Taxonomy, spoilage, and virulence characteristics of *Kaistella* species isolated from fish

by

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DECLARATION

I, **Masabata Lydia Gavu**, declare that the thesis **Taxonomy, spoilage and virulence characteristics of** *Kaistella* **species isolated from fish**, that I herewith submit for the Doctoral Degree **PhD. Food Science** at the University of the Free State is my independent work, and I have not previously submitted it for a qualification at another institution of higher education.

Submitted in fulfilment of the requirements for the Doctoral Degree, **Ph.D. Food Science,** in the Department of **Microbiology and Biochemistry,** Faculty of **Natural and Agricultural Science** at the University of the Free State.

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LIST OF ABBREVIATIONS

A	Absorbance
AAI	Amino acid identity
ANOVA	Analysis of variance
ANI	Average nucleotide identity
ATCC	American Type Culture Collection, Manassas, Virginia
B.	*Bacillus*
BLAST	Basic local alignment search tool
Bp	Base pairs
C.	*Chryseobacterium*
^{o}C	Degrees Celcius
CaCl	Calcium chloride
CCUG	Culture Collection of the University of Göteburg, Sweden
CDS	Coding sequences
CFU	Colony forming units
CRA	Congo red agar
DNA	Deoxiribonucleotide acid
dDDH	Digital DNA-DNA hybridization
DDH	DNA-DNA hybridization
DSM	Deutsche Sammlung von Mikro-organismen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
e.g.	For example
et al.	*et alia*; and others
Etc.	*Et cetera*
F.	*Flavobacterium*
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fig.	Figure
g	gram
G+C	Guanine and cytosine
GDDC	Genome-to-genome distance calculator
h	Hours
HCl	Hydrochloric acid
IJSEM	International Journal of Systematic and Evolutionary Microbiology

KH_2PO_4	Potassium dihydrogen phosphate
K.	*Kaistella*
KCTC	Korean Collection of Type Cultures
KOH	Pottasium hydroxide
LMG	Laboratory of Microbiology, University of Ghent, Belgium
LPSN	List of Prokaryotic Names with Standing in Nomenclature
M	Molar
MCA	MacConkey agar
MEGA	Molecular evolutionary genetics analysis
min	Minutes
mg	Milligram
$MgSO.7H_2O$	Magnesium sulphate heptahydrate
mM	MilliMolar
MM9	Minimal media 9
MK-6	Menaquinone-6
MK-7	Menaquinone-7
ml	Millilitre
ML	Maximum likelihood
mm	Millimetre
Mol	Mole
Mol%	Mole percentage
mRNA	Messenger RNA
MP	Maximum parsimony
NB	Nutrient broth
NA	Nutrient agar
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
ND	Not determined
NH_4Cl	Ammonium chloride
NJ	Neighbour-joining
nm	Nanometer
OD	Optical density
OGRI	Overall genome related index
PCR	Polymerase chain reaction

PFGE	Pulsed-field gel electrophoresis
pH	Power of hydrogen
pp	Pages
P.	*Pseudomonas*
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
RAST	Rapid annotation with subsystems technology
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
R2A	Reasoners2 agar
SEM	Scanning electron microscopy
sp.	Species
^{T}	Type strain
TEM	Transmission electron microscopy
TCP	Tissue culture plate
™	Trademark
TM	Tube method
TSA	Trypticase soy agar
µl	Microlitre
µm	Micrometre
UFSBC	University of the Free State bacterial culture collection
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
V.	*Vibrio*
v/v	Volume per volume
w/v	Weight per volume
WGS	Whole-genome sequencing

Abstract

In previous studies at the University of the Free State, aerobic, Gram-negative bacteria were isolated from Cape hake (*Merluccius capensis*), intended for human consumption. Although some of these isolates could be identified, six isolates remained unidentified. The purpose of this study was to determine the genomic and phenotypic characteristics of these isolates to assign them to the correct genus, to describe novel species, if present, to determine the significance of these isolates in terms of pathogenicity and/or spoilage, and to isolate bacteriophages against the bacterial strains for possible biocontrol strategies.

Based on 16S rRNA gene sequences, phylogenetic analysis confirmed that the six unidentified bacterial strains used in this study represented members of the genus *Kaistella.* Four of these six isolates were further characterized to determine whether they were novel species. Using genomic and phenotypic techniques, the DNA G+C content of strains SH 11-4(b), SH 19-2(b), SH 20-4 and SH 40-3 supported their affiliation with the genus *Kaistella*. Digital DNA-DNA hybridization, average nucleotide identity and amino acid identity values, and phenotypic characteristics demonstrated that strains SH 11-4(b), SH 19-2(b) and SH 40-3 represented novel species of the genus *Kaistella*. Results for strain SH 20-4 confirmed that it was not a novel species but represented another member of *Kaistella carnis*. The names of the novel species were proposed as *Kaistella merluccii* SH 11-4(b), *Kaistella piscis* SH 19-2(b), and *Kaistella frigidipiscis* SH 40-3.

The potential pathogenicity and/or food spoilage capability of the six *Kaistella* fish isolates and reference strains were then evaluated by determining the production of siderophores, the production of a variety of enzymes that function as virulence factors, evaluating their antimicrobial resistance patterns, their ability to form biofilms, as well as the determination of their resistance to antibiofilm compounds. All the *Kaistella* isolates produced Siderophores indicating their ability to sequester iron for survival. Gelatinase, whose expression has been linked to enhanced biofilm formation, was produced in the most significant amounts by most organisms in this study. '*Kaistella merluccii*' SH 11-4(b) could be regarded as a potential pathogen since it produced more than 4/8 virulence enzymes. *Kaistella* strains SH 11-3(a) and '*K. frigidipiscis*' SH 40-3 were the most resistant to antimicrobials. The antimicrobial resistance/susceptibility of the *Kaistella* and *Chryseobacterium* species in this study was determined using the Kirby-Bauer disc diffusion susceptibility method. The antimicrobial tests concluded that the fluoroquinolone and cephem antimicrobials would be the most effective in treating *Kaistella* infections.

Kaistella carnis SH 20-4 was regarded as a strong biofilm former since it gave positive results for biofilm formation using three different methods, while strains SH 11-3(a) and '*K. merluccii*' SH 11-4(b) were the least successful at forming biofilms.

This study revealed that 100 mM D-glucose was the most effective biofilm inhibition compound against the *Kaistella* test strains. Organisms whose biofilms showed the most resistance towards the inhibition compounds included *K. carnis* SH 20-4 and '*K. frigidipiscis*' SