hossain et al., 2020; Dhanapala et al.,2021; **Paper I**).

Previous studies have suggested that the resistance patterns of *Aeromonas* are species-specific (Fosse et al., 1996; Chen et al., 2012). Considering the diversity of the *Aeromonas* and strain variation, antimicrobial susceptibility patterns of the *Aeromonas* spp. can vary. In general, *Aeromonas* spp. are intrinsically resistant to ampicillin except for *A. trota*. *Aeromonas* are also resistant to other penicillins, but not to penicillins with extended-spectrum such as mecilinams (Janda and Abbott, 2010; **Paper I**). *Aeromonas* are generally resistant to first generation cephalosporin, but rather sensitive to higher generation cephalosporin (Yano et al., 2015; Dahanayake et al., 2020). However, a high resistance rate (69.6 %) toward third- and fourth generation cephalosporins has been observed in *Aeromonas* isolated from aquatic animals in Asia (Schar et al., 2021). In addition, resistance or reduced susceptibility toward cefotaxime and ceftriaxone was observed in *Aeromonas* isolated from RTE seafood (**Paper I**).

Aeromonas are somewhat sensitive to carbapenems; however, recent studies have shown the high occurrence of carbapenem-resistant *Aeromonas* strains from seafood sources (De Silva et al., 2019; **Paper I**). The high prevalence of carbapenem resistant *Aeromonas* from aquatic animals was reported in Asia (Schar et al., 2021), and other countries such as Colombia and Australia (Sinclair et al., 2016; Rosso et al., 2019). Increased resistance of *Aeromonas* spp. to β-lactam antibiotics (e.g. penicillins, cephalosporin, or carbapenems) should carefully be taken as a major issue due to the activity of inducible β-lactamase (Janda and Abbott, 2010, Fernández-Bravo and Figueras, 2020). Moreover, the occurrence of erythromycin-resistant *Aeromonas* strains, or trimethoprim-sulfamethoxazol resistant *Aeromonas* from seafood sources has been reported (Yano et al., 2015, Odeyemi and Ahmad, 2017; De Silva et al., 2018;2019; **Paper I**). A high resistance rate to tetracycline, florfenicol, or oxolinic acid has been observed in the *Aeromonas* from aquatic environments (Esteve et al., 2015; De Silva et al., 2018; Syrova et al., 2018; **Paper I**).

5.4. Multidrug resistance

The susceptibility profiles of bacteria have often been a subject of investigation to determine their multidrug resistance and evaluate their potential risk to public health. In literature, multidrug resistance generally means 'resistant to more than one antimicrobial agent'. However, the definition of multidrug resistance is vague, and no consensus on the definition of multidrug resistance has been

made yet until now (Falagas and Karageorgopoulos, 2008; Magiorakos et al., 2012). A group of international experts has proposed the definition of multidrug resistance as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). This definition has been applied by several studies (Sweeney et al., 2018; Wolfensberger et al., 2019; **Paper II**). Recently, Rafailidis and Kofteridis (2022) proposed to add that this non-susceptibility (in the current definition) is at most to the total number of all antimicrobial categories minus two, so that the definition reads: multidrug resistance is defined as non-susceptibility to at least one agent in three or more antimicrobial categories and up to (and including) the total number of all antimicrobial categories minus two.

In addition, the study by Magiorakos et al. (2012) critically reviewed the issues of multidrug resistance definitions. The main issues were based on how to create and select antimicrobial categories, how to select agents to be tested, and lastly, how to define antimicrobial resistance within the antimicrobial categories. According to the guidelines published by the CLSI, EUCAST and FDA, the list of antimicrobial categories and agents to be tested has been proposed for some of the significant bacteria in public health, such as *Staphylococcus aureus,* and *Enterococcus* spp. However, there is no proposed or established lists exist for other less significant bacteria such as *Aeromonas* spp.

Furthermore, multiple antibiotic resistance (MAR) index has been suggested as a cost-effective and valid method that used in source tracking of ARB from previous studies (Sandhu, 2016; Ayandele et al., 2020). MAR indexing was first introduced for the source tracking of faecal contamination of food from the previous study by Krumperman (1983). They suggested that MAR index can be defined as the ratio between the number of antibiotics to which a particular isolate is resistant, and the total number of antibiotics tested for the isolate. MAR index values greater than 0.2 indicate a high risk of contamination where antibiotics are frequently used, and these isolates are considered multidrug resistant. Some studies have used MAR index to determine multidrug resistant strains of *Aeromonas* (Odeyemi and Ahmad, 2017; Dahanayake et al. 2020; **Paper I**).

5.5. Antimicrobial Resistant Genes (ARGs) detection and database issues

Information on the distribution of ARGs and MGEs in bacterial genomes is critical to assess the potential risk of AMR to human health (Martínez et al., 2015; Zhang et al., 2022). In the last few years, research has focused on targeting a small fraction of ARGs using PCR-based methods to predict the resistance determinants of certain bacteria (De silva et al., 2018; Dahanayake et al., 2020). The development of next-generation sequencing (NGS) technologies has enabled a more comprehensive analysis of the distribution of ARGs and MGEs present in bacterial genomes, which becomes a powerful tool for understanding the spread and emergence of AMR (Hendriksen et al., 2019; Zhang et

al., 2021). This approach is also called WGS-based AST, as stated in **Chapter 5.3**. The ARG detection requires reliable annotation databases of the sequenced genomes accompanying phenotypic susceptibility data (Hendriksen et al., 2019). According to the study by Hendriksen et al. (2019), at least 47 online databases have been developed for ARGs detection including ResFinder (Zankari et al., 2012; Bortolaia et al., 2020) (used in **Paper II**), Comprehensive Antibiotic Research Database (CARD) (McArthur et al., 2013; Alcock et al., 2023), Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) (Gupta et al., 2014), ABRICATE (Seeman, 2016), and NCBI AMRFinder (Feldgarden et al., 2019;2021) (used in **Paper II**). In addition, some databases have particularly been designed for the detection of MGEs, *e.g.* PlasmidFinder (Carattoli et al., 2014), MobileElementFinder (Johansson et al., 2021) (used in **Paper II**).

Among several databases, ResFinder, CARD, and NCBI AMRFinder are probably the most widely used databases that regularly updated. However, the outputs from these databases often disagree with each other (Maboni et al., 2022; Papp and Solymosi, 2022). ResFinder focuses on acquired resistance genes, while CARD and AMRfinder covers both acquired resistance genes and point mutations. AMRfinderPlus, the most recently developed database, also includes the stress, heat, and biocide resistance and virulence factors of some organisms (Feldgarden et al., 2021). Both ResFinder and CARD are easily accessible through a web-interface, whereas some bioinformatic skill is required to have access to AMRfinder database since it is exclusively accessible by a Linux-operating system. A recent paper by Maboni et al. (2022) evaluated the efficiency of three databases (AMRfinder, Resfinder, and CARD) by comparing the ARGs profiles. They suggested that AMRFinder and ResFinder provided easy output interpretation and a lower number of overcalled ARGs, compared to the CARD, which gave many false positive results probably due to the less stringent cut-off thresholds. In **paper II**, the ARGs profile obtained by three databases was also compared and the final gene profile was obtained from both AMRfinder and ResFinder. On the contrary, CARD has been suggested as the most appropriate database for prediction of both mutation and acquired resistance genes, when the outputs from six databases were compared (except for AMRFinder) by Papp and Solymosi (2022). Thus, it is critical to compare the outputs from at least two databases to accurately identify AGRs in bacterial genomes for research.

Moreover, both ResFinder and CARD have shown a high sensitivity (> 97 %) to correctly identify ARGs associated with resistance phenotype, as well as a high specificity (> 98 %) to correctly identify the absence of ARGs related to susceptible phenotype for some foodborne pathogen like *Salmonella enterica*. (McArthur et al., 2013; Bortolaia et al., 2020). AMRFinder has also shown a high consistency (98.4 %) between predicted genotype and resistance phenotype for *S. enterica*, *Campylobacter* spp., and *E. coli* (Feldgarden et al., 2019). Accordingly, AMRFinder and ResFinder showed a good prediction

of resistance phenotypes for *E. coli* strains (Maboni et al., 2022). On the contrary, some discrepancies were observed between pheno- and genotypes for *Aeromonas* strains using AMRFinder (**Paper II**). Previous studies have suggested correlation between pheno- and genotypes depends on the type of antibiotics, the bacterial species tested, and the associated mechanism of resistance (Maunsell et al., 2021). In fact, the efficiency of these tools has been assessed mainly using the data of some foodborne pathogens, and thus there is no definite evidence of its applicability to other bacteria. Besides, incorrect data on phenotypic AST is often the reason for such discrepancies (Hendriksen et al., 2019). Zankari et al. (2013) showed that repeating phenotypic AST could resolve some discrepancies found between pheno- and genotypes predicted using ResFinder. Moreover, clinical breakpoints established by the CLSI or EUCAST do not always correspond to the presence or absence of resistance genes (Tyson et al., 2017). Considering current database issues, more standardisation of ARGs detection pipelines and databases, as well as additional phenotypic data on bacterial species other than foodborne pathogens, are required for its broader application and possible transition of AMR diagnostic using WGS-based approach (Hendriksen et al., 2019; Maboni et al., 2022).

6. Conclusions and future perspectives

This work has summarised the existing knowledge and the most recent findings on mesophilic *Aeromonas* present in Norwegian RTE seafood, with respect to AMR, virulence factor, and growth kinetics. First, this work has demonstrated the occurrence of mesophilic *Aeromonas* in different types of Norwegian RTE seafood and confirmed the power of using both MLPA and ANI analysis for correct identification of *Aeromonas* at the species level. *Aeromonas* strains present in RTE seafood were considered potentially pathogenic by carrying some of the virulence-associated toxin genes as well as other genes encoding virulence factors related to adherence, motility, secretion systems and toxins. In addition, this work has revealed the occurrence of multidrug resistant *Aeromonas* present in RTE seafood and the presence of multiple ARGs in their genomes, and particularly, those strains carrying ARGs in their MGEs can potentially act as a vector of transferring ARGs to other bacteria species and possibly to humans. Moreover, this work has shown that mild food processing factors that commonly applied in the production of RTE seafood may not guarantee the total inhibition of potentially pathogenic and multidrug resistant *Aeromonas*. Cold smoking, particularly PCS, is suggested as a promising strategy to prevent and control the growth of *Aeromonas,* while further studies are still needed to evaluate and confirm the antimicrobial effects of PCSs against *Aeromonas* in actual seafood matrixes. These findings suggest that consumption of RTE seafood with potentially pathogenic and multidrug resistant *Aeromonas* may be a risk factor to the frequent consumers of RTE seafood, and it can be a potential transmission route of AMR (or ARGs) from aquatic animals or environments to humans. Furthermore, *Aeromonas* circulating in the food chain could be potential reservoirs of both ARGs and virulence genes, and they might play a significant role in the emergence and transmission of multidrug resistant pathogens in the food chain. Thus, it is important to collect more information on virulence genes, ARGs and MGEs in *Aeromonas* residing in the food chain as well as to carefully monitor the AMR caused by *Aeromonas* under the *One Health* perspective. Additionally, to prevent and control the growth of *Aeromonas* in RTE seafood, more studies are needed to expand our knowledge on growth prediction as well as production of spoilage metabolites under mild processing factors in RTE seafood. Such information can be useful to optimise current mild processing technology and to develop appropriate technology to prevent the spoilage caused by *Aeromonas* and to ensure food safety.

Besides, in this work, the development of WGS technology and WGS-based databases such as VFDB or AMRFinder, has enabled us to perform a comprehensive analysis on virulence factors and AMR of *Aeromonas*. Nevertheless, current knowledge on virulence factor of *Aeromonas* are rather limited to clinically significant species, such as *A. hydrophila*. It is still challenging to differentiate the pathogenic strains from non-pathogenic strains, without ultimate virulence determinants to define a truly virulent

strain. Further studies should aim to collect more information on distribution of virulence factors in pathogenic *Aeromonas* strains and to investigate novel virulence factors and regulatory effects, for a more extensive evaluation of *Aeromonas* pathogenicity. Moreover, some discrepancies found between phenotypic resistance and predicted genotype resistance profiles suggested the limitation of relying on current WGS-based databases for the prediction of AMR among *Aeromonas*. A comprehensive analysis on AMR of *Aeromonas* can be done when information on both pheno- and genotypic resistance of *Aeromonas* are obtained. Thus, more data on phenotypic resistance and distribution of ARGs among *Aeromonas* population residing in different environments would be required not only for the improvement of current ARGs detection database and its broader application, but also for a better understanding of AMR caused by *Aeromonas*.

7. Reference

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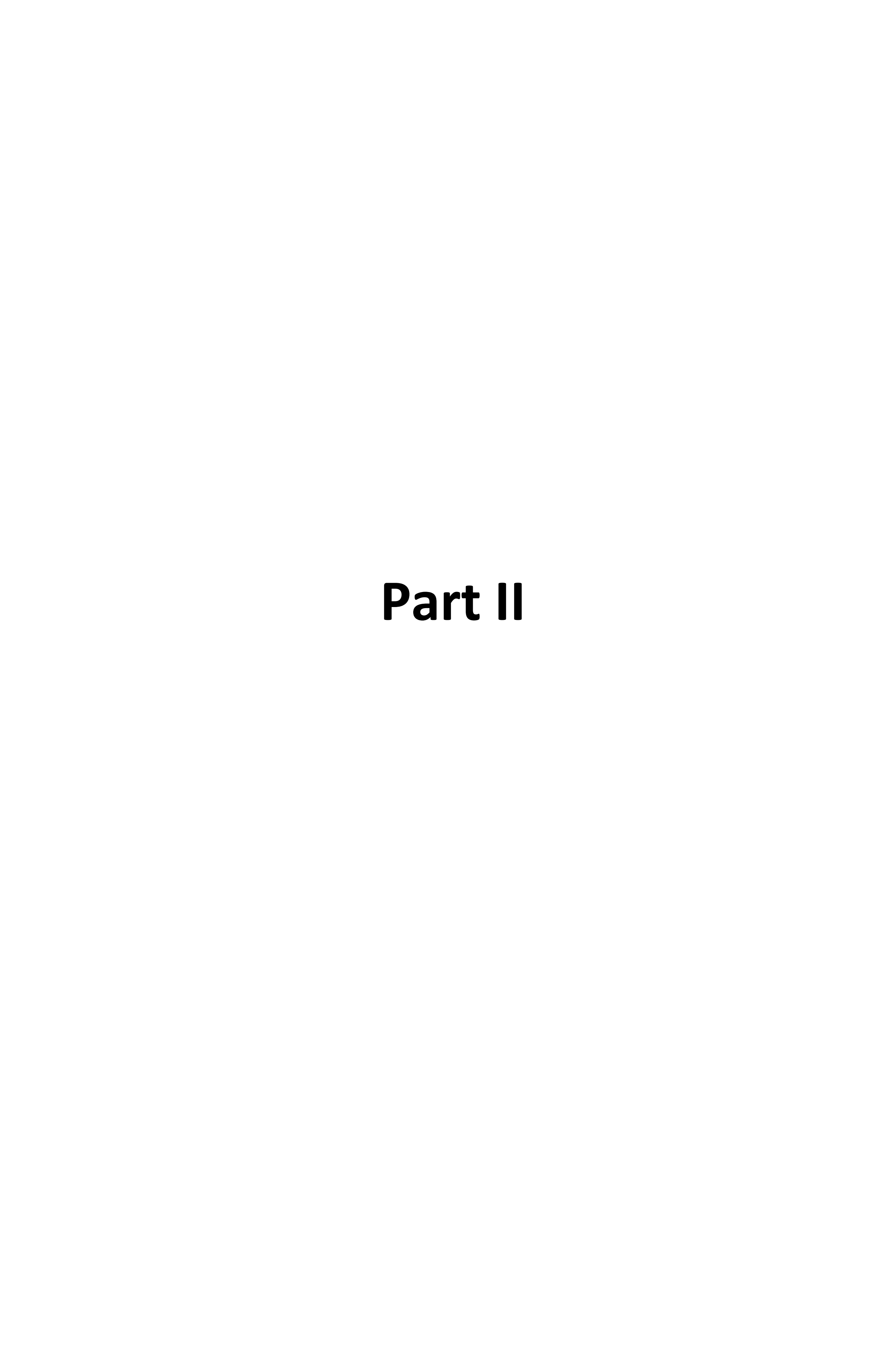

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Paper I

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ORIGINAL ARTICLE

Aeromonas spp. isolated from ready-to-eat seafood on the Norwegian market: prevalence, putative virulence factors and antimicrobial resistance

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Abstract

Aims: We aim to investigate the prevalence, putative virulence factors and antimicrobial resistance of mesophilic *Aeromonas* isolated from ready-to-eat (RTE) seafood available on the Norwegian market, and to assess the potential risks by consuming RTE seafood to consumers.
Methods and Results: The prevalence of mesophilic *Aeromonas* in 148 RTE seafood was investigated and the highest prevalence was found in retail sushi (17%), followed by oysters (10%), fresh salmon loins (10%) and scallops (4%). Among 43 *Aeromonas* isolates, 75% of them were identified as *A. media*, 23% as *A. salmonicida* and 2% as *A. bestiarum* based on partial *gryB* gene sequencing. *Aeromonas* isolates were potentially pathogenic due to the presence of four virulence genes: *alt* (73%), *hylA* (22%), *aerA* (17%) and *act* (6%). In addition, all isolates were resistant to ampicillin and erythromycin. Most of the isolates (98%) were multidrug resistant.
Conclusions: The occurrence of potentially pathogenic and multidrug-resistant *Aeromonas* strains in RTE seafood implies a potential risk to consumers. Our finding suggests that RTE seafood could be a potential vehicle for the transfer of virulent and multidrug-resistant *Aeromonas*.
Significance and Impact of the Study: To our knowledge, this is the first study to report multiple antibiotic resistance in *Aeromonas* associated with RTE seafood in Norway.

Introduction

Increased attention has been given to seafood as a source of essential nutrients, not only due to the high value of proteins, but also as a unique source of micronutrients and long chain omega-3 fatty acids (FAO 2016). With the current trend of consuming food considered fresh and natural, a wide variety of ready-to-eat (RTE) seafood, for example, sushi and sashimi, has gained popularity (Carlucci *et al.* 2015; Herrera 2016). To meet the demands of the consumers, minimal processing technology, e.g. non-thermal processing, has been developed in the food industry to produce fresh-like products with longer shelf life and increased product safety (Olatunde and Benjakul 2018). However, as RTE seafood is normally consumed without further heat treatment, consumption of RTE seafood could pose an increased microbial risk to the consumer and can be linked to seafood-associated outbreaks (Oliveira *et al.* 2011; Herrera 2016). Special attention is needed to foodborne pathogens such as *Listeria monocytogens* and *Aeromonas* species, which could multiply in perishable RTE seafood under refrigeration (Hoel *et al.* 2017a; EFSA BIOHAZ Panel *et al.* 2018).

Aeromonas spp. are ubiquitous aquatic bacteria and highly prevalent in environments such as fresh-, brackish and marine water, and also found as an inhabitant of aquatic animals (Martin-Carnahan and Joseph 2005). The genus *Aeromonas* belongs to the family Aeromonadaceae, and is a group of Gram-negative, rod-shaped, oxidase positive, catalase positive and facultatively anaerobic

bacteria (Colwell *et al.* 1986; Martin-Carnahan and Joseph 2005). The *Aeromonas* species have been traditionally classified into two major groups: one is mesophilic and motile species, typified by *Aeromonas hydrophila* which are associated with various human disease, and the other is psychrophilic and non-motile species, represented by *A. salmoncida* which mainly cause diseases in fish (Martin-Carnahan and Joseph 2005; Janda and Abbott 2010). The taxonomy of the genus *Aeromonas* has undergone numerous changes and is still controversial (Fernández-Bravo and Figueras 2020). Single evolutionary marker such as 16S ribosomal RNA, *gryB* (B-subunit of DNA gyrase), *rpoD* (σ^{70} factor), *rpoB* (β-subunit, DNA-dependent RNA polymerase) and *dnaJ* (heat shock protein 40) genes (Yanez *et al.* 2003; Soler *et al.* 2004), or multilocus sequence analysis (MLSA) of several genes (Martinez-Murcia *et al.* 2011; Beaz-Hidalgo *et al.* 2015) have been used for species identification and for phylogenetic analysis of *Aeromonas* species. Among all 36 species that have been discovered so far, about 19 species are considered emerging pathogens often linked to human infection (Fernández-Bravo and Figueras 2020).

Aeromonas spp. are potential foodborne pathogens and cause gastrointestinal as well as extraintestinal infections to humans (Tomás 2012). The occurrence of *Aeromonas* spp. has been frequently reported in water (Pablos *et al.* 2011), and food including meat (Neyts *et al.* 2000), vegetables (McMahon and Wilson 2001) as well as RTE seafood (Ullmann *et al.* 2005; Di Pinto *et al.* 2012; Hoel *et al.* 2015). The high prevalence of *Aeromonas* in different types of food and their ability to grow in food during refrigeration give rise to further concern over consumer health risk (Park and Ha 2014; Hoel *et al.* 2018). Some foodborne outbreaks caused by *A. hydrophila* were associated with the consumption of oysters (Carlos Abeyta *et al.* 1986), Swedish salad (Krovacek *et al.* 1995), salad ingredient (Zhang *et al.* 2012) and drinking water (Ventura *et al.* 2015).

The pathogenicity of *Aeromonas* is complex and multifactorial, and could be attributed by their several factors, such as structural components (capsules, lipopolysaccharide, S-layers, flagella, pili), extracellular proteins (exotoxins, enzymes) and secretion systems (Tomás 2012; Fernández-Bravo and Figueras 2020). Among them, genes encoding exotoxins including haemolysin (*hlyA*), aerolysin (*aerA*), cytotoxic heat-labile enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*), cytotonic heat-stable enterotoxin (*ast*) and Shiga-like toxins (*stx*), are well-known virulence factors and likely related to clinical symptoms during *Aeromonas* infection (Wong *et al.* 1998; Albert *et al.* 2000; Sha *et al.* 2002; Alperi and Figueras 2010; Palma-Martínez *et al.* 2016). For rapid and practical detection of potentially pathogenic *Aeromonas* strains, several PCR assays targeting virulence-associated toxin genes have been established (Kingombe *et al.* 2010; Ottaviani *et al.* 2011). The potential virulence traits of *Aeromonas* isolates from food or water have been assessed by screening the presence of virulence-associated toxin genes (Ottaviani *et al.* 2011; Pablos *et al.* 2011; Hoel *et al.* 2017b).

Moreover, antimicrobial resistance is an emerging threat to public health around the world. The extensive usage of antimicrobial drugs to humans and animals could lead to the development and spread of resistant bacteria (WHO 2014). In recent years, the transmission of resistant bacteria through the food chain has become a growing concern (Capita and Alonso-Calleja 2013). The transfer of resistant bacteria to humans via food or water could occur if the resistant bacteria is present in food or water (Verraes *et al.* 2013). Thus, the consumption of contaminated food products or water with multi-resistant bacteria is likely implicated in human infections (Capita and Alonso-Calleja 2013). In Norway, the usage of antimicrobial drugs in humans and animals is restrictive, and the prevalence of antimicrobial resistance of bacteria among humans, and in animals and food is lower compared to other countries (NORM/NORM-VET 2018). In other countries, multidrug-resistant *Aeromonas* have been isolated from different types of seafood (Yano *et al.* 2015; De Silva *et al.* 2019; Hossain *et al.* 2020). However, there is little information on antimicrobial resistance of *Aeromonas* isolated from Norwegian seafood.

Previously, high prevalence of mesophilic *Aeromonas* in retail sushi boxes from Norway and the presence of potentially pathogenic *Aeromonas* strains from the retail sushi were well documented (Hoel *et al.* 2015; Hoel *et al.* 2017b). However, the overall occurrence of mesophilic *Aeromonas* in various Norwegian RTE seafood is not known. In this study, the occurrence of mesophilic *Aeromonas* in various RTE seafood, as well as species identity and phylogenetic relationship of isolated *Aeromonas* sp. were investigated. In addition, the distribution of putative virulence factors and antimicrobial resistance of *Aeromonas* isolates were screened to assess the potential risks to consumer health.

Materials and methods

Sampling and Isolation of *Aeromonas* spp.

Five different types of RTE seafood including 30 retail sushi boxes, 24 oysters (21 *Ostrea edulis* and 3 *Crassostrea gigas*) and 24 scallops (*Pecten maximus*), 30 fresh salmon loins and 30 cold-smoked salmon (*Salmo salar* L.) were purchased from retail markets in Trondheim, Norway. In addition, seven oysters (six *Ostrea edulis*, and one

Crassostrea gigas) were collected from the west coast of Norway by the Norwegian Food Safety Authority and shipped to the laboratory. In total, 148 samples were collected and analysed from June to October 2019.

Presumptive *Aeromonas* sp. were isolated and quantified from all samples according to Nordic Committee on Food Analysis (NMKL) method No. 150 (NMKL 2004). In brief, a representative sample (10 g) from each product was aseptically cut and transferred to a sterile stomacher bag and diluted to 1 : 10 with sterile peptone water (1·0 g of bacteriological peptone and 8·5 g of NaCl per litre) and homogenized for 60 s in a Stomacher 400 lab blender (Seward Medical, Worthington, UK). The homogenate was serially diluted up to fourfold and streaked on starch ampicillin agar (SAA) supplemented with ampicillin (10 mg l^{-1}) (Sigma-Aldrich, Oslo, Norway) and incubated at 37°C for 24 h. Typical yellow colonies of *Aeromonas* spp. were quantified. Maximum 10 colonies per sample were cultivated in SAA for confirmation followed by an oxidase test on the DrySlide oxidase kit (BD, Oslo, Norway). All presumptive isolates were cultivated on tryptone soy agar (TSA) (Oxoid, Oslo, Norway) at 37°C and then stored in tryptone soy broth (TSB) (Oxoid) containing 20% glycerol at –80°C until further analysis.

DNA extraction and PCR amplification

Genomic DNA was extracted from 1 ml of overnight cultures grown in TSB at 37°C using the protocol for Gram-negative bacteria in the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Oslo, Norway). All PCR reactions were performed with 25 μl containing 1× PCR buffer (1·5 $mmol^{-1}$ $MgCl_2$), 200 μmol^{-1} of each nucleotide, 0·4 μmol^{-1} each primer, 2·5 U Taq polymerase (Qiagen, Oslo, Norway) and 50–100 ng DNA template. PCR amplification was as follows: initial denaturation at 95°C for 15 min, 30 cycles of denaturation at 95°C for 30 s, annealing for 30 s at the temperature according to the primers in Table 1, and extension at 72°C for 60 s, followed by a final extension at 72°C for 7 min. PCR products were visualized by electrophoresis in a 1·5% agarose gel (SeaKem, Lonza Group Ltd, Basel, Switzerland) in 1× TAE buffer.

Partial *gryB* gene sequencing and phylogenetic analysis

For *Aeromonas* species identification, primers *gryB3F* and *gryB14R* were used to amplify an approximately 1100 nt fragment of the *gryB* gene by PCR (Table 1). *Aeromonas hydrophila* (CCUG 14551), *A. caviae* (CCUG 25939), *A. veronii* biovar *veronii* (CCUG 27821), *A. veronni* biovar *sobria* (CCUG 30360) and *A. salmonicida* (SU2, environmental isolated from sushi, (Hoel *et al.* 2017b)) were included as reference strains. PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific). DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany), and the sequences were compared with available sequences (>97% nucleotide BLAST similarity) in GenBank database in National Center for Biotechnology Information (NCBI).

Multiple sequence alignments were performed using CLUSTAL W program (Thompson *et al.* 1994). The corresponding type sequence of all isolates and reference sequence were included in the alignment. Reference sequences of *A. bestiarum* strain CDC 9533-76T (AJ868362), *A. media* strain CECT 4232T (AY101782) and *E. coli* strain KCTC 2441 (EU014649) were retrieved from the Genbank database. Pairwise distances of all *gryB* sequences including the reference sequences were calculated by using Kimura's two-parameter model (Kimura 1980). Identical sequences based on distance score were not included in a phylogenetic analysis. The phylogenetic tree was constructed by using the neighbour-joining method (Saitou and Nei 1987) with MEGA X program (Kumar *et al.* 2018). Bootstrapping (1000 replicates) was performed to evaluate the phylogenetic tree topology based on repeatability and accuracy (Felsenstein 1985). Only bootstrap values more than 70% usually correspond to a probability of more than 95% that obtained phylogeny is real (Baxevanis and Ouellette 2001). The phylogenetic tree using the maximum likelihood method was also created to confirm the tree topology.

Phenotypic and genetic characterization of potential virulence factors

Haemolytic activity was determined on 5% cattle blood agar plates (Analysesenteret, Trondheim, Norway). Each isolate was cultivated on TSA at 37°C for 24 h. One colony of each isolate from overnight culture was streaked on the blood agar and incubated at 37°C for 24 and 48 h. A clear zone around the colonies was classified as β-haemolysis.

The presence of seven virulence-associated genes: haemolysin (*hlyA*), aerolysin (*aerA*), cytotoxic enterotoxin (*act*), cytotonic enterotoxins (*ast, alt*) and Shiga-like toxins (*stx-1, stx-2*) was screened with the DNA template of all *Aeromonas* isolates by PCR. *Aeromonas hydrophila* (CCUG 14551) was used as positive control for the *hlyA*, *aerA*, *act*, *ast* and *alt* genes and *E. coli* (CCUG 29197) was used as positive control for the *stx-1* and *stx-2* genes.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was conducted by a disk diffusion method according to the recommendation of the

Table 1 Primers used for PCR amplification and sequencing

Target gene	Nucleotide sequence (5′–3′)	Annealing temp (°C)	Size (bp)	Reference
gryB	TCCGGCGGTCTGCACGGCGT TTGTCCGGGTTGTACTCGTC	52	1100	Yanez *et al.* (2003)
hlyA	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	61	597	Wong *et al.* (1998)
aerA	GC(A/T)GA(A/G)CCC(A/G)TCTATCC(A/T)G TTTCTCCGGTAACAGGATTG	55	252	Santos *et al.* (1999)
act	GAGAAGGTGACCACCAAGAAGA AACTGACATCGGCCTTGAACTC	58	232	Kingombe *et al.* (1999)
alt	TGCTGGGCCTGCGTCTGGCGGT AGGAACTCGTTGACGAAGCAGG	58	361	Kingombe *et al.* (2010)
ast	GACTTCAATCGCTTCCTCAACG GCATCGAAGTCACTGGTGAAGC	58	536	Kingombe *et al.* (2010)
stx-1	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	58	180	Paton and Paton (1998)
stx-2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	58	255	Paton and Paton (1998)

Clinical and Laboratory Standards Institute (CLSI 2016). In brief, fresh inoculum of each isolate was evenly spread by a sterile cotton swab on Mueller-Hinton agar (MHA) (Oxoid). Maximum five antibiotic disks per plate were placed and incubated at 35°C for 16–18 h. After incubation, the diameter of inhibition zone around the disks was measured and the degree of susceptibility was categorized as sensitive, intermediate or resistant according to CLSI criteria (CLSI 2014, 2016). Each isolate was tested for their resistance against 15 antimicrobials belonging to 9 antimicrobial classes including Penicillins: Ampicillin (10 μg), Mecillinam (10 μg), Cephalosporins: Cefotaxime (30 μg), Ceftriaxone (30 μg), Quinolones: Ciprofloxacin (5 μg), Oxolinic acid (2 μg), Tetracyclines: Doxycycline (30 μg), Tetracycline (30 μg), Carbapenems: Imipenem (10 μg), Meropenem (10 μg), Aminoglycosides: Gentamicin (10 μg), Tobramycin (10 μg), Macrolides: Erythromycin (15 μg), Amphenicols: Florfenicol (30 μg) and trimethoprim/sulfamethoxazole (1·25/23·75 μg) (Oxoid).

In addition, multiple antibiotic resistance (MAR) index was calculated for each isolate. The MAR index is defined as 'a/b', where 'a' is the number of antibiotics to which a particular isolate is resistant, and 'b' is the total number of antibiotics tested for the isolate (Krumperman 1983). The isolate was considered multiple antibiotic resistant, if the resistance to at least 3 (≥3) antibiotics among 15 antimicrobial agents (MAR index >0·2) was observed.

Results

The prevalence of *Aeromonas* species

The prevalence of mesophilic *Aeromonas* spp. in 148 RTE seafood products is summarized in Table 2. The highest prevalence of *Aeromonas* spp. was found in retail sushi boxes (17%), followed by oysters (10%), salmon loins (10%) and scallops (4%). *Aeromonas* spp. were not detected in cold-smoked salmon. Overall, the mean prevalence of *Aeromonas* spp. in 148 sample was about 8%. The mean level of *Aeromonas* spp. present in the seafood samples was about 2·5 log CFU per gram (1·0–6·3 log CFU per gram). The highest level of contamination (6·3 log CFU per gram) was detected in one oyster sample.

In total 43 presumptive *Aeromonas* isolates were collected from four different seafood: 18 from retail sushi, 18 from oyster, 4 from scallops and 3 from salmon loins. All isolates were identified as *Aeromonas* sp. based on partial *gryB* gene sequencing. Nucleotide sequences of *gryB* amplicons were determined for 43 isolates and 5 reference strains. The lengths of nucleotide sequences were 725–1080 nt (96% of all sequences >1063 nt). Among 43 *Aeromonas* isolates, 75% of the isolates were identified as *A. media*, 23% as *A. salmonicida* and 2% as *A. bestiarum* based on partial *gryB* gene sequencing (Table 3). *A. media* was the most prevalent species among all *Aeromonas* isolates.

Table 2 The prevalence of mesophilic *Aeromonas* species in 148 ready-to-eat seafood products

	Number of positive samples (%)	Mean (range) level (log CFU per gram)
Retail sushi (mixture)	5/30 (17)	1·5 (1·0–2·2)
Oysters	3/34 (10)	5·5 (4·7–6·3)
Salmon loins	3 /30 (10)	1·6 (1·0–2·7)
Scallops	1 /24 (4)	1·6
Smoked salmon	0 /30 (0)	0
Total	12/148 (8)	2·5 (1·0–6·3)

Phylogenetic analysis of *Aeromonas gryB* sequences

The *gryB* gene sequences from all *Aeromonas* strains and reference strains were aligned, and percentage nucleotide substitutions were calculated for a continuous stretch of 961 nt (see Table S1). The sequence similarity between all *Aeromonas* strains ranged from 90·7 to 99·7%, and the overall mean similarity was around 92%. At the intraspecies level, the rate of nucleotide substitution ranges from 0·9 to 4·0% for *A. media*, 0·3 to 2·0% for *A. salmonicida*, and 0·7% for *A. bestiarum*. The interspecies sequence divergence was higher and >5·0% for all pairs of species. The exception was found between *A. salmonicida* and *A. bestiarum* with substitutions ranging from 2·0 to 4·0%.

After the removal of duplicate sequences, in total 21 nucleotide sequences were aligned to construct a neighbour-joining tree (Fig. 1). All sequences of interest belonging to the genus *Aeromonas* were more closely related to each other than they were to the outgroup reference strain *E. coli*. All isolates of interest clustered with their respective reference sequence. Two main clusters were observed in the constructed phylogenetic tree. One main cluster of the tree included the species *A. hydrophila*, *A. caviae* and *A. veronii*, while the other main cluster included *A. salmonicida*, *A. bestiarum* and *A. media*. In addition, a similar tree was obtained by the phylogenetic analysis using maximum-likelihood method, confirming the robustness of the neighbour-joining tree topology (see Fig. S1).

Putative virulence factors of *Aeromonas*

The presence of seven virulence-associated genes in *Aeromonas* isolates was screened by PCR assays. The distribution of the virulence-associated genes in different *Aeromonas* species is summarized in Table 4. The reference strain *A. hydrophila* (CCUG 14551) harboured all genes, except *stx-1* and *stx-2*. The reference strain *E. coli* (CCUG 29197) harboured *stx-1* and *stx-2*. Of the 43 strains, 32 (74%) were positive with at least one virulence-associated gene. The highest prevalence (74%) was observed for *alt* gene which was detected in all three species. *A. salmonicida* harboured the highest number of virulence genes, being positive for *hlyA, aerA, act* and *alt*. The *hlyA* and *aerA* was detected in 10 (100%) and 8 (72%) strains of *A. salmonicida* but not detected in any of *A. media* and *A. bestiarum*. The combination of *alt/act* genes was detected in one strain of each *A. salmonicida* and *A. bestiarum*. Moreover, 65% of *A. media* harboured the *alt* gene only, while none of the virulence genes was detected in the rest (35%) of *A. media*. All isolates gave negative results for the *ast*, *stx-1* and *stx-2*.

In addition, β-haemolysis was observed in 27% of the isolates (Table 4). All *A. salmonicida* and *A. bestiarum* were haemolytic. On the other hand, all *A. media*, except one strain, were non-haemolytic.

Table 3 The prevalence of different mesophilic *Aeromonas* species among the isolates from ready-to-eat seafood

	Number (%) of the strain identified			
	A. media	*A. salmonicida*	*A. bestiarum*	Total (%)
Retail sushi	18 (100)	0	0	18 (100)
Oysters	11 (61)	7 (39)	0	18 (100)
Salmon loins	0	2 (67)	1 (33)	3 (100)
Scallops	3 (75)	1 (25)	0	4 (100)
Total	32 (75)	10 (23)	1 (2)	43 (100)

Antimicrobial susceptibility of *Aeromonas*

The susceptibility of 43 mesophilic *Aeromonas* isolates to 15 antimicrobial agents was tested by the disk diffusion method, and the pattern of antimicrobial susceptibility is shown in Fig. 2 (see Table S2 for the disk diffusion zone diameter of each isolate). All isolates were resistant to ampicillin and erythromycin. Resistance to florfenicol and oxolinic acid was observed in 88 and 28% of the 43 isolates respectively. In addition, reduced susceptibility to oxolinic acid was also observed in 63% of the isolates. Two isolates were resistant to cefotaxime, while reduced susceptibility to cefotaxime and ceftriaxone was observed in 55 and 56% of the isolates respectively. Moreover, resistance (11%) or reduced susceptibility (11%) to imipenem was observed in most of *A. salmonicida* and one *A. bestiarum* isolates. One *A. salmonicida* strain was resistant to both imipenem and meropenem, and another two *A. salmonicida* strains showed reduced susceptibility to tobramycin. On the other hand, all isolates were susceptible to melicinam, ciproflaxin, doxycycline, tetracycline, gentamycin and trimethoprim-sulfamethoxazole.

Moreover, MAR was calculated for each isolate (see Table S2 for MAR index of each isolate) and the occurrence of MAR index for all isolates from different sources is shown in Fig. 3. Regardless of the origin, the MAR index for most of isolates (98%) was higher than and equal to 0·2 (mean MAR index = 0·22). In addition, MAR index above 0·26 was observed in 28% of the isolates. Among the four types of RTE seafood, more than 70% of the isolates originated from sushi boxes showed MAR index over 0·26. Moreover, the highest MAR value (0·33) was obtained from two strains of *A. media* and *A. salmonicida* isolated from sushi box and oyster respectively. In addition, the lowest value (0·13) was found from one strain of *A. salmonicida* isolated from oyster.

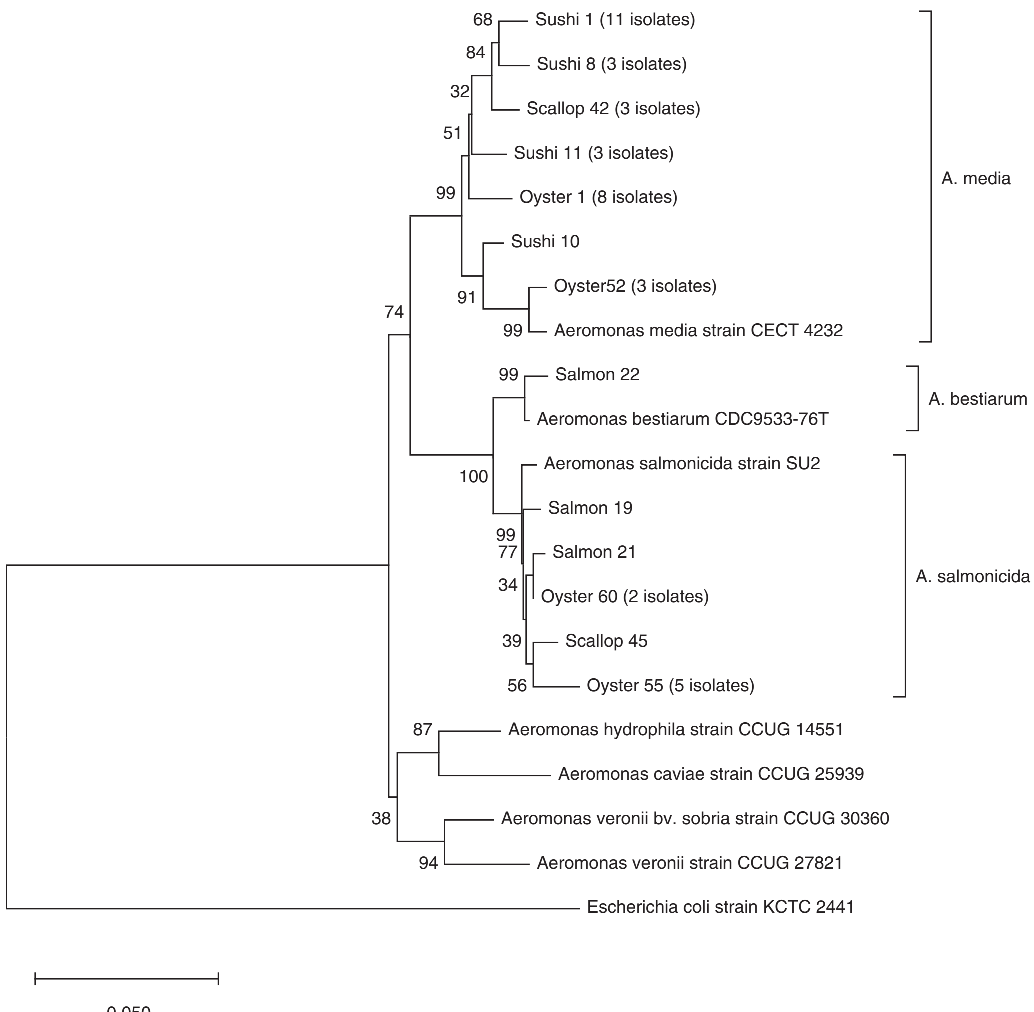

Figure 1 Neighbour-joining tree based on *gryB* sequences showing the inter- and intraspecies relationship of *Aeromonas* isolated from sushi, oysters, salmon and scallops (with number of identical sequences indicated). The scale bar indicates the evolutionary distance of 0·05 nucleotide substitution per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. This analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option).

Discussion

In this study, the prevalence of mesophilic *Aeromonas* spp. in various RTE seafood available on the Norwegian market was investigated. The overall prevalence of the *Aeromonas* spp. in 148 RTE seafood products was 8% (12/148). The prevalence is relatively low, in contrast to the high prevalence (70%, 41/58) of mesophilic *Aeromonas* spp. previously reported in retail sushi products in Norway (Hoel *et al.* 2015). Likewise, in a study conducted in Italy, around 70% (57/81) of RTE seafood including sushi (90·5%), sea salad (91·7%), surimi (85·7%) and peeled shrimp (75%), were contaminated with *Aeromonas* spp. (Di Pinto *et al.* 2012). In addition, the prevalence of *Aeromonas* spp. was reported to be 32% (27/84) of the retail seafood including raw seafood

Table 4 The distribution of potential virulence factors in *Aeromonas* isolates from ready-to-eat seafood

	Number (%) of positive isolates								
	n	*hlyA*	*aerA*	*act*	*alt*	*ast*	*stx-1*	*stx-2*	*β*-hemolysis
A. media	32	0	0	0	21 (65)	0	0	0	1 (3)
A. salmonicida	10	10 (100)	8 (72)	1 (10)	10 (100)	0	0	0	10 (100)
A. bestiarum	1	0	0	1 (100)	1 (100)	0	0	0	1 (100)
Total	43	10 (23)	8 (19)	2 (7)	32 (74)	0	0	0	12 (27)

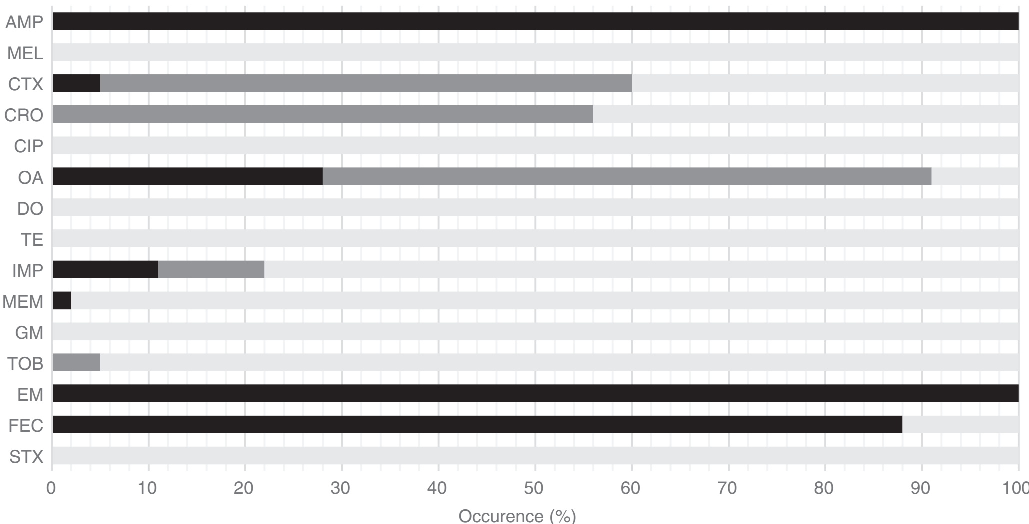

Figure 2 Antimicrobial susceptibility patterns of the 43 *Aeromonas* strains to 15 antibiotics including AMP: ampicillin, MEL: mecillinam, CTX: cefotaxime, CRO: ceftriaxone, CIP: ciprofloxacin, OA: oxolinic acid, DO: doxycycline, TE: tetracycline, IMP: imipenem, MEM: meropenem, GM: gentamycin, TOB: tobramycin, EM: erythromycin, FEC: florfenicol, STX: trimethoprim/sulfamethoxazole. Key to susceptibility pattern: ■: resistant, ■: intermediate, ■: susceptible.

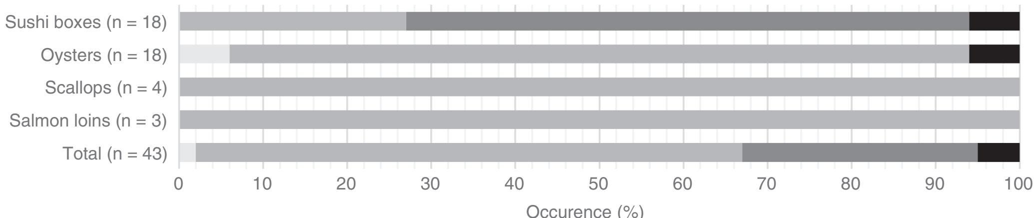

Figure 3 The occurrence of multiple antibiotic resistance (MAR) index of the *Aeromonas* strains isolated from different RTE seafood (Sushi boxes, salmon loins, oysters and scallops). Key to MAR index: ■: 0·33, ■: 0·26 ■: 0·2, ■: 0·13. The MAR index of each 0·13, 0·2, 0·26 and 0·33 means the observed resistance towards 2, 3, 4 and 5 antibiotics among 15 antibiotics respectively.

products, sushi and smoked salmon in a German study (Ullmann *et al*. 2005). This dissimilarity may result from several factors such as different methods of isolation, sampling sites, types and sources of the products, different processing and storage conditions, seasonal variation and geographical variation (FAO and WHO 2016).

Moreover, the levels of *Aeromonas* found in food and water vary (Palumbo *et al.* 1985; Janda and Abbott 2010). In our study, the mean level of mesophilic *Aeromonas* spp. detected in all RTE seafood was about 2·5 log CFU per gram (ranges from 1·0 to 6·3 log CFU per gram). Oysters were the most contaminated with *Aeromonas* spp. (mean level of 5·5 log CFU per gram) while the levels of *Aeromonas* spp. in sushi, salmon loins and scallops ranged from 1·0 to 2·7 log CFU per gram. Total aerobic counts (TAC) of the oyster samples contaminated with *Aeromonas* spp. were above 8 log CFU per gram (data not shown). A previous study reported similar level of *Aeromonas* (up to 5×10^5 CFU per gram) in raw shucked oyster, and TAC (up to $3{\cdot}7 \times 10^8$ CFU per gram) (Palumbo *et al.* 1985). Nevertheless, in a foodborne outbreak linked to the consumption of raw oyster, the average level of *Aeromonas* recovered from the oysters was estimated to be the most probable number 9·3 per 100 g (Carlos Abeyta *et al.* 1986). The level of *Aeromonas* was found to be around 2–3 log CFU per gram in another outbreak linked to a salad ingredient (Zhang *et al.* 2012). Although the foodborne outbreaks caused by *Aeromonas* are rare and the infectious dose is not well defined, the probability of infection is not negligible and there may be a risk of acute enteric illness in a susceptible host, when *Aeromonas* is present (Teunis and Figueras 2016). Thus, the presence of *Aeromonas* sp. in the selected RTE seafood products may indicate a potential hazard or risk to consumers. In particular, the high level of *Aeromonas* found in oyster implies the precaution of consuming raw oysters.

It is critical to perform accurate identification of bacteria to species level for evaluation of microbial hazard or risk (López-Campos *et al.* 2012). In this study, *Aeromonas* isolates were identified at species level based on partial *gryB* gene sequencing. We identified three different *Aeromonas* species; *A. media, A. salmonicida* and *A. bestiarum*. Among the *Aeromonas, A. hydrophila* is the most studied foodborne pathogen and frequently found in food from other studies (Ullmann *et al.* 2005; Ottaviani *et al.* 2011; De Silva *et al.* 2018; De Silva *et al.* 2019). High occurrence (68%) of *A. hydrophila* from retailed seafood products was reported in one German study (Ullmann *et al.* 2005). On the other hand, the most prevalent species in the seafood products investigated in the present study was *A. media* (75%). Previously, high prevalence of *A. media* in drinking water was reported (Pablos *et al.* 2011), whereas many studies have shown very low prevalence of *A. media* in seafood or other types of food (Ottaviani *et al.* 2011; Hoel *et al.* 2017b; De Silva *et al.* 2018; De Silva *et al.* 2019). Moreover, *A. salmonicida* strains which were isolated by incubating the samples at 37°C in this study were considered as atypical and mesophilic. Typical *A. salmonicida* are well-known fish pathogens, and characterized as psychrophilic, and non-motile species, while mesophilic and mostly motile species of *A. salmonicida* are classified as atypical and potentially human pathogenic (Austin *et al.* 1998). The high occurrence of atypical *A. salmonicida* was observed in seafood sources, such as retail sushi (Hoel *et al.* 2017b), scallops (De Silva *et al.* 2018) and mussels (Hossain *et al.* 2020).

The mean similarity of partial *gryB* gene sequence of *Aeromonas* was 92%, which is similar to other studies (92–93%) (Yanez *et al.* 2003; Hoel *et al.* 2017b). Consequently, considerable divergence (branch length) was observed between all *Aeromonas* species in the neighbour-joining tree (Fig. 1). Overall grouping of the *Aeromonas* strains in the tree, including two major sub-branching of the tree (*A. hydrophila, A. caviae* and *A. veronii*/*A. salmonicida, A. bestiarum* and *A. media*) was in accordance with the previous phylogenetic study based on *gryB* gene sequence (Yanez *et al.* 2003; Soler *et al.* 2004). Most isolates of interest belonged to one major branch (upper part of tree). Interestingly, an insertion of 3 bp (triplet AGC) was only detected in *gryB* gene sequence of *A. salmonicida* and *A. bestiarum*. These two species are clustered together in a sub-branch of the tree, and separately clustered from *A. media* which do not contain the triplet AGC. It has been suggested that the insertion is the exclusive character of this group of species (Yanez *et al.* 2003).

In one sub-branch of the tree, two independent clusters closely related to the *A. media* type strain with bootstrap value 99% were observed. In detail, oyster 52 and sushi 10 representative isolates clustered with *A. media* type strain, while the rest of isolates (sushi 1, 8, 11, scallop 42 and oyster 1) formed an independent cluster. The sequence divergence between oyster 52 isolate and *A. media* type strain was only 0·9%, while oyster 52 isolate showed higher sequence divergence of 3·3% to the rest of isolates, except for sushi 10 (2·4%). Considering the high probability of these two clusters based on bootstrap value, it might represent a distinct phylogenetic relationship among *A. media* species based on *gryB* gene sequences.

In addition, the clearly separated cluster of *A. salmonicida* and *A. bestiarum* with 100% branch support was observed, although the interspecies sequence divergence between *A. salmonicida* and *A. bestiarum* (2·0–4·0%) is lower than among other species (>5·0%). This result confirms the discriminatory power of *gryB* gene sequence by differentiating *A. salmonicida* and *A. bestiarum*, since it was impossible based on 16S rRNA gene sequencing (Martinez-Murcia *et al.* 2005). Moreover, more than 5% of intraspecies sequence divergence confirms the power of *gryB* gene to investigate phylogenetic relationships among closely related *Aeromonas* species.

Moreover, we determined the putative virulence traits of the *Aeromonas* strains by screening virulence-associated genes encoding specific toxins; including *alt* and *ast* (cytotonic enterotoxin), *act* (cytotoxic enterotoxin), *aerA* (aerolysin), *hlyA* (haemolysin), and *stx-1* and *stx-2* (Shiga-like toxins). Although the presence of virulence-associated genes in the *Aeromonas* does not directly imply that they can cause clinical symptoms of infection, there is some evidence of a correlation between the *Aeromonas* isolates harbouring toxin genes and their virulence traits (Albert *et al.* 2000; Sha *et al.* 2002; Ottaviani *et al.* 2011). The cyotoxic enterotoxin and aerolysin are pore-forming toxins, and the haemolytic, cytotoxic and enterotoxin activities of Act has previously been demonstrated (Xu *et al.* 1998; Chopra and Houston 1999). Later, the role of three enterotoxins of Act, Alt and Ast in causing *A. hydrophila*-induced gastroenteritis in an animal model was established, with the greatest contribution from cytotoxic enterotoxin Act, followed by cytotonic enterotoxin Alt and Ast (Sha *et al.* 2002). Thus, *Aeromonas* strains containing the genes encoding these enterotoxins are likely to have pathogenic potential, and screening for specific enterotoxin genes is suggested to be an effective way of detecting and characterizing *Aeromonas* virulence factors (Wang *et al.* 2003).

In this study, the majority of the *Aeromonas* strains were potentially pathogenic due to the presence of virulence-associated genes. Regardless of their origin, the pattern of detected genes seemed species-dependent. *Aeromonas salmonicida* strains possessed the highest number of toxin genes (either *hlyA, aerA, alt,* or *hlyA, aerA, act*), while most *A. media* strains only harboured the *alt* genes. Overall, at least one toxin gene was observed in 74% of the strains. The *alt* gene encoding heat-labile cytotonic enterotoxin was highly prevalent (32/43), whereas *ast* gene encoding heat-stable cytotonic enterotoxin was not detected in any of the isolates. Similar result was found in the study by Di Pinto *et al.* (2012), where the majority of *Aeromonas* isolates from different types of RTE seafood harboured *alt* gene but not *ast* gene. Likewise, one study by Pablos *et al.* (2009) found that all *Aeromonas* strains isolated from drinking water were positive with *alt* genes, but all of them were negative with *ast* genes. The study by Albert *et al.* (2000) indicated that the *Aeromonas* isolates positive with both *alt* and *ast* genes might synergistically cause severe diarrhoea, or associate with watery diarrheal, while the isolates positive with only *alt* gene were linked to induce loose stools. On the other hand, *act* gene was only detected in two strains of each *A. salmonicida* and *A. bestiarum* in this study. In contrast, other studies found high prevalence of *act* genes among the *Aeromonas* isolated from seafood (Yano *et al.* 2015; Hoel *et al.* 2017b). As suggested in other studies, heterogeneity in the distribution of virulence-associated genes can be found due to the diversity of *Aeromonas* strains isolated from various sources or geographical variation (Albert *et al.* 2000; Yano *et al.* 2015).

Moreover, of the 10 *A. salmonicida* strains, *hlyA* and *aerA* were detected in 10 and 8 strains, respectively, and all *A. salmonicida* had β-haemolysis on the blood agar. In a study by Wong *et al.* (1998), the involvement of both *hlyA* and *aerA* genes in the haemolytic activity of *A. hydrophila* was highlighted. A subsequent study confirmed that the correlation between both genes and the cytotoxicity of *Aeromonas* (Heuzenroeder *et al.* 1999). β-haemolysis was also observed by two strains of *A. bestiarum* and *A. media*, which did harbour neither *hlyA* nor *aerA* genes. We hypothesized that the β-haemolysis of *A. bestiarum* is most likely caused by the presence of *act* genes, and the haemolytic activity expressed by one *A. media* strain may result from the expression of alternative haemolysin gene variants.

Shiga-like toxins, as important virulence factors in the pathogenesis of gastroenteritis and haemolytic uremic syndrome (HUS), could be produced by enterohaemorrhagic *Escherichia coli* (EHEC), and other bacteria including *Aeromonas* spp. (Haque *et al.* 1996). Occasionally, *Aeromonas*-induced infection has been associated with HUS (Figueras *et al.* 2007). The *stx-1* and *stx-2* genes encoding Shiga-like toxins have been detected in clinical and environmental isolates of *Aeromonas* (Alperi and Figueras 2010; Palma-Martínez *et al.* 2016). In this study, the *stx-1* and *stx-2* genes were not detected in any of the *Aeromonas* isolates. So far, the cases where these genes were detected from foodborne *Aeromonas* have not been reported.

Furthermore, the antimicrobial susceptibility of the *Aeromonas* strains was determined. In general, the resistance patterns of the isolates were rather dependent on the class-specific antibiotic, but independent of the type species and species source. The resistant profiles were comparable to those of the *Aeromonas* spp. in other studies (Esteve *et al.* 2015). In general, *Aeromonas* spp. are resistant to ampicillin, thus ampicillin is commonly used for the culture media for a selective isolation of *Aeromonas* spp. (Huddleston *et al.* 2007). Accordingly, resistance to ampicillin was observed in all isolates, although none of the isolates were resistant to mecillinam. The resistance of the *Aeromonas* spp. to penicillins with extended spectrum seems to be low (Janda and Abbott 2010). The resistance to erythromycin was also observed in all isolates (100%). Similarly, high prevalence (62–86%) of erythromycin-resistant strains was found in the foodborne isolates from shrimp and scallops (De Silva *et al.* 2018; De Silva *et al.* 2019) as well as in clinical isolates (Esteve *et al.* 2015).

The resistance to first-generation cephalosporins has frequently been reported, but *Aeromonas* spp. are generally susceptible to higher generation cephalosporins (Yano *et al.* 2015; Dahanayake *et al.* 2020). In our study, only two isolates were resistant to cefotaxime, but reduced susceptibility to both cefotaxime and ceftriaxone was observed in around 55% of the isolates. Occasionally, increased resistance to third-generation cephalosporins has been also observed in clinical isolates (Zhou *et al.* 2019).

Although the *Aeromonas* are rather sensitive to carbapenems, recent studies have found the high occurrence of resistant *Aeromonas* strains to these agents from environmental and clinical isolates (Esteve *et al.* 2015; De Silva *et al.* 2019). High resistance to imipenem (90%) and meropenem (75%) was observed from the *Aeromonas* isolated from scallops (De Silva *et al.* 2019). In our study, the resistance or reduced susceptibility to imipenem was observed in 22% of the isolates, mainly in *A. salmonicida* strains. One *A. salmonicida* strain was resistant to both imipenem and meropenem. Esteve *et al.* (2015) pointed out that the levels of resistance to imipenem were almost four times higher than those previous reported. While the resistance to third-generation cephalosporin and carbapenems was not observed in most isolates, many strains showed reduced susceptibility to both agents. Special concern over the usage of these antibiotics may be needed in the near future, since the increased resistance of *Aeromonas* spp. to beta-lactam antibiotics (e.g. penicillins, cephalosporin, carbapenems) has been highlighted as one major concern due to the activity of inducible beta-lactamase (Janda and Abbott 2010; Fernández-Bravo and Figueras 2020).

Moreover, the resistance to aminoglycosides has been occasionally reported, but the levels of resistance are usually very low (Aravena-Román *et al.* 2012; Esteve *et al.* 2015). All isolates in this study were susceptible to gentamycin and tobramycin, except two strains. In addition, none of the isolates were resistant to trimethoprim-sulfamethoxazole, even though the high occurrence of resistant strains to trimethoprim-sulfamethoxazol has been reported from the *Aeromonas* isolated from aquatic sources (Yano *et al.* 2015; Odeyemi and Ahmad 2017).

While most of the antibiotics tested in this study were selected based on the human usage of antibiotics in Norway, florfenicol and oxolinic acids were included since they are the two antibiotics which can legally be used in aquaculture industry in Norway to treat the bacterial fish disease (NORM/NORM-VET 2018; Love *et al.* 2020). High resistance to both agents was observed, and 88 and 28% of the isolates were resistant to florfenicol and oxolinic acid respectively. In addition, reduced susceptibility to oxolinic acid was also observed in 63% of the isolates. Relatively lower resistance to florfenicol (0–7%) (Cízek *et al.* 2010; Syrova *et al.* 2018) and similar resistance to oxolinic acid (11–27%) (Esteve *et al.* 2015; Syrova *et al.* 2018) were observed from the *Aeromonas* isolated from aquatic sources. The occurrence of resistance to florfenicol and oxolinic acid observed in this study could partially be explained by the long-term use of these two agents in Norwegian aquaculture, even though the usage of antibiotics in aquaculture has significantly decreased since 1980s (NORM/NORM-VET 2018). To date, the use of antibiotics in aquaculture as well as human medicine in Norway is very restricted compared to other European countries (EMA 2019).

On the other hand, with the recent global growth of aquaculture, the significance of antibiotic usage in aquaculture to human health and food safety has become of special concern, this is due to the possible impacts on the development and spread of resistance from aquatic environment into human pathogen as well as the antimicrobial residues in food products (Okocha *et al.* 2018). Although the role of antibiotic usage in aquaculture in the development and dissemination of antimicrobial resistance genes into human pathogens is not fully understood, some studies have reported that genetic elements and antimicrobial resistance determinants for quinolones and tetracyclines were shared between aquatic bacteria and human pathogens, and they were likely transferred from the aquatic bacteria (Cabello *et al.* 2013). Thus, further studies on resistance-associated genes to florfenicol and oxolinic acid might be required to predict the potential impacts on human health.

Whereas the usage of antibiotics in aquaculture in Norway is low and limited to these two antibiotics, tetracycline antibiotics are widely used in other countries including Chile, United States, etc. (Love *et al.* 2020). In fact, tetracyclines are important antibacterial agents not only for the aquaculture industry, but also for human infections (Chopra and Roberts 2001). Accordingly, the resistance to the tetracycline antibiotics is frequently reported from the *Aeromonas* from aquatic environments (De Silva *et al.* 2018; Syrova *et al.* 2018) as well as in clinical isolates (Esteve *et al.* 2015; Zhou *et al.* 2019). Even though tetracyclines are ranked as top three antibiotics used for human infection in Norway (NORM/NORM-VET 2018), none of our isolates were resistant to tetracycline and doxycycline.

Multiple antibiotic resistance index was firstly used to identify the high-risk sources of faecal contamination of food by MAR indexing *E. coli* strains from environments by Krumperman (1983). They found that multidrug-resistant *E. coli* with MAR index over 0·2 were mostly originated from high risk of contamination where many

antibiotics were frequently applied. Nevertheless, Krumperman (1983) emphasized that MAR index with the threshold of 0·2 is arbitrary, and the index of samples in the range between 0·20 and 0·25 requires careful scrutiny. In our study, MAR index for most of isolates (98%) was higher than 0·2, which might also indicate the origin of most isolates is a high-risk source of antimicrobial contamination. Among five types of seafood sources in the present study, the isolates with MAR index over 0·26 were highly prevalent (more than 70%) in sushi boxes. This may indicate that sushi ingredients have a higher risk of antimicrobial contamination than other seafood sources. Since sushi boxes were the mixture of various ingredients such as seafood, vegetables and rice, *Aeromonas* isolated from sushi boxes might be originated from other sources, for example, imported seafood or various vegetables.

Even though different types of antimicrobial agents were tested in other studies, the high occurrence (70–95%) of multi-resistant *Aeromonas* (MAR index >0·2) was reported in other geographical regions, for example, from marketed fish and prawns in India (Vivekanandhan *et al.* 2002), shrimps (De Silva *et al.* 2018) and cockles (Dahanayake *et al.* 2020) in South Korea, as well as from various aquatic sources in Malaysia (Odeyemi and Ahmad 2017). However, this index should be interpreted with precaution. In fact, *Aeromonas* spp. are intrinsically resistant to ampicillin, and they were highly resistant to two antibiotics (florfenicol and oxolinic acid) which have been used in aquaculture for several decades. Except these antibiotics, we could reveal a low level of resistance against the antimicrobial agents investigated in the present study.

Overall, our results demonstrate the occurrence of potentially pathogenic *Aeromonas* strains in RTE seafood, particularly with high prevalence of *A. media* and atypical *A. salmonicida*. To our knowledge, this is the first study to report MAR in *Aeromonas* associated with RTE seafood on the Norwegian market. The occurrence of multidrug-resistant *Aeromonas* might indicate that RTE seafood could be a potential vehicle of antibiotic-resistant determinants, although further studies on characterization of antibiotic resistance genes would be necessary to understand the resistance mechanisms of the *Aeromonas* spp. The presence of potentially virulent and multidrug-resistant *Aeromonas* in RTE seafood implies a potential microbial risk to consumers when consuming perishable RTE seafood without heat treatment.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author contributions

H.J.L. performed the experiments, analysed the data, and wrote the manuscript. S.H. contributed to the experimental planning and gave technical advice on experiments. A.J. contributed to the experimental planning and supervised the project. S.H., A.J., B.T.L. and J.L. discussed the results and critically commented and revised the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree by maximum likelihood method based on *gryB* sequences showing the inter- and intraspecies relationship of *Aeromonas* isolated from sushi, oysters, salmon loins and scallops (with number of identical sequences indicated).

Table S1. Nucleotide substitution comparison (%) for *gryB* sequence of 43 *Aeromonas* isolates.

Table S2. Disk diffusion zone diameter and MAR index for 43 *Aeromonas* isolates.

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Correction

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Correction to: Aeromonas spp. isolated from ready-to-eat seafood on the Norwegian market: prevalence, putative virulence factors and antimicrobial resistance

This is a correction to: H.-J. Lee, S. Hoel, B.-T. Lunestad, J. Lerfall, A.N. Jakobsen, Aeromonas spp. isolated from ready-to-eat seafood on the Norwegian market: prevalence, putative virulence factors and antimicrobial resistance, *Journal of Applied Microbiology*, Volume 130, Issue 4, 1 April 2021, Pages 1380–1393, https://doi.org/10.1111/jam.14865.

In the originally published article there was a typographical error in the name of the housekeeping gene. Throughout the article, this should read '*gyrB*' instead of '*gryB*'.

These details have been corrected only in this correction notice to preserve the published version of record.

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Whole genome sequence analysis of *Aeromonas* spp. isolated from ready-to-eat seafood: antimicrobial resistance and virulence factors

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Aeromonas are widespread in aquatic environments and are considered emerging pathogens in humans and animals. Multidrug resistant (MDR) *Aeromonas* circulating in the aquatic environment and food production chain can potentially disseminate antimicrobial resistance (AMR) to humans *via* the foodborne route. In this study, we aimed to investigate AMR and virulence factors of 22 *Aeromonas* strains isolated from ready-to-eat (RTE) seafood. A multilocus phylogenetic analysis (MLPA) using the concatenated sequences of six housekeeping genes (*gyrB*, *rpoD*, *gyrA*, *recA*, *dnaJ*, and *dnaX*) in the 22 *Aeromonas* genomes and average nucleotide identity (ANI) analysis revealed eight different species; *A. caviae*, *A. dhakensis*, *A. hydrophila*, *A. media*, *A. rivipollensis*, *A. salmonicida*, *A. bestiarum*, and *A. piscicola*. The presence of virulence genes, AMR genes and mobile genetic elements (MGEs) in the *Aeromonas* genomes was predicted using different databases. Our data showed that the genes responsible for adherence and motility (Msh type IV pili, tap type IV pili, polar flagella), type II secretion system (T2SS) and hemolysins were present in all strains, while the genes encoding enterotoxins and type VI secretion system (T6SS) including major effectors were highly prevalent. Multiple AMR genes encoding β-lactamases such as *cphA* and bla_{OXA} were detected, and the distribution of those genes was species-specific. In addition, the quinolone resistance gene, *qnrS2* was found in a IncQ type plasmid of the *A. rivopollensis* strain A539. Furthermore, we observed the co-localization of a class I integron (*intI1*) with two AMR genes (*sul1* and *aadA1*), and a Tn521 transposon carrying a mercury operon in *A. caviae* strain SU4-2. Various MGEs including other transposons and insertion sequence (IS) elements were identified without strongly associating with detected AMR genes or virulence genes. In conclusion, *Aeromonas* strains in RTE seafood were potentially pathogenic, carrying several virulence-related genes. *Aeromonas* carrying multiple AMR genes and MGEs could potentially be involved in the dissemination and spread of AMR genes to other bacterial species residing in the same environment and possibly to humans. Considering a One-Health approach, we highlight the significance of monitoring AMR caused by *Aeromonas* circulating in the food chain.

KEYWORDS

***Aeromonas*, antimicrobial resistance, virulence factors, mobile genetic elements, whole genome sequences, multilocus phylogenetic analysis**

1. Introduction

Aeromonas are Gram-negative bacteria, ubiquitous in aquatic environments, including estuarine and brackish water (Martin-Carnahan and Joseph, 2005). Psychrophilic *A. salmonicida* and some mesophilic *Aeromonas* are responsible for fish diseases such as furunculosis and motile *Aeromonas* septicemia (MAS), while many species are opportunistic human pathogens (Janda and Abbott, 2010; Beaz-Hidalgo and Figueras, 2013). The genus *Aeromonas* comprises at least 31 species, and among them, *A. hydrophila*, *A. caviae*, and *A. veronii* are the major species that frequently involved in human gastroenteritis and extraintestinal infections (Fernández-Bravo and Figueras, 2020). Although the role of *Aeromonas* as a true enteropathogen has been controversial, several studies have suggested that more attention should be given to the genus *Aeromonas* as emerging foodborne pathogens (Teunis and Figueras, 2016; Wu et al., 2018; Hoel et al., 2019). *Aeromonas* have occasionally been recognized as the source of foodborne outbreaks (Zhang et al., 2012; Ventura et al., 2015). The occurrence of mesophilic *Aeromonas* in water and food including ready-to-eat seafood (RTE) has frequently been reported, and their pathogenic potential has been determined based on the analysis of virulence-associated toxin genes (Pablos et al., 2011; Hoel et al., 2017; Lee et al., 2021). The pathogenesis of *Aeromonas* is complex and multifactorial as several virulence factors related to adherence, motility, secretion, and toxins are involved (Tomás, 2012; Fernández-Bravo and Figueras, 2020).

Antimicrobial resistance (AMR) is an emerging threat to public health around the globe. The extensive use and abuse of antimicrobial drugs for humans and animals, and the spread of resistant bacteria within and between these sectors and the environment, has contributed to the increased emergence and spread of AMR (Mcewen and Collignon, 2018). Resistant bacteria residing in the food chain can spread to humans *via* the foodborne route (Founou et al., 2016; EFSA Panel on Biological Hazards (BIOHAZ), 2021). In the food chain, food animals including fish, vegetables, as well as food-producing environments are considered important reservoirs of resistant bacteria (EFSA Panel on Biological Hazards (BIOHAZ), 2021). Bacteria can be intrinsically resistant or acquire new resistance mechanisms by obtaining genetic materials located in mobile genetic elements (MGEs) such as plasmids or transposons, where the latter phenomenon is known as horizontal gene transfer (HGT; Piotrowska and Popowska, 2015; Founou et al., 2016).

The usage of antimicrobial drugs for humans and animals in Norway is very restrictive compared to most other countries (NORM/NORM-VET, 2020). Only two antimicrobial agents are legally used in aquaculture in Norway, and a substantial decrease in antimicrobial usage has been implemented in aquaculture since the top in 1987 (Love et al., 2020). However, antibiotic residues can reach aquatic environments, not only through use in aquaculture, but also through routes like agricultural run-off or improper wastewater treatment (Sanseverino et al., 2018). The occurrence of multidrug resistant (MDR) *Aeromonas* strains has been reported from RTE seafood on the Norwegian market (Lee et al., 2021) as well as other types of food including marine bivalves (Stratev and Odeyemi, 2016; Albini et al., 2022). In addition, *Aeromonas* have been shown to carry multiple AMR genes as well as MGEs, implying their potential to transfer AMR genes to other bacterial species (Piotrowska and Popowska, 2015; Dubey et al., 2022a,b).

Nevertheless, available information on AMR of *Aeromonas* is limited since it is based on phenotypic resistance patterns, or the screening of target AMR genes. Thus, the characterization of AMR genes and MGEs of MDR *Aeromonas* would be necessary to understand their resistance mechanisms and assess their potential to spread AMR genes. With the development of whole genome sequencing (WGS) technology and bioinformatics tools, it is possible to perform a more comprehensive analysis on obtaining AMR and virulence gene profiles in bacterial genomes. In recent years, comparative genomic analysis on the AMR or virulence genes has enabled us to evaluate the potential role of *Aeromonas* in spreading AMR to other bacteria, as well as to predict the pathogenic potential of the *Aeromonas* (Dubey et al., 2022a,b; Erickson et al., 2023; Song et al., 2023). However, to our knowledge, such analysis has not been conducted on the *Aeromonas* residing in the food chain, particularly in relation to RTE seafood. Therefore, in this study, whole genome sequences of 22 *Aeromonas* isolated from RTE seafood were obtained to explore the presence of all AMR and virulence genes in their genomes. In addition, the presence of MGEs was examined to investigate their potential to disseminate AMR or virulence genes to other bacterial species.

2. Materials and methods

2.1. Bacterial collection

In total, 79 *Aeromonas* isolates were investigated in this study. Among the 79 isolates, 26 were previously isolated from retail sushi products (Hoel et al., 2015), and 43 were isolated from different types of RTE seafood including retail sushi, salmon loins, oysters, and scallops (Lee et al., 2021). All of these 69 isolates were identified as *Aeromonas* spp. based on partial *gyrB* gene sequencing in previous studies (Hoel et al., 2017; Lee et al., 2021). In addition, ten presumptive *Aeromonas* isolates originating from a salmon processing environment (SPE) were donated by Thomassen et al. (2022, 2023), and subjected to *gyrB* gene sequencing for species identification in the present study.

2.2. Species identification by partial *gyrB* gene sequencing

Genomic DNA was extracted from 1 mL of overnight cultures grown in Tryptone Soy Broth (TSB; Oxoid, Oslo, Norway) at 37°C using the protocol for Gram-negative bacteria in the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Oslo, Norway). For PCR amplification, the primers gyrB3F (5′-TCCGGC GGTCTGCACGGCGT-3′) and gyrB14R (5′-TTGTCCGGGTTGT ACTCGTC-3′) were used to amplify an approximately 1,100 bp *gyrB* gene (Yáñez et al., 2003). All PCR reactions were performed with 25 μL containing 1 x PCR buffer (1.5 mM $MgCl_2$), 200 μM of each nucleotide, 0.4 μM each primer, 2.5 U Taq polymerase (Qiagen, Oslo, Norway) and 50–100 ng DNA template. PCR amplification was as follows: initial denaturation at 95°C for 15 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 7 min. PCR products were visualized by electrophoresis in a 1.5% agarose gel

(SeaKem, Lonza Group Ltd., Basel, Switzerland) in 1 × TAE buffer. PCR products were purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific). DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany), and the sequences were compared with available sequences (>97% nucleotide BLAST similarity) in the GenBank database in the National Center for Biotechnology Information (NCBI). A phylogenetic analysis was conducted based on the *gyrB* gene sequence of the 79 isolates and 11 relevant reference strains by the neighbor-joining (NJ) method with bootstrapping (1,000 replicates) using MEGA 11 version 11.0.10 (Tamura et al., 2021), according to the method described by Lee et al. (2021). The list of the reference strains is shown in Supplementary Table S1A.

2.3. Antimicrobial susceptibility testing (AST)

Among the 79 isolates, AST was performed for 36 isolates from Hoel et al. (2015) and Thomassen et al. (2022, 2023), while the antimicrobial susceptibility profile of 43 *Aeromonas* isolates was obtained from the previous study by Lee et al. (2021). AST was conducted by a disk diffusion method according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2016). In brief, the fresh inoculum of each isolate was evenly spread by a sterile cotton swab on Mueller-Hinton agar (MHA; Oxoid, Oslo, Norway). A maximum of five antibiotic disks were placed on each plate on the surface before incubation at 35°C for 16–18 h. After incubation, the diameter of the inhibition zone around the disks was measured, and the degree of susceptibility was categorized as sensitive, intermediate, or resistant according to CLSI criteria (CLSI, 2014, 2016). The resistance pattern of each isolate was examined against 15 antimicrobials belonging to 9 antimicrobial classes including aminoglycosides: gentamicin (10 µg) and tobramycin (10 µg), amphenicols: florfenicol (30 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg), carbapenems: imipenem (10 µg) and meropenem (10 µg), cephalosporins: cefotaxime (30 µg) and ceftriaxone (30 µg), macrolides: erythromycin (15 µg) and oxolinic acid (2 µg), penicillin: ampicillin (10 µg) and mecillinam (10 µg), quinolones: ciprofloxacin (5 µg), tetracyclines: doxycycline (30 µg) and tetracycline (30 µg; Oxoid). Based on the antimicrobial susceptibility pattern, MDR strains were defined as being resistant to at least one antimicrobial agent in three or more antimicrobial classes (Magiorakos et al., 2012).

2.4. WGS and genome assembly

To select the isolates of interest for WGS analysis, the 79 *Aeromonas* isolates were first divided into eight different groups representing eight different *Aeromonas* species based on the *gyrB* gene sequences. Depending on their source of isolation and antimicrobial susceptibility pattern, 22 *Aeromonas* isolates were chosen for WGS. Total genomic DNA was extracted from 1 mL of overnight cultures grown in TSB (Oxoid) at 37°C, using the Genomic Micro AX Bacteria Gravity kit (A&A biotechnology, Poland) according to the manufacturer's protocol. The quality of the DNA was checked on agarose gel, and DNA concentrations were estimated by spectrophotometric measurement using BioTek PowerWave XS (Winooski, VT, United States), Take3 plate and Gen5 2.0 software (BioTek Instruments Inc., Winooski, VT, United States). DNA samples were shipped on ice overnight to the Norwegian Sequencing Center (Ullevål University Hospital, Oslo, Norway) for WGS. Sequencing libraries were prepared using the Nextera DNA Flex Library Prep kit (Illumina, United States). Sequencing was performed using Illumina MiSeq platform (Illumina, United States), with 2 × 300 bp chemistry. The raw paired-end reads were cleaned and quality trimmed using BBDuk[1] and assembled using SPAdes v3.15.4 (Bankevich et al., 2012). Draft genome assemblies were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016). The genome assemblies of the 22 isolates were deposited in the NCBI Genbank with accession numbers (JAOPLB000000000-JAOPLW000000000) (Supplementary Table S1B).

2.5. Multilocus phylogenetic analysis (MLPA) and average nucleotide identity (ANI) analysis

The housekeeping gene sequences of 10 reference strains (either type strains or representative strains) were retrieved from NCBI Genbank (Supplementary Table S1A). For MLPA, six housekeeping gene sequences (*gyrB*, *rpoD*, *gyrA*, *recA*, *dnaJ*, and *dnaX*) were extracted from each of the 22 *Aeromonas* genomes by blasting the nucleotide sequences of 10 reference strains against the assembled genomes. Multiple sequence alignments of each housekeeping gene were performed using CLUSTAL W (Thompson et al., 1994) implemented in MEGA11. The aligned sequences were then concatenated and re-aligned to concatenated sequences (4,172 bp). Genetic distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980) and a phylogenetic tree was constructed by the NJ method with bootstrapping (1,000 replicates) using MEGA 11 (Tamura et al., 2021). To confirm the tree topology, a phylogenetic tree was also created using the maximum-likelihood (ML) method with bootstrapping (100 replicates). To verify the taxonomy, ANI values between the genomes including 22 isolates and eight reference strains were calculated using FastANI (Jain et al., 2018). ANI matrix was clustered by scripy's UPGMA and an ANIclustermap was created using the ANIclustermap pipeline (Shimoyama, 2022). The list of the reference genomes used for ANI analysis is included in Supplementary Table S1C.

2.6. Prediction of virulence genes, AMR genes, and MGEs

Genes associated with pathogenic bacteria virulence factors were identified by using VFanalyzer based on the virulence factors database (VFDB; Liu et al., 2019), where the threshold for virulence factor detection was set at 80%. The profile of virulence factors was visualized using the package pheatmap in R studio v.4.2.2 (R Core Team, 2022). The presence of AMR genes in bacterial genomes was predicted using the NCBI AMRFinderPlus v3.10.45 (Feldgarden et al., 2019), and

1 https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbduk-guide/

ResFinder v4.1 (Bortolaia et al., 2020) with default settings (the threshold for AMR gene identification was set at 90%). PlasmidFinder v2.1 (Carattoli et al., 2014) was used to identify plasmids in the genome assemblies where the threshold for plasmid identification was set at 80%. MGEs were identified using MobileElementFinder v1.0.3 (Johansson et al., 2021) with default settings (minimum sequence identity of 90%). Circular maps of chromosomes and plasmids were visualized using the Proksee software (Grant et al., 2023).

3. Results

3.1. MLPA and ANI value

The phylogenetic tree based on the *gyrB* gene sequences (a continuous stretch of 929 bp) of the 79 isolates and relevant reference strains was constructed by the NJ method for species identification (Supplementary Figure S1). The MLPA of the 22 *Aeromonas* isolates selected for WGS and relevant reference strains was performed based on the concatenated sequences (4,172 bp) of six housekeeping genes (*gyrB*, *rpoD*, *gyrA*, *recA*, *dnaJ* and *dnaX*) by the NJ method (Figure 1). An identical tree-topology was obtained by the phylogenetic analysis using the ML method, confirming the robustness of the NJ tree (data not shown). The constructed tree comprised two main clusters; one cluster included the species *A. salmonicida*, *A. piscicola*, *A. bestiarum*, and *A. popoffii*, and the other cluster included the species *A. hydrophila*, *A. dhakensis*, *A. caviae*, *A. media*, *A. rivipollensis*, and *A. paramedia* [one of the *A. media* species complex suggested by Talagrand-Reboul et al. (2017)]. All isolates of interest clustered to the respective reference strains. In addition, the same clustering of the isolates to the reference strains, with one exception, was observed in further phylogenetic analysis based on each of the housekeeping gene sequence *gyrB* (908 bp), *rpoD* (657 bp), *gyrA* (709 bp), *recA* (598 bp), *dnaJ* (800 bp) and *dnaX* (500 bp; data not shown). One exception was the clustering of SU58-3 with *A. piscicola* observed in the tree based on the *dnaX* gene sequence, while each of the other five gene sequences of SU58-3 clustered with *A. bestairum*.

Considering the ANI cutoff value of ≥ 96% for strains belonging to the same species, the ANI values between the 22 isolates and reference genome (Figure 2) supported the phylogenetic clustering observed in the tree shown in Figure 1. Our ANI analysis between 22 isolates and reference genomes confirmed the eight different species among our isolates. In addition, an ANI value of 99.9–100% was observed between the two isolates A533 and A536 from retail sushi product in 2015, as well as among the three isolates SU3, SU9 and SU15 from retail sushi in 2019, indicating that those isolates share high enough genomic similarity to be considered identical strains. Thus, only one genome representing the identical strains (A533 and SU3, respectively) was included in the final dataset of 19 *Aeromonas* strains for the prediction of virulence and AMR genes.

3.2. Virulence factors

Major virulence factors examined in this study include five categories; (i) adherence: the genes encoding type I and type IV pili, (ii) motility: the genes encoding polar and lateral flagella, (iii) immune evasion: the gene encoding capsules and lipopolysaccharide (LPS) O-antigens, (iv) secretion systems: the genes encoding type II secretion system (T2SS), type III secretion system (T3SS), and type VI secretion system (T6SS), and (v) toxins: the genes encoding cytotoxic and cytotonic enterotoxins, and exotoxins. Over 250 genes encoding multiple virulence factors were identified among the 19 *Aeromonas* strains, and virulence gene profiles of the 19 genomes were compared to the profiles of two reference genomes: *A. hydrophila* subsp. *hydrophila* ATCC 7966, which is a well-characterized type strain originally isolated from a tin of milk with a fishy odor (Seshadri et al., 2006), and *A. salmonicida* subsp. *salmonicida* A449, which is a type strain isolated from a brown trout with furunculosis (Reith et al., 2008) (Figure 3). Overall, the strains belonging to *A. piscicola*, *A. bestiarum*, *A. salmonicida*, *A. hydrophila*, and *A. dhakensis* contained more virulence factors than the strains belonging to *A. caviae*, *A. media*, and *A. rivipollensis*.

3.2.1. Adherence and motility

In this study, the presence of three distinct type IV pili was examined in 19 *Aeromonas* strains and those included were (i) mannose-sensitive haemagglutinin (Msh) pili, (ii) Tap pili, and (iii) Flp pili. Two reference strains contained the genes encoding all three type IV pili. Msh pili was also present in all 19 strains, while only ten contained a complete 17 gene cluster (*msh-A,-B,-C,-D,-E,-F,-G,-H,-I,-I1,-J,-K,-L,-M,-N,-O,-P,-Q*) encoding the Msh pili and those were the strains *A. hydrophila* A537, *A. rivipollensis* OY1, SU3, SC42, A539, *A. salmonicida* SU2 OY56, LJP441, and *A. bestiarum* SU6, SU58-3. The rest of the strains lacked some of the genes such as *mshA* or *mshQ*. In addition, over 14 genes including *tapD* encoding Tap pili, were found in all 19 strains, while Flp type IV pili were only detected in the *A. salmonicida*, *A. piscicola* and *A. bestiarum* strains (except for SL22). Like two references strains, type I pili were detected in 12 strains belonging to *A. dhakenis*, *A. caviae*, *A. piscicola*, *A bestiarum*, and *A. salmonicida* (except for SL21).

Moreover, over 50 genes encoding polar flagella were detected in all 19 strains as well as in two reference strains. Two major flagellin genes required for optimal polar flagella functions, *flaA* and *flaB*, were identified in most of the strains except for four strains *A. rivipollensis* A539, SU3, SC42, and *A. caviae* SU4-2. Other genes encoding flagellar motor components including *fliG*, *fliM*, *fliN*, *motX*, *motY*, *pomA*(A_2), and *pomB*(B_2) were found in all 19 strains. On the contrary, about 36 genes encoding lateral flagella including *laf* genes were detected in eight strains including *A. rivipollensis*, SU3, SC42, OY1, *A. salmonicida* SU2, OY59, LJP441, *A. bestiarum* SL22, and *A. piscicola* LJP308. The genes encoding the lateral flagella were observed in the reference strain *A. salmonicida* A449, but not in the *A. hydrophila* ATCC 7966.

3.2.2. Immune evasion

Most of the *Aeromonas* strains contained genes encoding capsules except for *A. salmonicida* LJP441. Genes encoding the LPS O-antigens were identified in 11 strains, including *A. dhakensis* A533, *A. hydrophila* A537, *A. media* OY52, *A. rivipollensis* A539, SU3, SC42, *A. salmonicida* SU2, OY56, OY59, *A. bestiarum* SL22 and *A. piscicola* LJP308. On the contrary, none of the reference strains had the genes encoding the capsules or LPS O-antigens.

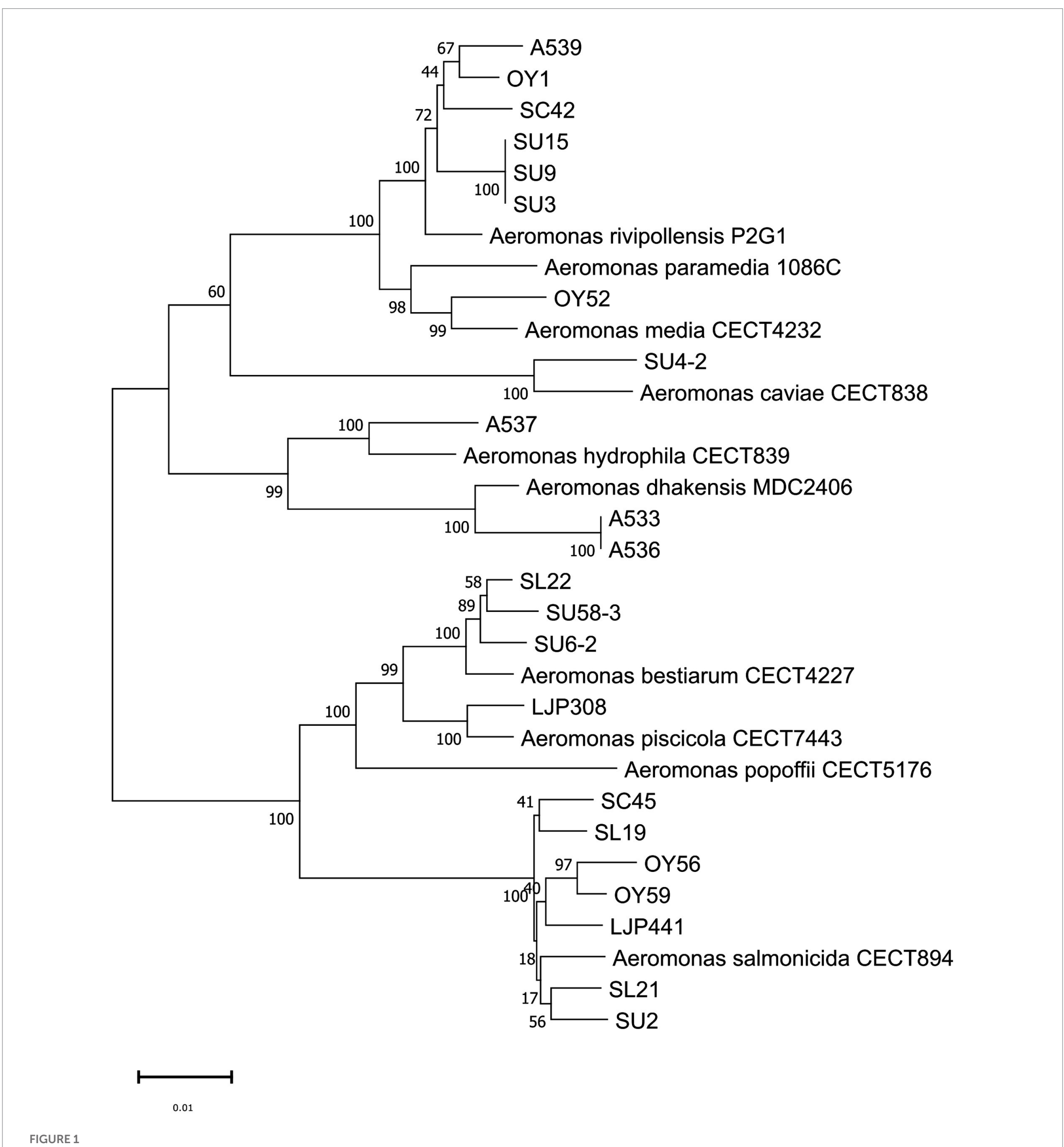

FIGURE 1
Multilocus phylogenetic analysis (MLPA) based on the concatenated sequence of six housekeeping genes (*gyrB*, *rpoD*, *gyrA*, *recA*, *dnaJ*, and *dnaX*, in total 4,172bp) by the neighbor-joining method showing the relationship of 22 *Aeromonas* isolates and 10 reference strains. Numbers at nodes indicate bootstrap values (1,000 replicates) and the scale bar, 0.01 indicates the evolutionary distance of nucleotide substitution per site. The sequence data of the reference strains was retrieved from NCBI Genbank (Supplementary Table S1).

3.2.3. Secretion systems

Genes encoding T2SS were detected in all examined strains including two reference strains. Like the *A. hydrophila* ATCC7966, most of the strains did not contain the genes encoding T3SS. Only three strains including *A. bestiarum* SL22, *A. piscicola* LJP308 and *A. salmonicida* OY59 had over 40 genes encoding T3SS, like the *A. salmonicida* A449; however, the bifunctional toxin gene, *aexT*, was only detected in the reference strain. Moreover, over 20 genes encoding T6SS, including major effectors such as *hcp (or hcp1)*, *vgrG1*, *vasH*, or *vasK*, were detected in most of *Aeromonas* strains, except for three strains including *A. rivipollensis* A539, SC42, and *A. caviae* SU4-2. Both reference strains contained the genes encoding T6SS; however, some of the effector genes such as *hcp* and *vgG* were absent in the genome of *A. salmonicida* A499.

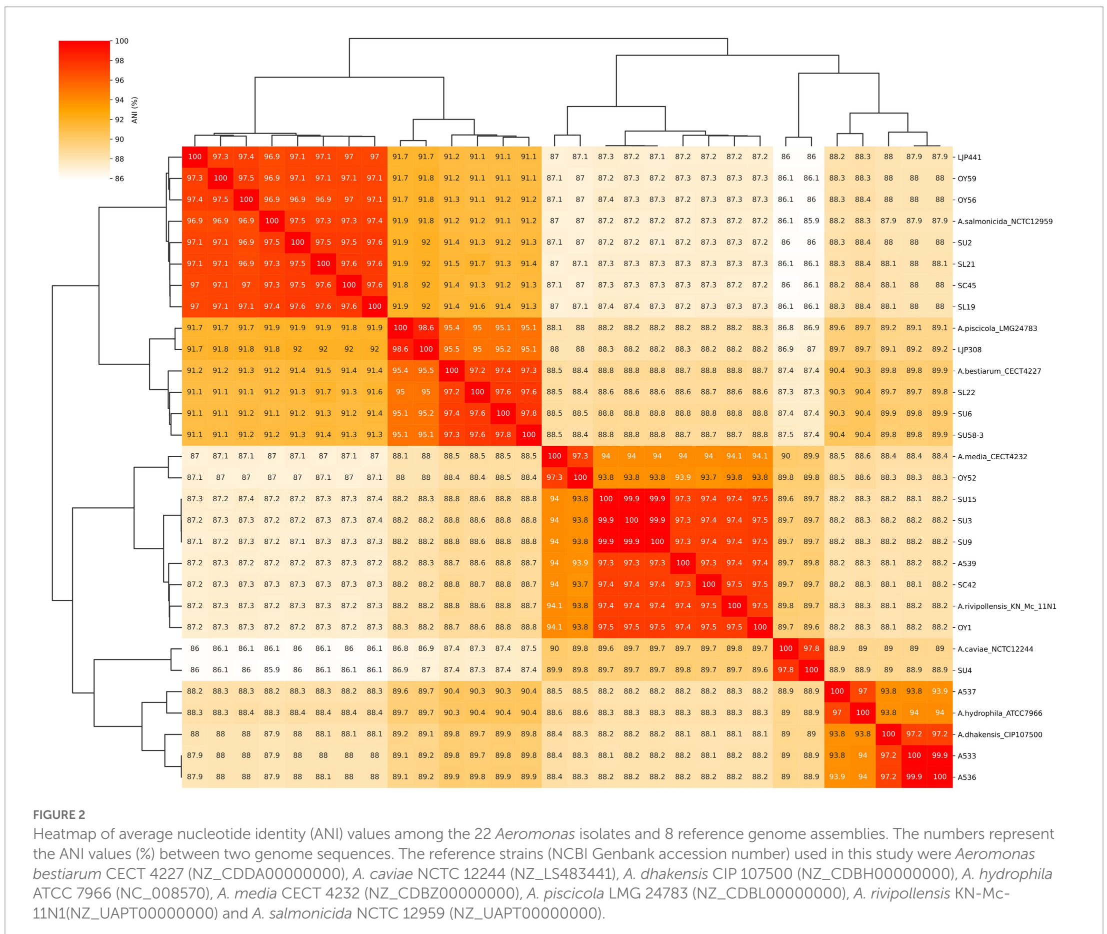

FIGURE 2
Heatmap of average nucleotide identity (ANI) values among the 22 *Aeromonas* isolates and 8 reference genome assemblies. The numbers represent the ANI values (%) between two genome sequences. The reference strains (NCBI Genbank accession number) used in this study were *Aeromonas bestiarum* CECT 4227 (NZ_CDDA00000000), *A. caviae* NCTC 12244 (NZ_LS483441), *A. dhakensis* CIP 107500 (NZ_CDBH00000000), *A. hydrophila* ATCC 7966 (NC_008570), *A. media* CECT 4232 (NZ_CDBZ00000000), *A. piscicola* LMG 24783 (NZ_CDBL00000000), *A. rivipollensis* KN-Mc-11N1(NZ_UAPT00000000) and *A. salmonicida* NCTC 12959 (NZ_UAPT00000000).

3.2.4. Toxin genes

All 19 *Aeromonas* strains contained the genes encoding hemolysin HlyA (*hlyA*), hemolysin III, and thermostable hemolysin, like both reference strains. Genes encoding the extracellular heat-liable hemolysin (*ahh1*) and the aerolysin AerA/cytotoxic enterotoxin Act (*aerA/act*) were also detected in both reference strains and most of our strains, except for the *A. caviae*, *A. media* and *A. rivipollensis*. The heat-stable cytotonic enterotoxin gene (*ast*) was found in the *A. hydrophila* ATCC 7966 as well as nine strains including *A. hydrophila* A537, *A. bestiarum* SU6, SU58-3, SL22, *A. piscicola* LJP308 and *A. salmonicida* SL19, SL21, SC45, OY56. In addition, six genes encoding the repeat toxins (RTX: *rtx-A,-B,-C,-D,-E,-H*) were detected in the *A. hydrophila* ATCC 7966 and ten strains including *A. hydrophila* A537, *A. dhakensis* A533, *A. bestiarum* SU6, SU58-3, and all of the *A. salmonicida* strains except for OY59. Three strains of *A. rivipollensis* SU3, SC42, OY1 possessed only the *rtxA* gene. Furthermore, the exotoxin A (ETA) gene, *toxA* was detected in nine strains including *A. dhakensis* A533, *A. salmonicida* SL19, SL22, OY56, OY59, LJP441, *A. bestiarum* SU6, SU58-3, and *A. piscicola* LJP308, unlike the reference strains.

3.2.5. Other virulence factors

The *adeG* gene encoding efflux pump autoinducer, related to biofilm formation was detected in *A. bestiarum* strain SU58-3. Iron uptake genes, *basG* or *basB* were detected in two strains *A. dhakensis* A533 and *A. hydrophila* A537. The catalase-peroxidase gene, *katG*, associated with stress adaptation was detected in all of the *A. caviae*, *A. media* and *A. rivipollensis* strains. The *neuB2* gene encoding O-linked flagellar glycosylation was detected in two strains *A. media* OY52 and *A. salmonicida* SU2. More detailed information on virulence gene profile is available in Supplementary Table S3.

3.3. AMR

Phenotypic antimicrobial susceptibility profiles of 79 *Aeromonas* isolates (Supplementary Table S2) showed that all isolates were resistant to ampicillin as expected. Resistance to erythromycin, florfenicol and oxolinic acid was observed in 57, 48, and 22% of the strains, respectively. Reduced susceptibility to oxolinic acid was observed in 34% of the isolates. In addition, resistance (10%) or reduced susceptibility (11%) to imipenem was mostly observed in

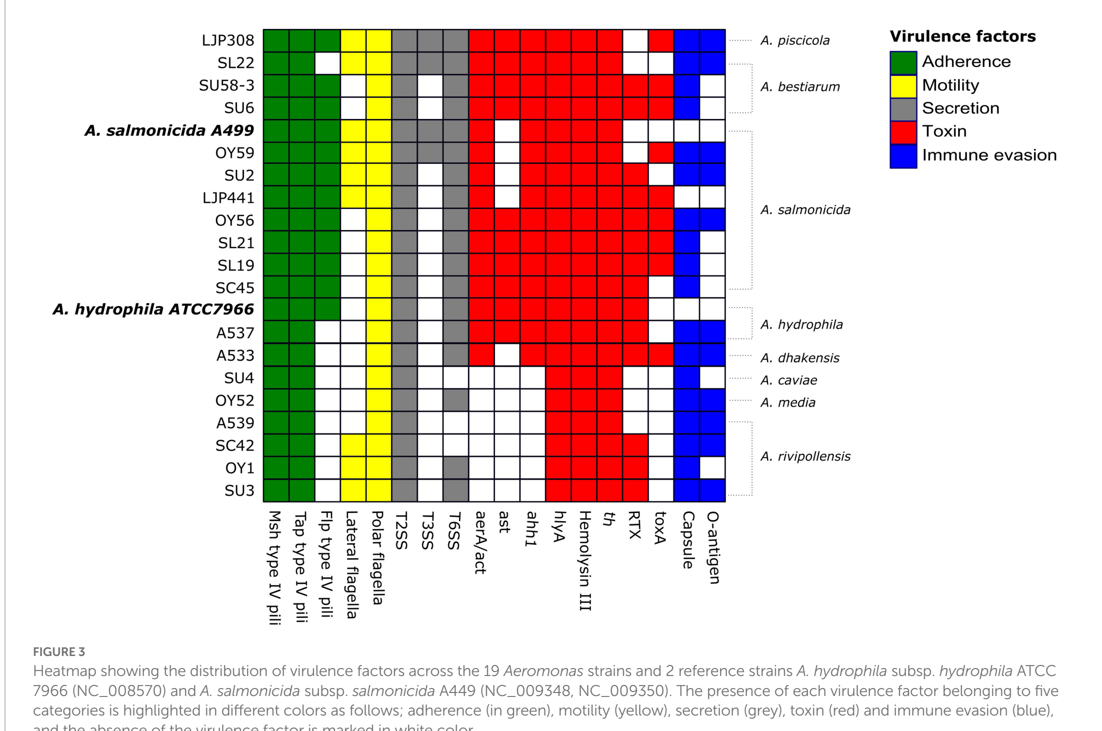

FIGURE 3
Heatmap showing the distribution of virulence factors across the 19 *Aeromonas* strains and 2 reference strains *A. hydrophila* subsp. *hydrophila* ATCC 7966 (NC_008570) and *A. salmonicida* subsp. *salmonicida* A449 (NC_009348, NC_009350). The presence of each virulence factor belonging to five categories is highlighted in different colors as follows; adherence (in green), motility (yellow), secretion (grey), toxin (red) and immune evasion (blue), and the absence of the virulence factor is marked in white color.

A. salmonicida, *A. bestiarum* and *A. dhakensis* strains. Resistance to cefotaxime was found in the *A. rivipollensis* strain SU2 and SU3, while reduced susceptibility to cefotaxime and ceftriaxone was observed in about 30% of the strains. One *A. salmonicida* strain (OY59) was resistant to both imipenem and meropenem, and another two *A. salmonicida* strains (OY60 and 61) showed reduced susceptibility to tobramycin. The *A. caviae* strain SU4-2 showed reduced susceptibility to trimethoprim/sulfamethoxazole. None of the *Aeromonas* strains were resistant to mecillinam, ciprofloxacin, doxycycline, tetracycline, or gentamycin. Moreover, about 58% of the *Aeromonas* strains were considered MDR. Most MDR strains were originally isolated from RTE seafood, and they were mainly resistant to ampicillin (penicillins), erythromycin (macrolides), florfenicol (amphenicols) and oxolinic acid (quinolones). On the other hand, none of the strains originated from the SPE was considered MDR.

Furthermore, WGS confirmed that all 19 *Aeromonas* strains contained multiple AMR genes in their genomes and revealed the presence of different classes of β-lactamases (Table 1). The class B metallo-β-lactamases (MBL) group was dominated by *cphA1* and *cphA5* found in the *A. salmonicida*, *A. bestiarum*, and *A. piscicola* strains, as well as *cphA2* detected in the *A. dhakensis* and *A. hydrophila* strains. However, genes belonging to class B MBL group were not detected in any of the *A. caviae*, *A. media* and *A. rivipollensis*. Genes belonging to class C β-lactamases (bla_{AQU}, bla_{MOX}, and *cepS*) were detected only in four strains including *A. caviae* SU4-2, *A dhakensis* A533, *A. hydrophila* A537, and *A. media* OY52. All 19 *Aeromonas* strains contained different types of bla_{OXA} genes belonging to the class D β-lactamases group. Identified bla_{OXA} type genes were species-specific as following; bla_{OXA-12} (in *A. dhakensis*, *A. hydrophila*), $bla_{OXA-780}$ (in *A. caviae*), $bla_{OXA-427}$ (in *A. media* and *A. rivipollensis*), *and* $bla_{OXA-956}$ (in *A. salmonicida*, *A. bestiarum*, and *A. piscicola*). The class A β-lactamases group including extended spectrum β-lactamases (ESBL) was not found in any of the *Aeromonas* strains. Other than β-lactamases, the sulfonamide resistance gene, *sul1* and the aminoglycoside resistant gene, *aadA1* were detected in the *A. caviae* SU4-2, and the quinolone resistance gene, *qnrS2* was detected in the *A. rivipollensis* A539. In addition, two genes encoding a major facilitator superfamily (MFS) efflux pump protein, *tet(E)* and *qacEΔ1*, were found in the *A. caviae* SU4-2 while only *tet(E)* was detected in the *A. hydrophila* A537.

3.4. MGEs

The presence of MGEs including plasmids, transposons, and insertion sequence (IS) elements was examined in the 19 *Aeromonas* strains. *A. rivipollensis* A539 was the only strain that contained a plasmid, which belonged to the IncQ1 group. A circular map of the plasmid (6,535 bp) shows the presence of two replication proteins *repA* and *repC*, the mobilization protein *mobA*, as well as the quinolone resistance gene, *qnrS2* (Figure 4A).

All *Aeromonas* strains except for *A. piscicola* LJP308 carried at least one transposase as a part of their IS elements, which belongs to different IS families such as IS1595, IS3, IS481, IS4, IS1634, IS5, IS21, IS30, IS66, IS110, IS200/IS605, IS256, IS630, and ISAs1 (Supplementary Table S4). Among the 19 strains, the *A. caviae* strain

TABLE 1 Genotypic and phenotypic antimicrobial resistance (AMR) profile of 19 *Aeromonas* strains isolated from RTE seafood and a salmon processing environment (SPE).

Identified species	Source of isolation	Isolate ID	Genotypic AMR profile					Phenotypic AMR profile	
			β-lactamases			Efflux pump proteins	Other genes	Resistant	Intermediate
			Class B	Class C	Class D				
Aeromonas caviae	Retail sushi	SU4-2	–	bla_{MOX-15}	$bla_{OXA-780}$	*tet(E), qacEΔ1*	*sul1, aadA1*	AMP	STX
Aeromonas dhakensis	Retail sushi	A533	*cphA2*	$bla_{AQU,}$	$bla_{OXA-12\ (950)}$	–	–	AMP, OA, IPM	–
Aeromonas hydrophila	Retail sushi	A537	*cphA2*	*cepS*	$bla_{OXA-12\ (951)}$	*tet(E)*	–	AMP, OA, EM	–
Aeromonas rivipollensis	Retail sushi	A539	–	–	$bla_{OXA-427}$	–	*qnrS2*	AMP, OA	–
	Retail sushi	SU3	–	–	$bla_{OXA-427}$	–	–	AMP, CTX, OA, EM, FEC	CRO
	Scallop	SC42	–	–	$bla_{OXA-427}$	–	–	AMP, EM, FEC	CTX, CRO, OA
	Oyster	OY1	–	–	$bla_{OXA-427}$	–	–	AMP, EM, FEC	CRO, OA
Aeromonas media	Oyster	OY52	–	bla_{MOX-9}	$bla_{OXA-427}$	–	–	AMP, EM FEC	CTX, CRO, OA
Aeromonas bestiarum	Retail sushi	SU6-2	*cphA1*	–	$bla_{OXA-956}$	–	–	AMP	IPM
	Salmon loin	SL22	*cphA1*	–	$bla_{OXA-956}$	–	–	AMP, IPM, EM	–
	Retail sushi	SU58-3	*cphA1*	–	$bla_{OXA-956}$	–	–	AMP	–
Aeromonas piscicola	Inlet water (SPE)	LJP308	*cphA1*	–	$bla_{OXA-956}$	–	–	AMP, EM	–
Aeromonas salmonicida	Retail sushi	SU2	*cphA1*	–	$bla_{OXA-956}$	–	–	AMP	IPM
	Salmon loin	SL19	*cphA1, cephA3*	–	$bla_{OXA-956}$	–	–	AMP, IPM, EM	–
	Salmon loin	SL21	*cphA5*	–	$bla_{OXA-956}$	–	–	AMP, IPM, EM	–
	Scallop	SC45	*cphA5*	–	$bla_{OXA-956}$	–	–	AMP, IPM, EM	–
	Oyster	OY56	*cphA1*	–	$bla_{OXA-956}$	–	–	AMP, EM, FEC	OA, IPM
	Oyster	OY59	*cphA5*	–	$bla_{OXA-956}$	–	–	AMP, IPM, MEM, EM, FEC	OA
	Gutting machine (SPE)	LJP441	*cphA1*	–	$bla_{OXA-956}$	–	–	AMP	IPM

AMP: ampicillin, CTX: cefotaxime, CRO: ceftriaxone, OA: oxolinic acid, IMP: imipenem, MEM: meropenem, EM: erythromycin, FEC: florfenicol, and STX: trimethoprim/sulfamethoxazole.

SU4-2 carried the highest number (21) of IS elements, and they were widely distributed in the chromosome. In addition, composite transposons (*cn*) together with their respective IS elements were identified in 12 strains including *A. hydrophila* A537, *A. salmonicida* SL19, OY56, and all strains of *A. bestiarum* and *A. rivipollensis*. In contrast, a non-composite transposon (*Tn*) was only detected in the *A. caviae* strain SU4-2. A circular map of the *A. caviae* SU4-2 genome shows the presence of the transposon *Tn512*, containing the *mer* operon encoding mercury-resistance, as well as a class I integron (*IntI1*; Figure 4B). Two AMR genes *sul1* and *aadA1,* together with an efflux pump gene, *qacEΔ1*, were located between Tn*512* and *IntI.* In addition, a transcription repressor, *tet(R)*, was located next to a tetracycline efflux MFS efflux pump, *tet(E)*.

4. Discussion

In this study, 79 *Aeromonas* isolates from RTE seafood and a SPE were subjected to species identification using *gyrB* gene sequencing, and these isolates were tested for susceptibility against 15 antimicrobial agents. Based on the *gyrB* gene sequence identity, 22 isolates representing eight different *Aeromonas* species were selected for WGS considering their source of isolation and phenotypic resistance patterns. A MLPA was performed based on the concatenated sequences of six housekeeping genes in the whole genome sequences of 22 isolates for taxonomy classification. The whole genomes of 19 *Aeromonas* strains were further examined to predict the presence of virulence genes, AMR genes as well as MGEs using different databases.

Identification of *Aeromonas* strains at the species level is still controversial and challenging due to the genetic heterogenicity in the genus *Aeromonas* (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020). Molecular identification methods using 16S ribosomal RNA gene or housekeeping genes such as *gyrB* or *rpoD*, have been widely used for defining species and assessing the phylogenetic relationships within genus *Aeromonas* (Yáñez et al., 2003; Soler et al., 2004). Nevertheless, previous studies have shown the limitation of using a single gene sequence to infer the phylogeny of closely related *Aeromonas* species, due to the different phylogenetic

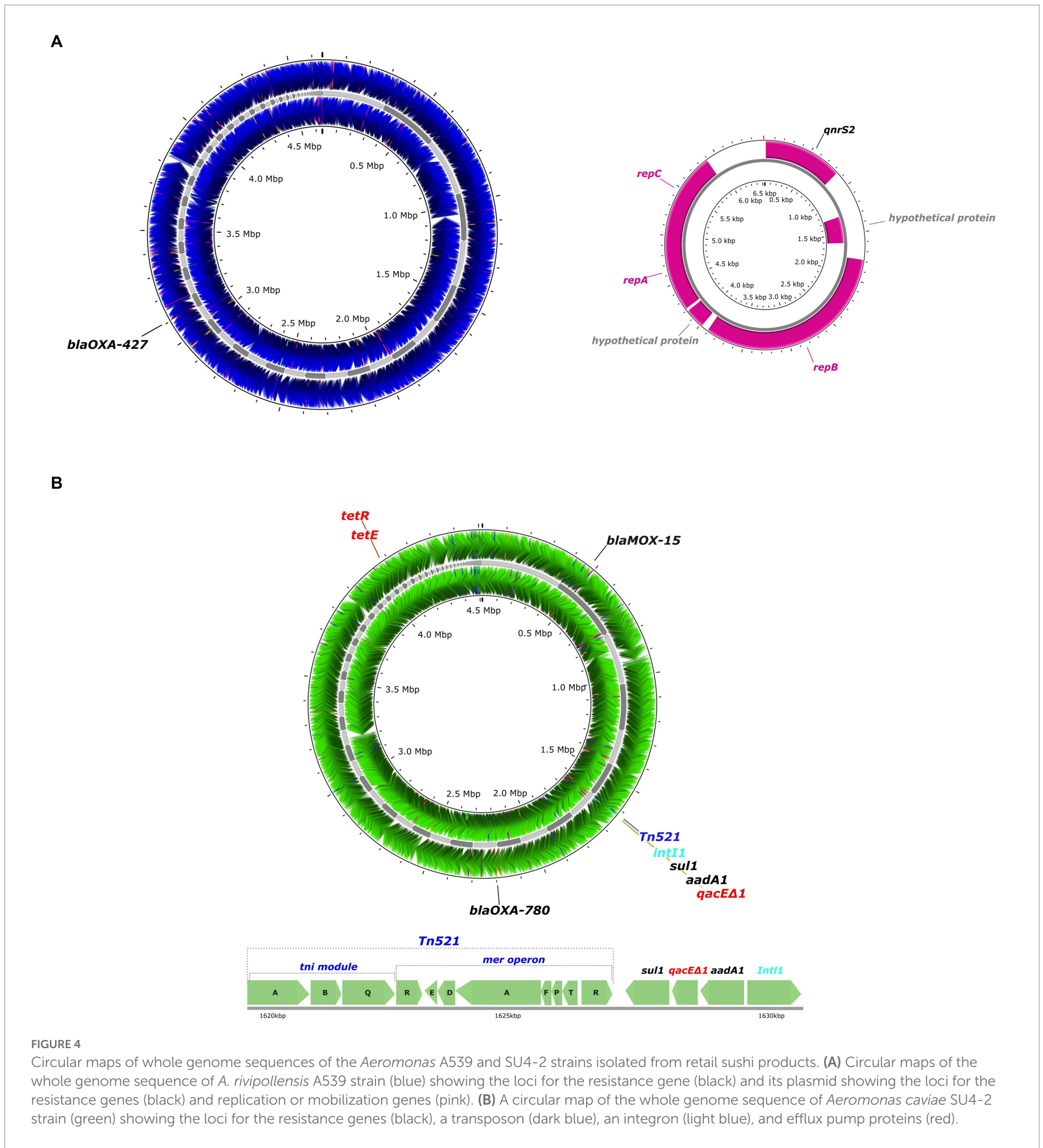

FIGURE 4
Circular maps of whole genome sequences of the *Aeromonas* A539 and SU4-2 strains isolated from retail sushi products. **(A)** Circular maps of the whole genome sequence of *A. rivipollensis* A539 strain (blue) showing the loci for the resistance gene (black) and its plasmid showing the loci for the resistance genes (black) and replication or mobilization genes (pink). **(B)** A circular map of the whole genome sequence of *Aeromonas caviae* SU4-2 strain (green) showing the loci for the resistance genes (black), a transposon (dark blue), an integron (light blue), and efflux pump proteins (red).

resolution of protein-coding genes or possible horizontal gene transfer or recombination (Navarro and Martínez-Murcia, 2018). Currently, a MLPA of concatenated sequences of five or more housekeeping genes has been recommended for the correct identification of *Aeromonas* species (Martínez-Murcia et al., 2011; Navarro and Martínez-Murcia, 2018). In recent years, WGS technology has become more common, and the number of available *Aeromonas* genome sequences in the NCBI database is constantly increasing (Fernández-Bravo and Figueras, 2020). ANI is considered as one of the most robust methods to compare genomic relatedness among strains, with organisms belonging to the same species showing ≥95–96% ANI value (Richter and Rosselló-Móra, 2009). Currently, an ANI value of 96% is the recommended cutoff to define species boundaries of *Aeromonas* (Colston et al., 2014). ANI analysis has been used for the taxonomic verification of *Aeromonas* species (Figueras et al., 2014; Beaz-Hidalgo et al., 2015).

In our study, the phylogeny of the 22 isolates were analyzed by the MLPA based on the concatenated sequences of six housekeeping genes. The constructed tree by MLPA showed the robust topology with high supporting values by both NJ and ML methods. The phylogenetic clustering of the 22 isolates to eight different *Aeromonas* species could be verified by the ANI value ≥96%. We also constructed

the six different phylogenetic trees based on each of the housekeeping gene sequences that extracted from the whole genome sequences. The species identity of 22 isolates based on each of the six gene markers and the concatenated sequences of the six gene markers was identical. One exceptional clustering of SU58-3 with *A. piscicola* was found based on the *dnaX* gene sequence. Nevertheless, we can conclude that the species identity of SU58-3 can be confirmed to be *A. bestiarum* by the ANI value of 97% between SU58-3 and *A. bestiarum*, while the ANI value was less than 95% between SU58-3 and *A. piscicola*. Additionally, the clustering of the *dnaX* gene sequence of the SU 58–3 with *A. piscicola* unlike other gene sequences suggested that using single gene sequence such as *dnaX* gene might not be specific enough to distinguish the closely related species such as *A. piscicola* and *A. bestiarum*.

Mesophilic *Aeromonas* strains are considered emerging pathogens in humans, causing gastrointestinal and extraintestinal infections with various clinical manifestations (Tomás, 2012; Fernández-Bravo and Figueras, 2020). *Aeromonas* pathogenicity is considered a multifactorial process, and the presence of several virulence factors can enable *Aeromonas* to adhere, invade, and destroy the host cells (Tomás, 2012; Fernández-Bravo and Figueras, 2020). Genes related to adherence, motility, immune evasion, secretion, and toxin production were the major virulence factors of *Aeromonas* strains examined in this study.

Aeromonas have two independent flagella systems for motility: polar flagella, required for the motility in a liquid environment (swimming) and lateral flagella, which is needed for the movement across a solid surface (swarming) (Kirov et al., 2002). While both flagella systems are involved in the early colonization of human intestinal cells as well as biofilm formation, not all mesophilic *Aeromonas* can produce a lateral flagella system (Kirov et al., 2004). In our study, 47% of 19 *Aeromonas* strains had the genes encoding the lateral flagella including *laf* genes, which are the major genes for lateral flagella biosynthesis (Canals et al., 2006a). However, the absence of the lateral flagella system in the reference strain *A. hydrophila* ATCC 7966 might imply that they might be not absolutely required for the virulence mechanism (Seshadri et al., 2006). Moreover, all 19 strains carried over 50 genes expressing polar flagella, while two major flagellin genes *flaA* and *flaB* were detected only in 15 strains. A previous study by Canals et al. (2006b) showed that the double mutant *flaA* and *flaB* caused the loss of polar flagella and reduced adherence and biofilm formation, implying that only those strains containing these two genes are likely to have the polar flagella with optimal functions.

Among type IV pili detected in mesophilic *Aeromonas* spp., Msh bundle-forming pili are known to be the major adherence system responsible for cell adherence, colonization, and biofilm formation (Kirov et al., 1999; Hadi et al., 2012). A complete gene cluster of Msh pili (*mshA-Q*) observed in ten strains in our study, have been characterized from *A. veronii bv. sobria* and these genes are required for the optimal function of cell adherence and biofilm formation (Hadi et al., 2012). In particular, *mshQ* is known to play an important role in the Msh pili biosynthesis of *A. hydrophila* (Qin et al., 2014). In the genome of *A. hydrophila* ATCC7966, some of the genes *mshA, mshk, mshP* were absent, implying that not all the 17 genes might be required for the adherence function of other species. In addition, genes encoding Tap pili were detected in all strains, including a TapD protein (t*apABCD*), which is known to be associated with the assembly and functionality of Msh pili as well as extracellular secretion of aerolysin and proteases, contributing to the T2SS (Pepe et al., 1996; Hadi et al., 2012). Moreover, the distribution of the genes encoding Flp pili was species-related, since those genes were only detected in the *A. piscicola*, *A. bestiarum*, and *A. salmonicida* strains. While very little is known about the specific role of Flp pili in mesophilic *Aeromonas*, the Flp pili of psychrophilic *A. salmonicida* strains have previously been identified and found not essential for their virulence (Boyd et al., 2008).

Bacterial cell surface polysaccharides including capsules, LPS O-antigens, and S-layers play important roles in immune evasion of many pathogens including *Aeromonas* (Rasmussen-Ivey et al., 2016). In this study, the genes encoding capsules and O-antigens were detected among our *Aeromonas* strains. The capsule covers the outer layers of the bacterial cell wall and is an important virulence factor of *Aeromonas* that helps prevent phagocytosis by host cells and acts as a barrier to toxin substances (Merino and Tomás, 2015). The O-antigen is the most surface-exposed LPS, acting as a colonization factor, and previous studies have shown that *Aeromonas* strains lacking O-antigen were unable to colonize hosts and have reduced expression of T3SS components (Merino et al., 1996; Vilches et al., 2004). Among 19 strains, most of had the gene encoding capsule as a virulence factor, whereas 11 strains representing each of the species except for *A. caviae* having both capsule and O-antigen, are likely involved in the virulence mechanisms of colonizing and invading host cells. However, none of the genes encoding capsule or O-antigen were detected in two reference genomes based on the VFDB. Considering that LPS O-antigen structure of the *A. salmonicida* strains A449 was previously characterized by the previous study (Wang et al., 2005), we cannot rule out the presence of those genes in both reference genomes. It might be that current database of VFDB is not fully updated with the genes associated with capsules or O-antigens of *Aeromonas*.

Of six secretion systems identified in Gram-negative bacteria, only T2SS, T3SS and T6SS were detected in the 19 strains. T2SS (detected in all strains) is an essential pathway for the pathogenesis of *Aeromonas*, since it is involved in the extracellular secretion of virulence factors such as DNase, protease, hemolysin and aerolysin (Sandkvist, 2001; Lowry et al., 2014). Both T3SS and T6SS are considered virulence markers of *Aeromonas* (Tomás, 2012). T3SS is a needle-like structure, injecting effectors directly into host cells, different T3SS effectors such as AexT, AexU, AopP, AopO and AopH have previously been identified in virulent *A. hydrophila* and *A. salmoncidia* strains (Burr et al., 2003; Sha et al., 2007). On the other hand, other studies have shown that both environmental and epidemic *A. hydrophila* strains lacked the genes encoding T3SS, implying that alternate secretion system is more critical for the virulence of *A. hydrophila* and probably other species as well (Seshadri et al., 2006; Pang et al., 2015). Like T3SS, T6SS could also inject their effectors into the host cells, and four effectors including Hcp (hemolysin coregulated protein), VgrG (valine-glycine repeat protein G), vasH (sigma factor 54 activator) and vasK are typical characteristics of the T6SS (Suarez et al., 2008, 2010). A previous study has showed that deletion of two genes *hcp1* and *vgG1* in virulent *A. hydrophila* strains significantly reduced their virulence (Tekedar et al., 2019). Besides, the T6SS has also been characterized from non-pathogenic *Aeromonas* strains (Pang et al., 2015; Rasmussen-Ivey et al., 2016). In our study, the T6SS including two genes (*hcp1* and *vgG1*) was highly prevalent among the

strains, implying their potential roles in pathogenicity and biofilm formation, while most of the strain lacked the genes encoding T3SS.

Two main types of enterotoxins are present in *Aeromonas* spp.: cytotoxic and cytotonic (Tomás, 2012). Cytotoxic enterotoxins include Act and aerolysin, known as the main virulence factors of *A. hydrophila*, and responsible for hemolytic, cytotoxic and enterotoxic activity (Chopra et al., 1993; Chopra and Houston, 1999). Two types of cytotonic enterotoxin include heat-liable Alt, and heat-stable Ast (Chopra et al., 1994). Hemolysins are cytotoxic and pore-forming toxins produced by pathogenic bacteria, and two hemolysin genes (*hlyA* and *aerA*) have been detected from all virulent *A. hydrophila* strains from the previous studies (Wong et al., 1998; Heuzenroeder et al., 1999). In addition, a previous study by Wang et al. (2003) showed that the combination of *aerA* and *ahh1* genes seemed to be the most cytotoxic genotype of hemolysins, identified from all virulent *A. hydrophila*. In our study, three hemolysin genes encoding hemolysinA (*hlyA*), thermostable hemolysins (*th*) and hemolysinIII (*hlyIII*) were detected in all strains. Both *aerA* and *ahh1* encoding aerolysin and extracellular hemolysin were detected in the *A. dhakensis*, *A. hydrophila*, *A. bestiarum*, *A. piscicola* and *A. salmonicida* strains, implying their potential for cytotoxic effects. Moreover, a RTX operon consisting of six genes (*rtxACHBDE*) were detected in some of the *A. hydrophila*, *A. dhakensis*, *A. bestiarum* and *A. salmonicida* strains, while only *rtxA* (exotoxin) was detected in the *A. rivipollensis* strains. A previous study by Suarez et al. (2012) showed that *rtxA* plays an important role in host cell rounding and apoptosis.

Our data showed the presence of multiple AMR genes in the *Aeromonas* strains regardless of the source of isolation. A large proportion of AMR genes detected in our *Aeromonas* strains belonged to the Ambler class B, C, and D *β*-lactamases, and species-specific distribution of *β*-lactamases genes was observed in accordance with previous observations (Fosse et al., 2003; Chen et al., 2012; Dubey et al., 2022a). *Aeromonas* spp. could produce *β*-lactamases which confer resistance to a broad spectrum of *β*-lactams antibiotic by hydrolyzing the four-membered *β*-lactam ring of antibiotics, and Ambler class B, C, and D *β*-lactamases are known as three major classes of chromosomally mediated β-lactamases detected in *Aeromonas* (Janda and Abbott, 2010).

Among the Ambler class B *β*-lactamases, the most prevalent gene among the 19 *Aeromonas* genomes was *cphA*. Previous research has reported that *cphA* gene is considered intrinsic among environmental *Aeromonas* spp., showing carbapenems-hydrolyzing activity (Balsalobre et al., 2009). In our study, cphA *β*-lactamases such as *cphA1*, *cphA2*, or *cphA5* were detected in the chromosome of several strains, which showed phenotypic resistance or reduced susceptibility to imipenem or meropenem (carbapenems). The presence of those genes in the *Aeromonas* strains is likely to confer their phenotypic resistance to carbapenems. In addition, we observed the species-specific distribution of *cphA* genes, in accordance with previous studies (Rossolini et al., 1995; Chen et al., 2012). The class B *β*-lactamase genes, particularly *cphA* seem to be prevalent among *Aeromonas* spp., as these genes have frequently been observed in both environmental and clinical isolates of *Aeromonas* (Rossolini et al., 1995; Balsalobre et al., 2009; Wu et al., 2012).

Of the 19 strains, only four strains contained the genes belonging to the Ambler class C *β*-lactamases, associated with the resistance mechanisms of many β-lactam antibiotics, including narrow spectrum cephalosporins, third generation cephalosporins, but less active on fourth generation cephalosporins (Bush et al., 1995). Class C *β*-lactamases have been identified in both chromosomes and plasmids of *Aeromonas* spp. originating from various sources (Piotrowska et al., 2017; Ebmeyer et al., 2019; Piccirilli et al., 2022). The class C *β*-lactamases genes detected in our study were bla_{MOX-9} (in *A. media*), bla_{MOX-15} (in *A. caviae*), bla_{AQU} (in *A. dhakensis*), and *cepS* (in *A. hydrophila*). Among these, bla_{MOX} variants from bla_{MOX-3} to bla_{MOX-12} have previously been found in both environmental and clinical isolates of *Aeromonas* spp. (Ye et al., 2010; Piotrowska et al., 2017). In addition, bla_{MOX-9} was previously found in the transposon of *A. media* species and considered as a mobile antibiotic resistance gene (Ebmeyer et al., 2019; Piccirilli et al., 2022), whereas this gene detected in our study was encoded in the chromosome of the *A. media* strain. Moreover, the presence of bla_{AQU} was observed in clinical isolates of *A. dhakensis* showing cefotaxime resistance (Wu et al., 2013), and *cepS* together with bla_{OXA-12} and *cphA7* genes were detected in clinical strains of *A. hydrophila* strains showing carbapenem resistance (Hilt et al., 2020). Both bla_{AQU} and *cepS* have also been detected in environmental isolates of *Aeromonas* spp. (Wang et al., 2021; Dubey et al., 2022a).

Among the Ambler class D *β*-lactamases, oxacillin-hydrolyzing type β-lactamases (OXAs) was observed in all 19 strains. OXAs can confer resistance not only to penicillin, but also to cephalosporins and carbapenems (Evans and Amyes, 2014). OXAs have been identified among several Gram-negative bacteria, including *Aeromonas* spp. (Poirel et al., 2010). The first OXA-like gene identified in the chromosome of *Aeromonas* spp. was designated as bla_{OXA-12}, and bla_{OXA-12} associated genes including new variants such as $bla_{OXA-427}$, $bla_{OXA-780}$, $bla_{OXA-830}$ and $bla_{OXA-956}$ are considered innate in *Aeromonas* spp. (Rasmussen et al., 1994; Chen et al., 2019). Accordingly, four different bla_{OXA} genes (bla_{OXA-12}, $bla_{OXA-427}$, $bla_{OXA-780}$, $bla_{OXA-956}$) detected in our study were chromosomally encoded in the *Aeromonas* genomes, and a species-related distribution was observed. Previous studies have also reported the presence of bla_{OXA} genes in the *Aeromonas* spp. isolated from environmental (Moura et al., 2012; Piotrowska et al., 2017) and clinical samples (Hilt et al., 2020; Tang et al., 2020).

Other than *β*-lactamases, the quinolone resistance gene *qnrS2* was present in the *A. rivipollensis* strain A539 showing phenotypic resistance to quinolones. Furthermore, the sulfonamide resistant gene *sul1* was detected in the *A. caviae* strain SU4-2, that phenotypically showed reduced susceptibility to trimethoprim/sulfamethoxazole. On the other hand, some discrepancies between genotypic and phenotypic resistance were observed. For instance, phenotypic resistance to erythromycin or florfenicol could not be predicted based on the AMR gene profiles, and no correlation was found between the aminoglycoside resistance gene, *aadA1* and the phenotypic resistance of the *A. caviae* strain SU4-2. Both AMRFinder and ResFinder databases have been constructed with high accuracy in predicting genotype–phenotype concordance for some foodborne pathogens; however, the outcomes may depend on the bacterial species, the type of antibiotics and the associated mechanism of resistance (Feldgarden et al., 2019; Bortolaia et al., 2020). In addition, incorrect phenotypic data might be the reason for discrepancies, since repeating phenotypic testing could have resolved most discrepancies between phenotypes and predicted genotypes using ResFinder (Zankari et al., 2013). Moreover, multidrug efflux pump proteins were detected from the two strains *A. caviae* SU4-2 and *A. hydrophila* A537. The MFS efflux pump gene, *qacEΔ1* linked to sulfonamide (*sul1*) and aminoglycoside (*aadA1*) resistance genes was observed in the *A. caviae* strain SU4-2,

while another efflux pump gene, *tet(E)* linked to the *tet(R)* was found in both *A. caviae* SU4-2 and *A. hydrophila* A537 strains. Similarly, the *qacEΔ1* together with other resistance genes (*sul1*, *aadA2*) were also detected in *A. caviae* and *A. hydrophila* strains isolated from fish (Dubey et al., 2022a). In addition, the *tet(E)* has previously been identified in *Aeromonas* (Dubey et al., 2022b; Erickson et al., 2023).

A pool of genes located in MGEs such as plasmids, transposons, and integrons are considered flexible and transferrable, and may be associated with virulence factors, toxic compounds as well as antibiotic resistance (Piotrowska and Popowska, 2015). Several studies have reported the presence of MGEs in *Aeromonas* strains isolated from aquatic environments and fish, and their association with resistance or virulence determinants (McIntosh et al., 2008; del Castillo et al., 2013; Dubey et al., 2022a; Song et al., 2023). In our study, an IncQ plasmid carrying *qnrS2* was detected in the *A. rivipollensis* strain A539 isolated from a retail sushi product. Other studies have suggested the strong relationship between the IncQ plasmid and *qnrS2*, where they have observed the IncQ plasmids harboring *qnrS2* in *Aeromonas* strains isolated from fish and water (Cattoir et al., 2008; Majumdar et al., 2011; Han et al., 2012). Moreover, we found the co-localization of a transposon Tn521 containing a mercury operon and a class I integron (*Int11*) with two AMR genes (*sul1, aadA1*) and an efflux pump protein (*qacEΔ1*) in the *A. caviae* strain SU4-2, implying co-resistance of mercury and antibiotic resistance. Co-resistance occurs when resistance determinants to heavy metals and antibiotics are harbored together in the same MGEs, and this co-localization could result in co-selection mechanisms of other genes in the same elements (Chapman, 2003; Baker-Austin et al., 2006). Some studies have reported the IncA/C plasmids of *Aeromonas* spp. carrying the mercury operon and AMR genes in the class I integron (McIntosh et al., 2008; del Castillo et al., 2013;). Besides, several transposons and IS elements detected in *Aeromonas* spp. have strongly been associated with *β*-lactamases in previous studies (Girlich et al., 2011; Picão et al., 2013). However, we found no strong association between other MGEs identified in our *Aeromonas* strains and AMR genes.

5. Conclusion

In the present study, 22 *Aeromonas* strains isolated from RTE seafood and a SPE were classified into eight different *Aeromonas* species based on the MLPA and ANI analysis. Most strains contained several genes encoding major virulence factors related to adherence, motility, immune evasion, secretion systems, and toxins, and in particular, we observed the high incidence of enterotoxins, T6SS and its major effectors. Multiple AMR genes encoding class B, C and D *β*-lactamases were found in all *Aeromonas* strains and their distribution was species-related. In addition, the presence of other AMR genes located in MGEs such as an IncQ type plasmid in the *A. rivipollensis* strain A539 and a transposon and a class I integron in the *A. caviae* SU4-2 indicates their potential to disseminate AMR genes to other bacteria. Considering that most *Aeromonas* strains were isolated from RTE seafood, our study suggests that *Aeromonas* strains circulating in the food chain could potentially be pathogenic and act as a vector for dissemination of AMR genes to other bacteria residing in the same environments. Thus, we highlight the importance of collecting more knowledge of AMR and mobilome in the genus *Aeromonas* to understand their ability to transfer AMR within the food chain, and the potential risk of AMR caused by *Aeromonas* circulating in the food chain should be carefully monitored.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material and in the NCBI database under accession number: PRJNA877469.

Author contributions

H-JL: manuscript preparation, methodology, bioinformatics analysis, editing, and submission. JS: methodology, bioinformatics analysis, and editing. JL: editing and supervision. B-TL, SH, and AJ: conceptualization, editing, and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1175304/full#supplementary-material

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Paper III

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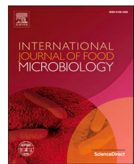
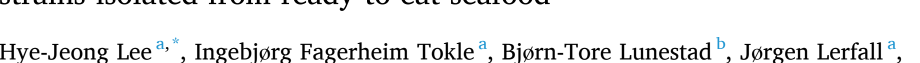


The effect of food processing factors on the growth kinetics of *Aeromonas* strains isolated from ready-to-eat seafood



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ABSTRACT

Aeromonas are ubiquitous aquatic bacteria and frequently isolated from seafood. There is growing awareness of *Aeromonas* as foodborne pathogens, particularly in connection with consumption of ready-to-eat (RTE) seafood. The aim of this study was to investigate the effect of food processing factors on the growth kinetics of eight *Aeromonas* strains (representing seven species) isolated from RTE seafood. The effect of low temperature (4 and 8 °C) in combination with different NaCl concentrations (0.5–6.5 %) or with two purified condensate smokes (PCSs; Red Arrow SmokEz VTABB and JJT01) at different concentrations (0–0.26 %) was studied in Trypton Soy Broth (TSB). In food processing, application of PCS is considered healthier than traditional smoking. Growth kinetics parameters of each strain were estimated by using a primary predictive model. Our result showed that the addition of 3.5 % NaCl at refrigeration temperature (4 °C) was not sufficient to inhibit the growth of *A. media*, *A. bestiarum*, *A. piscicola*, and *A. salmonicida*, while higher NaCl concentration (≥5.0 %) at 8 °C suppressed their growth. On the other hand, our result demonstrated the antimicrobial potential of using PCS at maximal allowed concentration (0.26 %) against *Aeromonas*. PCS concentration and phenol content were important factors influencing the growth kinetics parameters of *Aeromonas*. Moreover, the growth kinetics of three *Aeromonas* strains were further studied in commercially produced vacuum-packed fresh and cold-smoked salmon stored at 4 °C for 14 and 21 days, respectively. Our results demonstrate that vacuum packing combined with cold storage at 4 °C was insufficient to inhibit the growth of *Aeromonas* in fresh salmon, while the growth was inhibited in a minimally salted cold-smoked salmon (salt content of 1.8 %). Our study implies that mild food processing factors applied in the production of RTE seafood might not guarantee the total inhibition of *Aeromonas*. Even though further studies on evaluating the antimicrobial potential of PCSs in actual seafood matrixes are necessary, the present study suggests that PCS technology might be a promising approach to prevent the potential growth of *Aeromonas*.

1. Introduction

With the recent trend of consuming food perceived as healthier and more natural, consumption of ready-to-eat (RTE) seafood such as sushi, sashimi, and cold-smoked fish products has increased (Menozzi et al., 2020). In the production of RTE seafood, the growth of microorganisms is controlled by establishing a series of hurdles that challenge microbial growth to prolong the product's shelf life, an approach known as hurdle technology (Leistner, 2000). The hurdles applied are combinations of two or more mild processing technologies to retain high nutritional and sensory value. Mild processing technologies include a variety of processes e.g., chilling, salting, and smoking (Abel et al., 2022). RTE seafood are consumed without further cooking process, and proper refrigerated storage is thus needed to inhibit the growth of microorganisms (Herrera, 2016). However, mild processing technology combined with cold storage have shown to not completely prevent the growth of potential pathogenic microorganisms, such as *Listeria monocytogenes*, or *Aeromonas* spp. (EFSA BIOHAZ Panel et al., 2018; Hoel et al., 2019). Consequently, consumption of RTE seafood is often one of the major causes of foodborne diseases (EFSA and ECDC, 2021).

Aeromonas are ubiquitous aquatic bacteria, and some species are known as fish pathogens (Janda and Abbott, 2010). Additionally, there

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is a growing awareness of *Aeromonas* as foodborne pathogens (Hoel et al., 2019). This is due to their widespread occurrence in different types of water and food (Stratev et al., 2012), and their pathogenic potential by producing various virulence factors such as hemolysin, aerolysin, and cytotoxic enterotoxins. (Hoel et al., 2017; Nagar et al., 2011). The occurrence of potentially pathogenic *Aeromonas* possessing virulence-associated toxin genes has been reported in various RTE seafood (Hoel et al., 2015; Lee et al., 2021) as well as drinking water (Pablos et al., 2011). Several virulence-associated toxins of *Aeromonas* spp. are likely linked to clinical symptoms of *Aeromonas* infections such as gastrointestinal and extraintestinal infections to humans (Tomás, 2012). The consumption of oysters, salads and drinking water contaminated with *A. hydrophila* has been reported as the source of foodborne outbreaks (Abeyta et al., 1986; Ventura et al., 2015; Zhang et al., 2012). In addition, *Aeromonas* are commonly recognized as spoilage organisms in seafood (Jakobsen et al., 2020; Parlapani et al., 2013). The spoilage potential of *A. salmonicida* in vacuum-packed salmon has been previously reported (Jakobsen et al., 2020).

Microbial growth is influenced by several intrinsic and extrinsic factors, such as temperature, water activity, and pH. Refrigeration is one of the most common practices of controlling microbial growth in order to maintain the quality of food (Mercier et al., 2017). *Aeromonas* is known to grow at a wide range of temperatures from 0 to 45 °C, and the ability of some *Aeromonas* to grow under refrigeration temperature was highlighted in previous studies (Hoel et al., 2018; Vivekanandhan et al., 2003). Salt is a widely used preservative as well as a flavor enhancer. However, reducing salt intake to <5 g/day is recommended due to unhealthy effects of high sodium intake (WHO, 2013). The combination of low temperature and high salt contents can be a limiting factor for *Aeromonas* growth (Gram, 1991), while some *Aeromonas* can grow at the concentration of 4.0 % NaCl at 5 °C (Vivekanandhan et al., 2003). In addition, smoking is one of the oldest methods of preserving fish, and a traditional cold-smoking process includes salting, dehydration, and smoking, which establish combined hurdles (Leroi et al., 2000). To produce a cold-smoked salmon, filets with salt (<6.0 % in the water phase) are subject to wood smoking at 25–30 °C or subject to artificial smoke flavoring by applying atomized purified condensed smokes (PCS) (Løvdal, 2015). However, due to the potential health risk by polycyclic aromatic hydrocarbons (PAHs) from wood smoking, the use of atomized PCS has gained increased attention to produce smoked fish products with healthier and better sensory perspectives (Lingbeck et al., 2014; Waldenstrøm et al., 2021). PCS consist of several compounds including phenols, organic acids, and carbonyls, which are responsible for the antimicrobial effects in smoked products (Lingbeck et al., 2014). Previous studies suggested the antimicrobial potential of PCS against foodborne pathogens including *Aeromonas* (Lingbeck et al., 2014; Sofos et al., 1988; Suñen et al., 2003). Moreover, RTE seafood are usually packed under vacuum (VP) or modified atmosphere (MAP) and subjected to refrigeration to extend the shelf life (Aberoumand and Baesi, 2020). Previous studies showed that VP in combination with refrigeration at 4 °C is insufficient to inhibit *Aeromonas* spp. in RTE salmon and trout products (Jakobsen et al., 2020; Suñen et al., 2003). Although a high CO_2 concentration in MAP has shown better antimicrobial activity against *Aeromonas* than VP, MAP (60–67 % CO_2, initial headspace concentration) could not completely inhibit their growth in salmon nor cod stored at 4 °C (Hoel et al., 2022; Jakobsen et al., 2020).

The prevalence of potentially pathogenic *Aeromonas* was highlighted in different types of RTE seafood on the Norwegian market, which implies a potential risk of consuming perishable RTE seafood without heat treatment (Hoel et al., 2015; Lee et al., 2021). In order to prevent and control the growth of potentially pathogenic *Aeromonas* in RTE seafood, it is critical to understand the growth behaviors under different growth conditions. However, there is limited information about growth characteristics of *Aeromonas* under various processing factors applied in production of RTE seafood. In addition, available information on growth kinetics of *Aeromonas* is limited to some species such as *A. hydrophila*, *A. caviae*, and *A. sobria*. More knowledge on growth characteristics of *Aeromonas* would be beneficial for the practical development of advanced hurdle technology to produce the RTE seafood with higher quality and safety. Thus, we aimed to assess the growth kinetics of eight *Aeromonas* strains representing seven species isolated from RTE seafood subjected to various processing conditions. In the first part of the study, we investigated the effects of different food processing factors such as temperature, NaCl, and PCS. In the second part, the growth of three *Aeromonas* strains were studied in commercially available vacuum-packed fresh and cold-smoked salmon fillets stored at 4 °C for 14 and 21 days, respectively.

2. Materials and methods

2.1. Bacterial strains

Eight *Aeromonas* strains were isolated from retail sushi (*A. caviae* SU4-2, *A. dhakensis* A536, *A. hydrophila* A538, *A. media* SU10, *A. piscicola* SU58-3, and *A. salmonicida* SU2) and Atlantic salmon (*Salmo salar*) loins (*A. salmonicida* SL21 and *A. bestiarum* SL22) in previous studies (Hoel et al., 2015; Lee et al., 2021).

2.2. Salmon juice preparation

As a model system to mimic the nutrient composition of salmon, enriched salmon juice (SJ) was used as a growth medium. The SJ was prepared from fresh salmon loins obtained from a local retailer, according to the method described by Wiernasz et al. (2017) and modified by Stupar et al. (2021). In brief, 500 g of salmon loin was blended with 1 l of distilled water, boiled for 2 min, filtered through a 185 mm folding filter (Schleicher & Schuell, Dassel, Germany), and sterilized at 100 °C for 30 min. Sterile SJ was stored at −45 °C. Before use, 90 ml of SJ was supplemented with 10 ml of 1 M K_2HPO_4/KH_2PO_4 buffer solution (Merck, Darmstadt, Germany) at pH 6.7, 1 g of D-glucose (Merck, Oslo, Norway), and 1.5 g of NaCl (VWR, Oslo, Norway). After enrichment, the medium was filtrated through a 0.45 μm syringe filter (VWR).

2.3. Growth kinetics study in liquid culture systems

Growth kinetics parameters of the *Aeromonas* strains were investigated in two liquid culture systems (TSB and SJ) under different food processing factors; temperature (4 and 8 °C), NaCl (0.5–6.5 %) and purified condensed smoke (PCS, 0–0.26 %).

2.3.1. Temperature

Aeromonas were grown on a tryptone soya agar (TSA) (Oxoid, Oslo, Norway) at 30 °C for 48 h, and one colony was precultured in 10 ml tryptone soya broth (TSB (Oxoid)) at 15 °C for 24 h. From the preculture, 300 μl was inoculated into 30 ml TSB and SJ, respectively, to achieve the initial bacterial concentration of approximately 7 log CFU/ml. The pH of TSB and SJ was measured as 7.4 ± 0.02 and 6.7 ± 0.02, respectively, by using a pH meter (Testo, Germany). Optical density (OD) was measured at 600 nm with a UV spectrophotometer (UV-1800, Shimadzu Crop., Japan) immediately after inoculation. Three parallel cultures were grown for each strain, and was incubated at three different temperatures (4, 8, and 15 °C) over a certain period (69–220 h) and OD was measured until it reached the stationary phase to estimate growth rates. Each TSB and SJ without bacterial inoculum were used as a media control during the OD measurement, respectively.

2.3.2. Sodium chloride (NaCl)

NaCl (Merck) was dissolved into TSB (containing 0.5 % NaCl) in Scott bottles (1000 ml) to obtain the final NaCl concentration (2.0, 3.5, 5.0, and 6.5 %), and subjected to sterilization at 121 °C for 15 min. TSB without supplements (0.5 % NaCl) was used as a control group. The pH of TSB containing 2.0, 3.5, 5.0, and 6.5 % NaCl was measured as 7.28 ±

0.02, 7.18 ± 0.02, 7.18 ± 0.02, and 7.11 ± 0.02, respectively. Each strain was precultured in TSB as described in Section 2.3.1. Each bacterial inoculum (approximately 7 log CFU/ml) was prepared in TSB containing different concentrations of NaCl and stored at two different temperatures (4 and 8 °C). Three parallel cultures were grown for each strain and OD was measured until it reached the stationary phase. TSB containing different concentrations of NaCl without bacterial inoculum was used as a media control during the measurement.

2.3.3. *Purified condensed smoke (PCS)*

Two purified condensed smokes (PCS) (SmokEz VTABB RA12012 and JJT01 30764575) were purchased from Red Arrow™ (Manitowoc, WI, USA). The abbreviation VTABB and JJT01 are used in the later context. Each PCS concentrate was dissolved in TSB to obtain the final concentration (0.026, 0.13, and 0.26 %), where 0.26 % is the recommended maximum level (2.6 g/1 kg processed fish product) according to the manufacturer. The pH of TSB containing 0.026, 0.13 and 0.26 % VTABB was 7.38 ± 0.02, 7.21 ± 0.02 and 6.30 ± 0.02, respectively, and the pH of TSB containing 0.026, 0.13 and 0.26 % JJT01 was 7.38 ± 0.02, 7.21 ± 0.02 and 6.67 ± 0.02, respectively. TSB without supplements was used as a control group. Each strain was precultured in TSB as described in Section 2.3.1. Each bacterial inoculum (approximately 7 log CFU/ml) was prepared in TSB containing different concentrations of PCS and stored at two different temperatures (4 and 8 °C). Three parallel cultures were grown for each strain and OD was measured until it reached the stationary phase. TSB containing different concentrations of each PCS without bacterial inoculum was used as a media control during the measurement.

2.4. *Growth kinetics study in fresh and cold-smoked salmon*

Growth kinetics parameters of three *Aeromonas* strains (*A. bestiarum* SL22, *A. piscicola* SU58-3, and *A. salmonicida* SU2) were investigated in commercially produced vacuum-packed fresh salmon and cold-smoked salmon fillets stored at 4 °C for 14 and 21 days, respectively.

2.4.1. *Sample and bacterial inoculum preparation*

Fresh and cold-smoked salmon fillets (SALMA, Oslo, Norway) from farmed Atlantic salmon (*Salmo salar*) were purchased from a local retail. The cold-smoked salmon (SALMA) was lightly smoked and containing 1.8 g salt/100 g product. The salmon fillets were cut in 20 ± 2 g at five days post-harvest for the fresh salmon and six days post-harvest for the cold-smoked salmon. Each bacterial inoculum was prepared by growing the strain overnight on TSA, and one colony was transferred to a 250 ml conical flask containing 100 ml TSB. The culture was then grown at 8 °C at 230 rpm for 48 h to allow the bacteria to adapt to cold storage. Further, the culture was diluted with TSB to an OD value corresponding to a bacterial concentration of approximately log 3 CFU/ml. Each salmon piece was inoculated with 1 ml of its respective bacterial culture (strain SL22, SU58-3 or SU2), which was spread evenly on the surface with a sterile spreader. Uninoculated salmon (fresh and cold-smoked) was used as a control, resulting in eight experimental groups. Both inoculated and uninoculated salmon samples were air-dried on the bench for 30 min before vacuum packaging (VP). Each sample was packaged in 20 μm polyamide (PA)/70 μm polyethylene (PE) bag (120 × 80 mm, Star-Pack Productive, Boissy-l'Aillerie, France) with a Webomatic Supermax-C vacuum machine (Webomatic, Bochum, Germany). Air was evacuated to an end pressure of 10 mbar before sealing. The vacuum-packed fresh salmon and cold-smoked salmon were stored at 4 °C for 14 and 21 days, respectively.

2.4.2. *Microbiological analysis*

A representative sample (10 g) from each product was aseptically cut and transferred to a sterile stomacher bag and diluted 1:10 with sterile peptone water (1.0 g of bacteriological peptone and 8.5 g of NaCl/l) and homogenized for 60 s in a Stomacher 400 lab blender (Seward Medical, Worthington, UK). Serial dilutions of the homogenate were prepared for further analysis. For quantification of *Aeromonas* spp., the homogenate was streaked on starch ampicillin agar (SAA) supplemented with ampicillin (10 mg/l) (Sigma-Aldrich, Oslo, Norway) and incubated at 37 °C for 24 h according to Nordic Committee on Food Analysis (NMKL) method No. 150 (NMKL, 2004). Total aerobic plate count (APC) including black colonies of H_2S-producing bacteria, were quantified on Lyngby's iron agar (IA) (Oxoid) supplemented with 0.04 % L-cysteine (Sigma-Aldrich), and the plates were incubated at 22 °C for 72 h according to NMKL method No. 184 (NMKL, 2006). Sampling was performed at day 0, 2, 4, 6, 8, 10, 12 and 14 for fresh salmon, and at day 0, 3, 7, 11, 15, 17, 19 and 21 for cold-smoked salmon.

2.5. *Total phenolic content (TPC)*

2.5.1. *TPC of purified condensed smoke (PCS)*

Total phenolic contents (TPC) of the two PCSs (VTABB and JJT01) were analyzed in three replicates according to the method described by Singleton et al. (1999). For analysis, 1 ml of each PCS (0.26 % VTABB and 0.26 % JJT01) was added 1 ml Folin-Ciocalteu reagents (VWR International, France) and diluted with 5 ml deionized water. After 7 min at room temperature, 10 ml Na_2CO_3 (7 %, Alfa Aesar, Germany) was added before the mixture was diluted to 100 ml in a volumetric flask. Both sample solutions and standard solutions made from a serially diluted gallic acid (GA) monohydrate solution (CAS: 5995-86-8, Sigma-Aldrich, USA) were stored dark at room temperature for 2 h before measurement at 750 nm on the UV spectrophotometer. The results of standard solutions were plotted into a standard curve to calculate the total phenolic content of sample solutions. TPC was expressed as mg GA equivalents/100 ml or g sample.

2.5.2. *TPC of cold-smoked salmon*

The TPC of cold-smoked salmon (5 g) was also analyzed in three replicates according to the method described by Singleton et al. (1999). Before analysis, all samples were added to 20 ml of methanol:water (100:80) and homogenized for 5 min (11,000 rpm) with a IKA®T25 digital ULTRA TURRAX® homogenizer (GmBH & Co, Germany). Then the mixture was centrifuged for 15 min at 4800 rpm before the supernatant was filtrated through a 185 mm folded filter (Schleicher & Schuell, Dassel, Germany) into a conical flask. One milliliter of the filtrated supernatant was analyzed as described in Section 2.5.1.

2.6. *Growth kinetic parameters and statistical analysis*

Growth kinetic parameters were estimated from log-transformed bacterial concentrations. OD values were used to convert bacterial concentrations in the liquid culture systems to log CFU/ml, with a conversion factor of OD_{600} of 0.1 = 8.0×10^7 cells/ml. Bacterial concentrations deriving from salmon samples were calculated as log CFU/g. The primary growth model of Baranyi and Roberts (1994) (available at combase.cc) was fitted to the log-transformed data to estimate maximum growth rate (μ_{max}, (log CFU/h)), lag-phase duration (*h*), and maximum population density (Y_{max}, (log CFU/g)). Each value was calculated and expressed as mean values ± standard deviation (SD), together with the coefficient of determination (R^2) and standard error of the fit (SE). In addition, the maximum growth rates (μ_{max}, (log CFU/h)) obtained from the primary model of Baranyi and Roberts were modelled as a function of temperature, using a Ratkowsky square root model (Ratkowsky et al., 1982) (Eq. (1)).

$$\mu = (b(T - T_{min}))^2 \tag{1}$$

where *b* is the slope of the regression line, *T* is the storage temperature and T_{min} is the theoretical minimum temperature for growth. The software program SPSS Statistics (ver. 28, IBM) was used for all statistical analysis (including a one-way ANOVA, a two-way ANOVA, Tukey post

hoc, and *t*-test ($P = 0.05$)).

3. Results

3.1. Effect of temperature, NaCl, and PCSs on Aeromonas growth

3.1.1. Temperature

The effects of different temperatures (4, 8, and 15 °C) on growth kinetics parameters of eight *Aeromonas* strains were studied in TSB and SJ. In TSB, all eight strains were able to grow at 8 and 15 °C (Fig. 1a, c). At 4 °C, five strains were able to grow, whereas three strains of *A. hydrophila*, *A. dhakensis* and *A. caviae* did not grow (Fig. 1e). A significant decrease in maximum growth rates (μ_{max}) was observed as lower temperature was applied (a one-way ANOVA, $P < 0.001$), whereas lag phases (h) were not significantly affected by the temperature ($P = 0.078$). In salmon juice (SJ), the growth pattern of each strain was similar to the TSB at all temperatures, excluding *A. caviae* which did not grow at 8 °C (Fig. 1b, d, f).

When growth kinetics parameters of all strains were compared in the two liquid culture systems, μ_{max} were significantly higher in TSB than SJ (*t*-test, $P < 0.001$). For example, the μ_{max} of *A salmonicida* SU2 was two times higher in TSB ($\mu_{max} = 0.096 \pm 0.003$ log CFU/h) than in SJ ($\mu_{max} = 0.043 \pm 0.002$ log CFU/h) at 15 °C. However, there were no significant differences in lag phases and maximum population densities (Y_{max}) between the two media ($P = 0.188$ and $P = 0.235$, respectively). When the effect of temperature was further examined, significantly higher μ_{max} in TSB were observed at 8 and 15 °C for all strains ($P < 0.001$), but not at 4 °C ($P = 0.165$) (a two-way ANOVA, Tukey post hoc) (Fig. 2a). Significantly longer lag phases were observed in SJ compared to TSB at 4 °C ($P < 0.001$), whereas storage at 15 °C resulted in shorter lag phases in SJ (but not significant $P = 0.103$) as several strains had no detectable lag phase in SJ (Fig. 2b). In addition, Y_{max} was not significantly affected by the temperature.

For each strain, a Ratkowsky square root model was used to describe maximum growth rate (μ_{max}) as a function of storage temperatures (4, 8, and 15 °C) in TSB and SJ media (Table 1). In general, the slope of the regression line (b) was higher in TSB than SJ, indicating a stronger temperature response in TSB than in SJ. Particularly, *A. piscicola* and two strains of *A. salmonicida* showed a stronger temperature response in TSB than SJ. In TSB, and the strongest response to increased temperature was observed for *A. piscicola* compared to the other strains but the inter-species variation was relatively low in TSB. In SJ, a stronger temperature response was observed for *A. media* and *A. bestiarum* compared to the other strains. Three strains of *A. hydrophila*, *A. dhakensis* and *A. caviae* were excluded from the analysis due to no detectable growth at 4 °C.

3.1.2. Sodium chloride (NaCl)

The effects of NaCl at different concentrations (0.5–6.5 %) were studied in TSB at 4 and 8 °C. At 8 °C, all strains were able to grow at concentration ≤3.5 % NaCl (Fig. 3a, b), while none of them were able to grow at higher concentrations (≥5.0 %). *A. dhakensis* showed the longest lag phase (about 50 h) at 3.5 % NaCl (8 °C) compared to the other strains. At 4 °C, all tested strains were able to grow at concentration ≤3.5 % NaCl (Fig. 3c, d). At both temperatures, the concentration of 2.0 % NaCl did not significantly affect any of the growth parameters compared to the TSB (containing 0.5 % NaCl). On the other hand, an increase in NaCl concentration to 3.5 % resulted in significantly lower μ_{max}, longer lag phases and lower Y_{max} (a one-way ANOVA, $P < 0.001$). For instance, the μ_{max} of *A. bestiarum* ($\mu_{max} = 0.026 \pm 0.002$ log CFU/h) was two times higher in TSB (0.5 % NaCl) than at 3.5 % NaCl ($\mu_{max} = 0.009 \pm 0.001$ log CFU/h). In addition, significantly higher μ_{max} were observed at 8 °C compared to 4 °C at each NaCl concentration for all strains (a two-way ANOVA and *t*-test, $P < 0.001$) (Fig. 4a). For example, at 3.5 % NaCl, the μ_{max} of *A. media* at 8 °C ($\mu_{max} = 0.036 \pm 0.001$ log CFU/h) was four times higher than at 4 °C ($\mu_{max} = 0.009 \pm 0.001$ log CFU/h). A significantly shorter lag phase was observed at 8 °C than 4 °C at the concentration of 2.0 % and 3.5 % ($P < 0.01$), but not at 0.5 % ($P = 0.392$) (Fig. 4b). Moreover, significantly higher Y_{max} was observed at 8 °C than 4 °C at 3.5 % ($P < 0.001$) but not at 2.0 % ($P = 0.175$) and 0.5 % ($P = 0.171$).

3.1.3. Purified condensed smoke (PCS)

The effects of two PCSs (VTABB and JJT01) at different concentrations (0–0.26 %) were tested in TSB at 4 and 8 °C. The TPC of 0.26 % VTABB and JJT01 were measured as 10.9 ± 0.003 mg/100 ml and 4.7 ± 0.002 mg/100 ml, respectively. At 8 °C, the addition of VTABB at the maximal concentration (0.26 %) completely inhibited the growth of all *Aeromonas* strains. In case of JJT01, the growth of most strains was inhibited, except for two strains of *A. salmonicida* SU2 and *A. piscicola* with an extended lag phase (245 h and 351 h, respectively). When the concentration of both PCSs was reduced to 0.13 % at 8 °C, all strains were able to grow, except for *A dhakensis* which did not grow with VTABB (Fig. 5b). At lower concentration of 0.026 % VTABB, all strains could grow after 6 h of lag phase (Fig. 5a), whereas significantly longer lag phases (approximately 14 h) were observed with JJT01 ($P < 0.001$) (Supplementary Fig. A). At 4 °C, all tested strains were able to grow at lower concentrations (0.13 % and 0.026 %), excluding *A. media* and *A. bestiarum* which did not grow with VTABB at 0.13 % (Fig. 5c, d).

When the growth kinetics parameters for all strains were compared to growth in TSB (without supplements), the addition of each PCS at the lowest concentration (0.026 %) did not significantly affect any of the parameters. At the concentration of 0.13 %, significantly lower μ_{max} and longer duration of lag phase were observed for both PCSs (a one-way ANOVA, $P < 0.001$). For example, the μ_{max} of *A. salmonicida* SU2 in TSB ($\mu_{max} = 0.046 \pm 0.003$ log CFU/h) was two times higher than the rate in 0.13 % VTABB ($\mu_{max} = 0.024 \pm 0.003$ log CFU/h) at 8 °C. For both of PCSs, significantly higher μ_{max} was observed at 8 °C than 4 °C at each concentration (Fig. 6a, a two-way ANOVA and *t*-test, $P < 0.001$). For example, at 0.13 % VTABB, the μ_{max} of *A. salmonicida SL21* at 8 °C ($\mu_{max} = 0.026 \pm 0.005$ log CFU/h) was three times higher than the rate at 4 °C ($\mu_{max} = 0.008 \pm 0.002$ log CFU/h). On the other hand, the duration of lag phase was not significantly influenced by the temperature (Fig. 6b). Significant higher Y_{max} was observed at 8 °C than 4 °C at each concentration of VTABB ($P < 0.01$), whereas Y_{max} was not affected by the temperature in case of JJT01.

3.2. The growth of Aeromonas in salmon

3.2.1. Vacuum-packed fresh salmon loin

Three strains; *A. bestiarum* SL22, *A. piscicola* SU58-3 and *A. salmonicida* SU2 were inoculated in fresh salmon fillets respectively, and vacuum-packed and stored at 4 °C for 14 days. Uninoculated pieces of salmon fillet were packed and stored under the same conditions as a control group. The mean concentration of *Aeromonas* in the samples inoculated with *A. salmonicida* SU2 was 2.56 ± 0.41 log CFU/g at day 0. The number increased proportionally as a function of storage time and a mean concentration of 7.81 ± 0.37 log CFU/g was reached at 14 days (Fig. 7, dotted line). Even though all strains of *Aeromonas* were inoculated by the same procedure, *Aeromonas* were not detected in the samples inoculated with *A. bestiarum* and *A. piscicola* at day 0. Probably, these two strains were temporarily non-culturable on the starch ampicillin agar (SAA) due to the selective condition of the media. The growth of *A. bestiarum* and *A. piscicola* were detected from day 2 and 4, respectively and the highest concentrations (4.95 ± 0.13 and 4.58 ± 0.10 log CFU/g, respectively) were observed at day 8 and flattening out. No *Aeromonas* were detected in the control samples, except for day 10, where a concentration of 2.95 log CFU/g was detected in one of three parallels.

The mean concentration of APC in the samples inoculated with the *Aeromonas* strains ranged from 2.57 ± 0.21 to 3.22 ± 0.30 log CFU/g at day 0. The APC increased as a function of storage time, and the highest

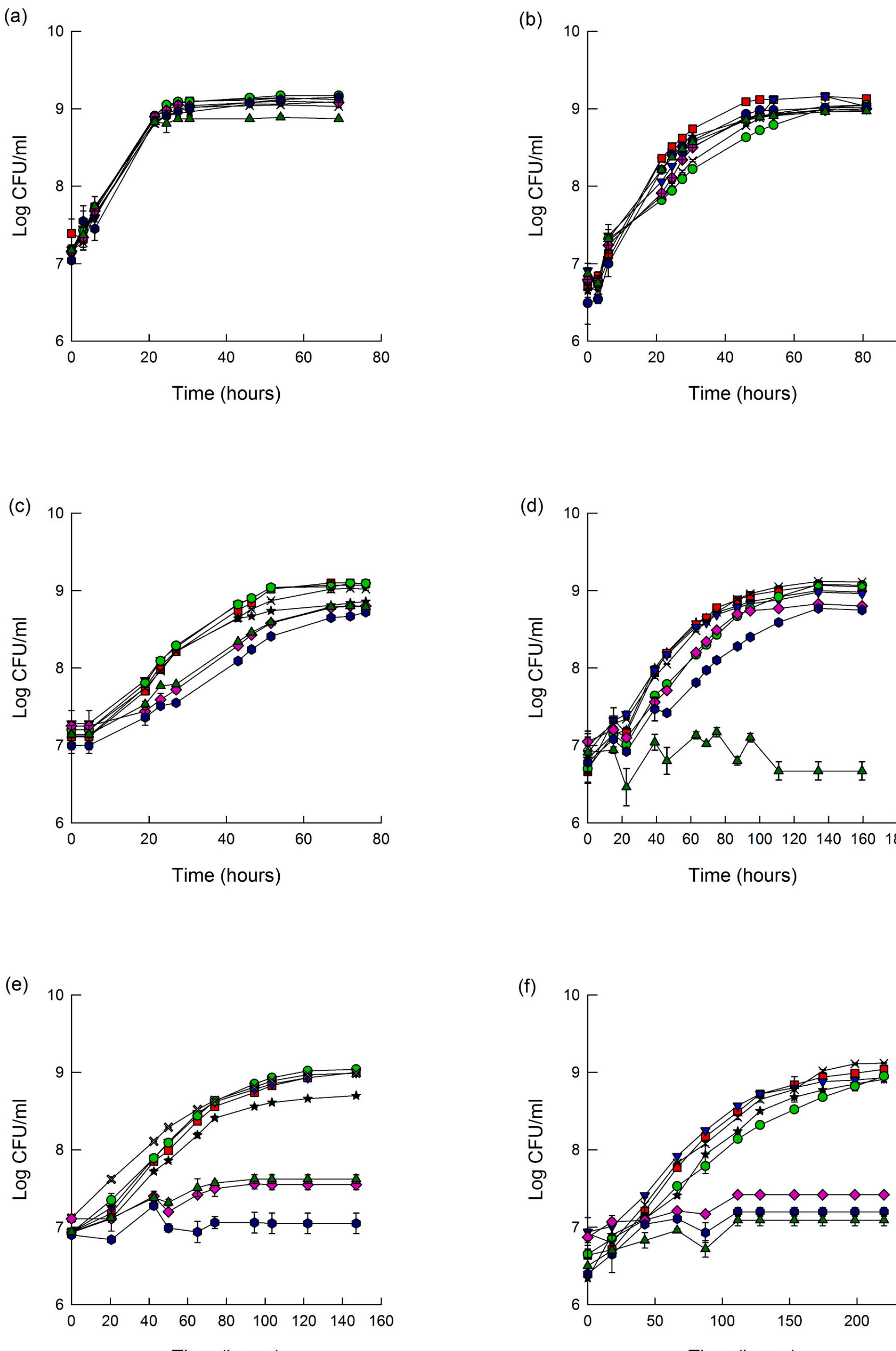
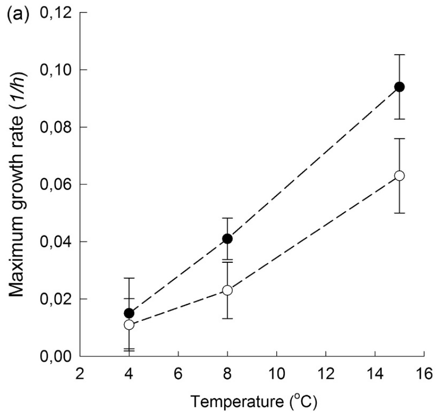


Fig. 1. Effect of temperature on the growth of eight *Aeromonas* strains in TSB and SJ model system. (a) TSB at 15 °C, (b) SJ at 15 °C, (c) TSB at 8 °C, (d) SJ at 8 °C, (e) TSB at 4 °C, (f) SJ at 4 °C. *A. media* (★ black), *A. bestiarum* (■ red), *A. piscicola* (▼ blue), *A. salmonicida* SU2 (● green), *A. salmonicida* SL21 (× gray), *A. hydrophila* (◆ pink), *A. dhakensis* (⬢ dark blue) and *A. caviae* (▲ dark green). Each point represents the mean bacterial count ($n = 3$), and vertical bars indicate ±SD.



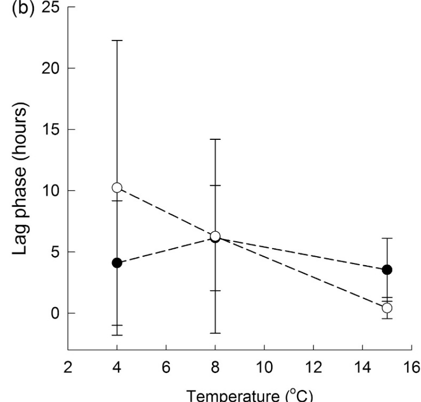


Fig. 2. Estimated marginal means of (a) maximum growth rate (μ_{max}) and (b) lag phase (h) of all eight strains in TSB (●) and SJ media (○) at three temperatures (4, 8 and 15 °C). Each point represents the mean (n = 24) of eight strains (n = 3 for each strain) and vertical bars indicate ±SE, calculated by a two-way ANOVA.

Table 1
Parameters of the square root model for the effect of temperature on the maximum growth rate of all eight strains in TSB and SJ media, where b (±SE) is the slope of the regression line, T_{min} (±SE) is the theoretical minimum temperature for growth, and R^2 is the fit of the model.

Media	Strain	b	T_{min} (°C)	R^2
TSB	*A. media*	0.013 ± 0.002	−8.8 ± 1.4	0.98
	A. bestiarum	0.011 ± 0.001	−10.6 ± 1.0	0.99
	A. piscicola	0.015 ± 0.001	−6.7 ± 0.5	0.99
	A. salmonicida SU2	0.014 ± 0.000	−7.1 ± 0.0	1
	A. salmonicida SL21	0.014 ± 0.000	−6.4 ± 0.0	0.99
SJ	*A. media*	0.013 ± 0.001	−6.1 ± 0.5	0.99
	A. bestiarum	0.012 ± 0.002	−7.3 ± 1.2	0.98
	A. piscicola	0.009 ± 0.001	−10.0 ± 1.1	0.98
	A. salmonicida SU2	0.008 ± 0.000	−11.4 ± 0.0	0.99
	A. salmonicida SL21	0.009 ± 0.000	−9.2 ± 0.0	0.99

concentrations (ranging from 6.52 ± 0.39 to 7.80 ± 0.30 log CFU/g) were observed after 14 days of storage. The quantification of APC in the inoculated samples resulted mainly from black colonies, representing H_2S-producing bacteria (Fig. 7, solid line). H_2S-producting bacteria had a similar growth pattern to the inoculated *A. salmonicida* SU2 (quantified on SAA), implying that observed black colonies on IA were *A. salmonicida* SU2. Moreover, a significant correlation was found between the concentration of *A. salmonicida* SU2 on SAA and the H_2S-producting bacteria on IA ($R^2 = 0.93$, $P < 0.001$). The correlation was weaker but also significant for *A. bestiarum* and *A. piscicola* ($R^2 = 0.56$, and $R^2 = 0.52$, $P < 0.01$, respectively). Thus, the quantification data of H_2S-producing bacteria was used to estimate the growth kinetic parameters of the *Aeromonas* strains (Table 2). The mean concentration of APC in the control group was 1.52 ± 0.52 log CFU/g at day 2 and developed to the level of 5.53 ± 0.19 log CFU/g at 14 days, but H_2S-producing bacteria were not detected, except for day 11, where a concentration of 3.04 log CFU/g was detected in one of three parallels.

3.2.2. Vacuum-packed cold-smoked salmon

The same strains (*A. bestiarum* SL22, *A. piscicola* SU58-3, and *A. salmonicida* SU2) were inoculated in cold-smoked salmon, and vacuum-packed and stored at 4 °C for 21 days. Uninoculated cold-smoked salmon was packed and stored in the same conditions as a control group. The mean concentration of TPC in the cold-smoked salmon was 1.49 ± 0.01 mg/100 g product. The mean concentration of *Aeromonas* in the samples inoculated with *A. salmonicida* SU2 was 2.73 ± 0.08 log CFU/g at day 0. *Aeromonas* concentration fluctuated over the storage period of 21 days, and no *Aeromonas* was detected at day 15 and 17 except for one of three parallels (2.95 log CFU/g). After 21 days, the highest concentration of 4.43 log CFU/g was detected in one of three parallels, indicating an increase of approximately 1.5 log compared to time zero. No *A. piscicola* and *A. bestiarum* were detected on the SAA throughout storage, except for some sporadic colonies of *A. piscicola* detected in one of three parallels at day 11 (3.73 log CFU/g), and 21 (3.04 log CFU/g) and *A. bestiarum* at day 7 (2.56 log CFU/g). This is most likely due to the non-culturable state of these two strains on the SAA, as previously noted for the fresh salmon samples. No *Aeromonas* were detected in the control samples during the storage.

Throughout the storage time, there were no significant changes in the mean concentration of APC in the cold-smoked salmon inoculated with each *Aeromonas* strain, even if there was a tendency toward a slight increase in the APC from day 0 to day 21. Similar to the fresh salmon sample, APC was represented by a significant portion of H_2S-producing bacteria, and H_2S-producting bacteria had a similar growth pattern to the inoculated *A. salmonicida* SU2. A significant correlation was observed between the concentration of *A salmonicida* SU2 on SAA and the H_2S-producting bacteria on IA ($R^2 = 0.58$, $P < 0.01$). However, no significant relation was found between H_2S-producing bacteria and storage time ($P = 0.455$). This might indicate poor growth of *A. salmonicida* SU2 on SAA over the storage time. The mean concentration of APC in the control sample was 1.2 ± 0.25 log CFU/g at day 3 and developed to 4.6 ± 1.47 log CFU/g after 15 days, and no H_2S-production bacteria was detected during the storage.

4. Discussion

In this study, the growth kinetics parameters of eight *Aeromonas* strains (*A. media*, *A. bestiarum*, *A. piscicola*, *A. hydrophila*, *A. dhakensis*, *A. caviae* and two strains of *A. salmonicida*) were studied under different growth conditions. First, the effect of different temperatures (4, 8 and 15 °C) was tested in liquid culture (TSB) and in a salmon juice (SJ) model. Then, the effect of NaCl concentrations (0.5–6.5 %), or two types of PCSs (VTABB and JJT01) in combination with low temperatures (4 and 8 °C) was investigated in liquid broth (TSB). Moreover, the growth kinetics parameters of three *Aeromonas* strains were investigated in inoculated fresh and minimally processed cold-smoked salmon fillets under vacuum packaging at 4 °C for 14 and 21 days, respectively.

The effect of temperatures (4, 8 and 15 °C) on the growth of *Aeromonas* was studied, as we hypothesized that moderate (8 °C) to severe (15 °C) temperature abuse of refrigerated food is likely to happen during transport and retail display. Our study presented that refrigeration could

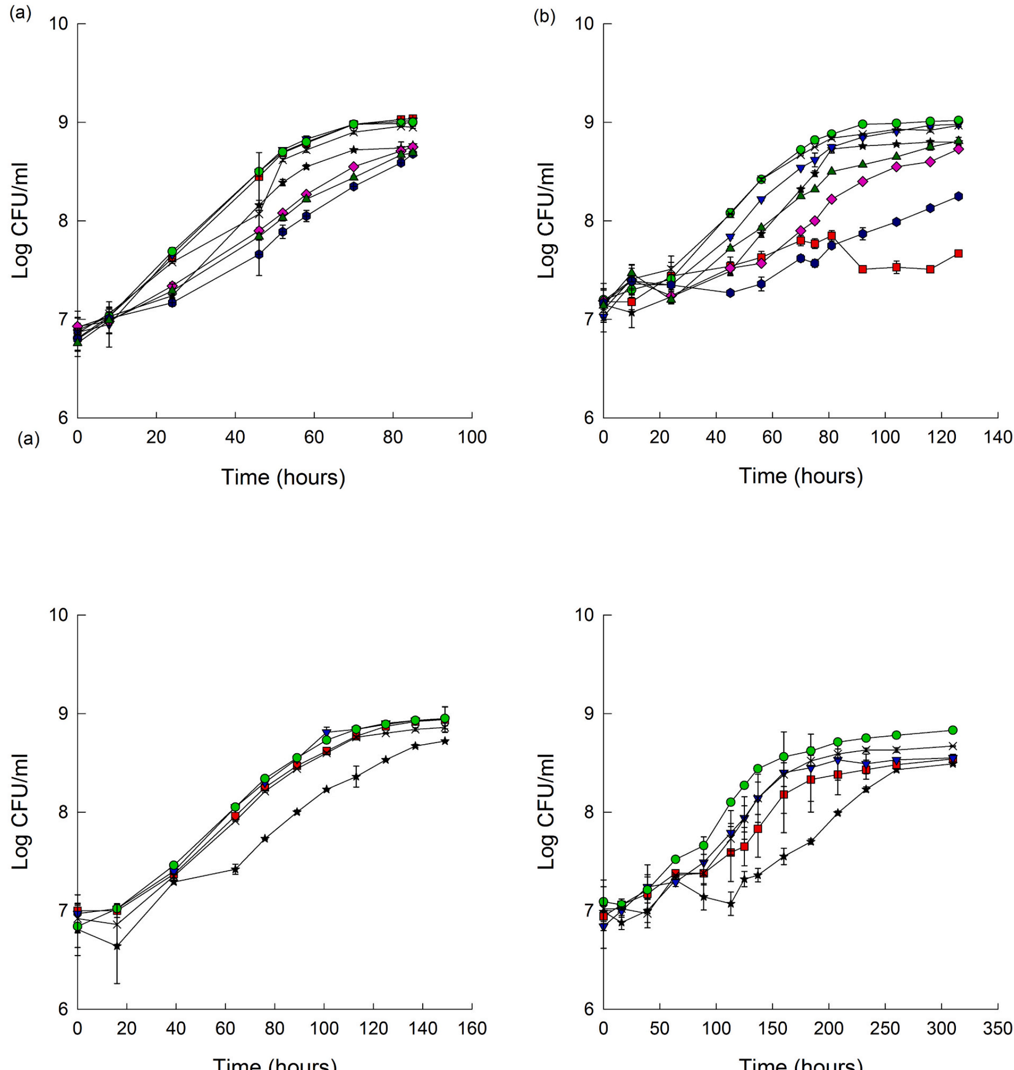
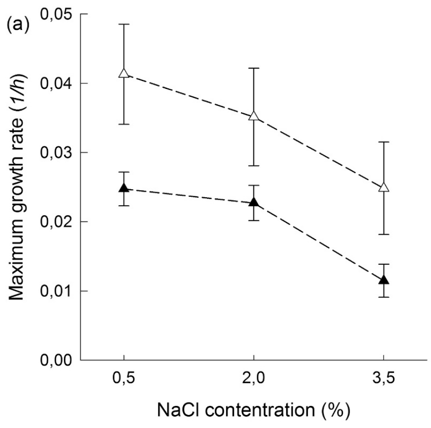


Fig. 3. Effect of NaCl concentration on the growth of eight *Aeromonas* strains in two different temperatures (4 and 8 °C). (a) 2.0 % NaCl at 8 °C, (b) 3.5 % NaCl at 8 °C, (c) 2.0 % NaCl at 4 °C, (d) 3.5 % NaCl at 4 °C. *A. media* (★ black), *A. bestiarum* (■ red), *A. piscicola* (▾ blue), *A. salmonicida* SU2 (● green), *A. salmonicida* SL21 (× gray), *A. hydrophila* (◆ pink), *A. dhakensis* (⬢ dark blue) and *A. caviae* (▴ dark green). Each point represents the mean bacterial count ($n = 3$), and vertical bars indicate ±SD.

not sufficiently inhibit the growth of tested *Aeromonas* strains, except for *A. hydrophila, A. dhakensis* and *A. caviae*. Our data showed that a temperature abuse scenario at 8 and 15 °C could enhance the growth of all tested *Aeromonas* strains, as an increase in temperatures resulted in significantly higher maximum growth rates with shorter lag phases for all strains, in accordance with previous studies (Kim et al., 2022; Zuccolotto et al., 2006). In addition, the *Aeromonas* strains in the present study displayed diversity in tolerance to temperature, particularly at refrigeration temperature (4 °C). *A. media*, *A. bestiarum*, *A. piscicola* and *A. salmonicida* showed ability to grow at 4 °C, whereas no growth of *A. hydrophila*, *A. dhakensis* and *A. caviae* was observed. The growth of *A. media*, *A. piscicola* and *A. salmonicida* at 4 °C was previously reported (Allen et al., 1983; Beaz-Hidalgo et al., 2009; Hoel et al., 2018), while the growth characteristics of *A. bestiarum*, *A. caviae*, and *A. dhakensis* at low temperature is poorly described. The presence of *A. hydrophila* in refrigerated food is frequently reported and their growth at refrigeration temperature has previously been highlighted (Palumbo et al., 1985; Vivekanandhan et al., 2003). On the other hand, a previous study demonstrated that most *A. hydrophila* isolated from either environment or clinical background could not grow or weakly grow at 4 °C (Zuccolotto et al., 2006). The ability of *A. hydrophila* at low temperature might be dependent on isolation source. *A. hydrophila*, *A. dhakensis*, and *A. caviae* are more often associated with clinical background; however, all *Aeromonas* strains in this study were environmentally isolated from seafood. Environmental isolates are known to be well adapted to low temperatures as low as 4 °C, where the growth of clinical isolates could



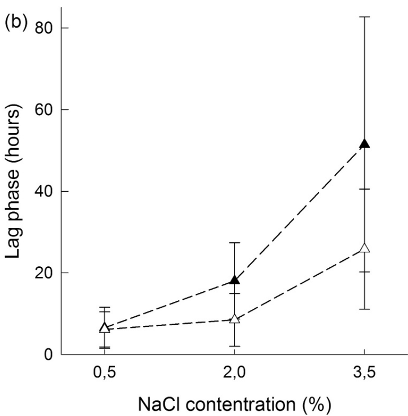


Fig. 4. Estimated marginal means of (a) maximum growth rate (μ_{max}) and (b) lag phase (h) of eight strains in different NaCl concentrations (0.5 %, 2.0 % and 3.5 %) at 4 °C (▲) and 8 °C (△). Each point represents the mean (n = 24 for 8 °C and n = 15 for 4 °C) of eight strains (n = 3 for each strain) and vertical bars indicate ±SE, calculated by a two-way ANOVA.

be inhibited (Mateos et al., 1993).

The SJ model was introduced to imitate the growth condition of salmon. In this study, most tested *Aeromonas* strains were able to grow in the SJ model at all tested temperatures, implying the potential growth of *Aeromonas* in salmon. However, the overall maximum growth rate was significantly lower in the SJ than in the TSB ($P < 0.001$), suggesting that the growth of *Aeromonas* in SJ was somewhat inhibited. The low composition of protein, peptides, and free amino acids were suggested to be possible factors limiting the antimicrobial activity of lactic acid bacteria in shrimp juice (Fall et al., 2010). Further study of defining physicochemical characteristics of salmon juice seems necessary to find the limiting factors to the growth of *Aeromonas*. As the growth kinetic parameters were significantly affected by the media, further studies were carried out in TSB media. Nevertheless, in both TSB and SJ, temperature was the most important factor influencing the growth kinetics of *Aeromonas*, particularly maximum growth rates. In addition, the inter-species diversity was observed in their growth kinetic parameters and their temperature responses in both medium.

Different NaCl concentrations (0.5, 2.0, 3.5, 5.0, and 6.5 %) were chosen to simulate the salt tolerance of *Aeromonas* at two different temperatures in liquid culture (TSB). Our data showed that all tested *Aeromonas* strains were able to grow at concentrations ≤3.5 % NaCl at 8 °C, but not at the higher concentrations (≥5.0 % NaCl). Higher concentrations of NaCl resulted in significant reduction in maximum growth rates and prolonged lag phases ($P < 0.001$), which is in accordance with a previous study (Palumbo et al., 1985). Previous studies also showed salt content-dependent growth of *Aeromonas,* where mesophilic *Aeromonas* including *A. media*, *A. bestiarum*, *A. piscicola* and *A. hydrophila* could grow in concentration up to 3.0 % NaCl, while only a few strains could grow in media with >5.0 % NaCl (Ali et al., 1996; Allen et al., 1983; Beaz-Hidalgo et al., 2009; Gram, 1991).

Our results demonstrated that the combination of 3.5 % NaCl and refrigeration temperature (4 °C) was insufficient to inhibit the growth of *A. media*, *A. bestiarum*, *A. piscicola* and *A. salmonicida*. Growth kinetic parameters of all tested strains were significantly influenced by salt concentration as well as temperature, as higher salt concentration with lower temperature resulted in significant reduction of maximum growth rates and longer lag phases ($P < 0.001$). Temperature-dependent salt tolerance of *Aeromonas* was described in the previous studies, where *Aeromonas* could grow uninhibitedly at the concentration of 5.0 % NaCl at 37 °C (Gram, 1991) and even at 6.0 % NaCl at 28 °C (Delamare et al., 2000), while no growth was observed at the concentration of 5.0 % NaCl at lower temperature (5–10 °C) (Gram, 1991; Vivekanandhan et al., 2003). In practice, to inhibit unwanted microorganisms, adequate amount of salt (<6.0 % in the water phase) together with preservatives such as sorbate, benzoate or smoke are needed to produce lightly preserved fish products such as cold-smoked salmon (Løvdal, 2015). Lower salt content is however desirable due to the unhealthy effects of high sodium intake, and for example, the final salt content of cold-smoked salmon is recommended to be <3 g NaCl/100 g product in Norway (Ministry of Health and Care Services, 2015). Considering the current trend of lowering salt content, establishing additional hurdles to the salt-tolerant bacteria is critical to control their growth in the processing of lightly salted product. Investigating the viability of *Aeromonas* between 3.5 and 5.0 % NaCl concentrations would be still needed to define a minimum inhibitory NaCl concentration for these strain at low temperature.

Moreover, our study demonstrated the antimicrobial potential of PCS (at maximal concentration) against *Aeromonas* in TSB, in accordance with previous studies (Sofos et al., 1988; Suñen et al., 2003). Phenol concentration and pH of the smoke extract are considered important factors, and previous studies demonstrated that higher level of phenols in smoke extracts and pH drop in smoked products were the reasons for the better antimicrobial activity (Suñen et al., 2003; Valø et al., 2020). The two times higher TPC content of VTABB might explain better inhibitory potential than JJT01. On the other hand, no inhibitory effect on *Aeromonas* growth was observed with lower TPC levels of both PCSs. Measured pH in liquid culture varied between 6.3 and 7.4; however, *Aeromonas* are usually sensitive to lower pH (<6.0) (Daskalov, 2006). Moreover, the growth of *Aeromonas* in PCS enriched medium was also temperature-dependent, as lower temperature resulted in significant reduction of maximum growth rates for all strains ($P < 0.001$).

Overall, our study demonstrated the inter-species variation in the ability to grow at different temperatures in combination with salt content or PCSs in liquid culture systems. The liquid culture systems such as TSB or SJ could be reproducible and useful to study inter-species diversity and screen the strains for further analysis. However, such system might be not directly comparable to the cold-smoking process used in a real product. For example, the smoking process using PCS are usually performed by atomization process, where compressed air is used to vaporize PCS in a closed chamber to produce the smoked products (Valø et al., 2020). In addition, a previous study reported that antimicrobial activity of smoke extracts in microbiological culture media was not comparable to a real food matrix (Hao et al., 1998). Evaluating the

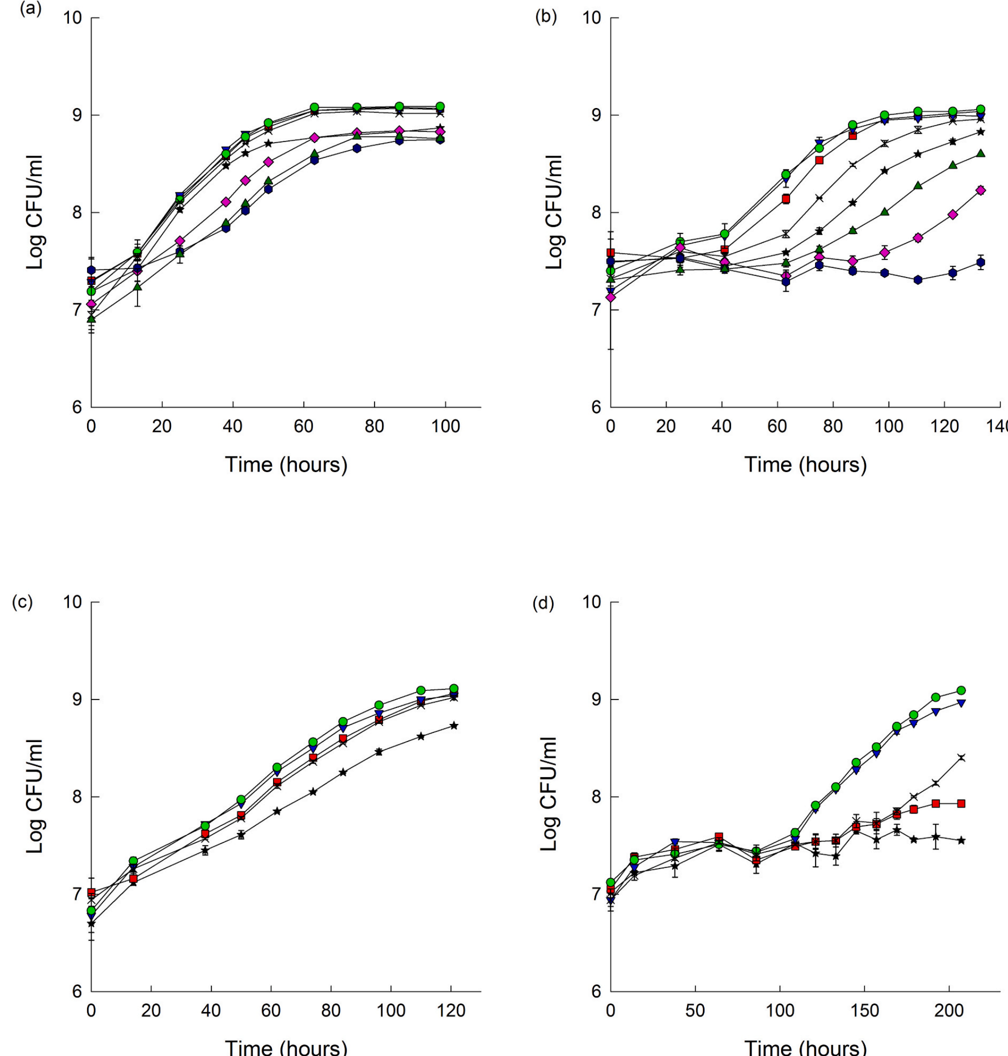
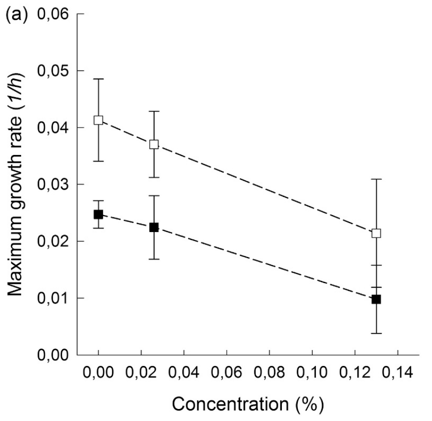


Fig. 5. Effect of adding VTABB at different concentrations on the growth of eight *Aeromonas* strains in two different temperatures (4 and 8 °C). (a) 0.026 % VTABB at 8 °C, (b) 0.13 % VTABB at 8 °C, (c) 0.026 % VTABB at 4 °C, (d) 0.13 % VTABB at 4 °C. *A. media* (★ black), *A. bestiarum* (■ red), *A. piscicola* (▼ blue), *A. salmonicida* SU2 (● green), *A. salmonicida* SL21 (× gray), *A. hydrophila* (◆ pink), *A. dhakensis* (⬢ dark blue) and *A. caviae* (▲ dark green). Each point represents the mean bacterial count ($n = 3$), and vertical bars indicate ±SD.

antimicrobial activity of PCS in a real product was suggested to be more relevant since a food environment is different from microbiological media and several compounds in smoke extracts could interfere with antimicrobial activity (Suñen et al., 2003). Further experiments in actual food matrixes such as PCS-treated salmon products would be required to confirm the antimicrobial potential of PCS against *Aeromonas*. In addition, as PCS is often applied in combination with salting in the cold smoking process, a follow-up study would be needed to investigate the combined effect of PCS and salt contents on *Aeromonas* growth.

Among eight *Aeromonas* strains, *A. bestiarum* SL22, *A. piscicola* SU58-3 and *A. salmonicida* SU2 which exhibited higher growth rates compared to the other strains in the liquid culture system were selected for growth kinetic studies in fresh and cold-smoked salmon products. SAA media is specifically recommended for *Aeromonas* isolation from food samples (NMKL, 2004); however, the selectivity and sensitivity of this media could be dependent on the species and isolation sources (Latif-Eugenín et al., 2016). It is worth noting that the growth of *Aeromonas* might have been somehow underestimated due to the temporarily non-culturable state of *Aeromonas* strains in the selective media (SAA). Nevertheless, our study demonstrated that vacuum packaging with cold storage (4 °C) could not sufficiently inhibit the growth of *Aeromonas* in VP salmon, in accordance with previous studies where they could detect the growth of *Aeromonas* in VP products stored at low temperature (Hudson et al., 1994; Jakobsen et al., 2020; Suñen et al., 2003). On the other hand, our data showed that the growth of the three *Aeromonas* strains was inhibited in cold-smoked salmon. Cold-smoked salmon is generally characterized by a NaCl concentration ranging from 2.5 to 3.5 % (*w*/w), and smoke treatment corresponding to 0.6 mg of phenol/100 g of



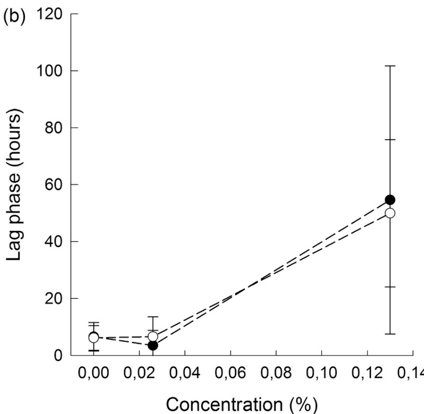


Fig. 6. Estimated marginal means of (a) maximum growth rate (μ_{max}) and (b) lag phase (h) of all eight strains in different concentrations (0 %, 0.026 % and 0.13 %) of VTABB at 4 °C (■) and 8 °C (□). Each point represents the mean (*n* = 24 for 8 °C and *n* = 15 for 4 °C) of eight strains (*n* = 3 for each strain) and vertical bars indicate ±SE, calculated by a two-way ANOVA.

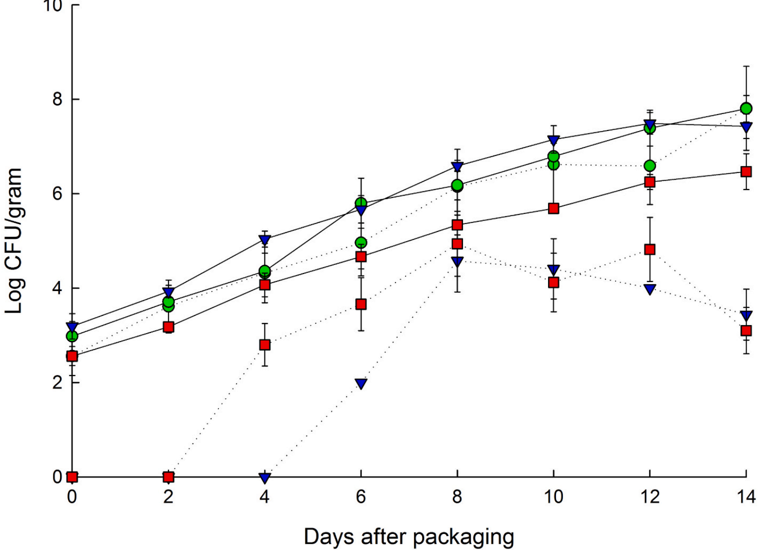


Fig. 7. Growth of H_2S-producing bacteria inoculated with *A. bestiarum* SL21 (■ red), *A. salmonicida* SU2 (● green), and *A. piscicola* SU58-3 (▾ blue) (solid line) quantified on iron agar (IA), and growth of *A. bestiarum* (■ red), *A. salmonicida SU2* (● green), and *A. piscicola* (▾ blue) (dotted line) quantified on starch ampicillin agar (SAA) in vacuum-packed salmon product stored at 4 °C for 14 days. Each point represents the mean bacterial count (n = 3), and vertical bars indicate ±SD.

product according to the French Standard NF V45-065. pH in cold-smoked salmon products is >5.0 and usually varies between 6 and 6.3 (Løvdal, 2015). Previous studies suggested the antimicrobial effect of salting and smoking in a cold-smoking process was proportional to the contents of salt and smoke (Leroi et al., 2000) and their effects were synergistic (Neunlist et al., 2005). In our study, the cold-smoked salmon contained a relatively low level of salt (1.8 g/100 g product) but contained a higher level of phenol (1.49 mg/100 g product) than the French Standard. Among the *Aeromonas* strains in the present study, *A. salmonicida* SU2 and *A. piscicola* SU58-3 were the most tolerant strains toward PCS treatment in liquid culture (containing 4.7 mg/100 ml), which is three times higher than the TPC level in the cold smoked salmon. Both strains were also able to grow in the concentration of 3.5 % NaCl at low temperature (4 °C) in liquid culture. Nevertheless, observed inhibition of these strains in the cold-smoked salmon might imply that phenol content alone was not the limiting factor influencing growth of these strains. It is most likely that the combined effect of high phenol, low salt contents and other processing factors such as low pH resulted in effective hurdles to inhibit growth of three *Aeromonas* strains in the cold-smoked salmon product.

5. Conclusion

In conclusion, we demonstrated that the combined effect of low temperature and 3.5 % NaCl was insufficient to inhibit the growth of *A. media*, *A. bestiarum*, *A. piscicola* and *A. salmonicida*, implying that mild

Table 2
Growth kinetic parameters for *Aeromonas* strains inoculated in vacuum-packed fresh salmon, estimated from the primary model of Baranyi and Roberts (1994). The quantification data of H_2S-producing bacteria on iron agar (IA) was used for growth prediction. Each sample point is represented as mean ($n = 3 \pm$ SD). Maximum growth rate (μ_{max}, log CFU/d), lag phase duration (d), maximum population density (Y_{max}, log CFU/g), R^2 (fit of model), SE (standard error of R^2) and NL: no lag.

Inoculated strain	μ_{max} (log CFU/d)	Lag phase (d)	Y_{max} (log CFU/g)	R^2	SE
A. salmonicida SU2	0.40 ± 0.03	NL	7.82 ± 0.28	0.98	0.225
A. piscicola SU58-3	0.43 ± 0.03	0.062 ± 0.045	7.48 ± 0.08	0.99	0.107
A. bestiarum SL22	0.33 ± 0.02	NL	6.48 ± 0.13	0.99	0.117

processing as applied in the production of RTE seafood may not guarantee the total inhibition of *Aeromonas*. On the other hand, cold-smoking process including the application of PCS could be an alternative approach to control the growth of *Aeromonas*. Our data could be used as a basis in the decision making for the practical development of cold-smoked salmon products with PCS technology Follow-up studies in a food matrix are still necessary to evaluate the actual antimicrobial activity of PCS against these bacteria. Moreover, our study presented that eight *Aeromonas* strains representing seven species displayed a variability in tolerance toward various processing factors, which might be useful for growth prediction of *Aeromonas* in RTE seafood. Our study highlighted that mild processing technology should be optimized to control the growth of potentially pathogenic *Aeromonas*, and further studies are required to expand our knowledge for growth prediction under mild processing factors in RTE seafood.

CRediT authorship contribution statement

Hye-Jeong Lee: Formal analysis, Writing-Original Draft & Editing, Visualization, Ingebjørg Fagerheim Tokle: Investigation, Formal analysis, Bjørn-Tore Lunestad: Supervision, Writing-Review & Editing, Jørgen Lerfall: Supervision, Writing-Review & Editing, Sunniva Hoel: Supervision, Conceptualization, Writing-Review & Editing, Anita Nordeng Jakobsen: Conceptualization, Supervision, Writing-Review & Editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2022.109985.

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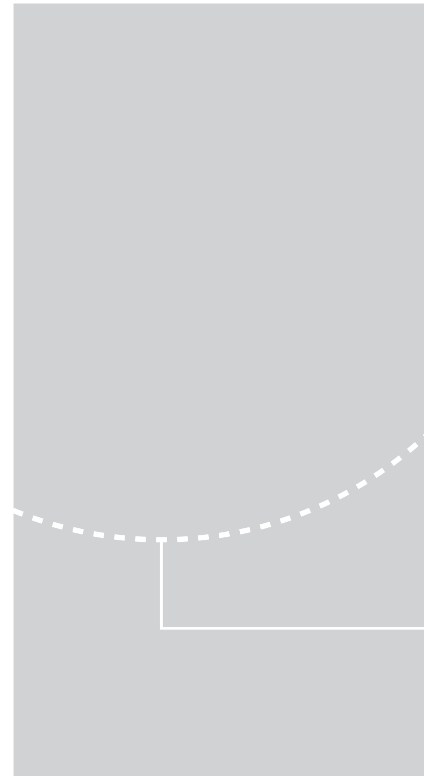

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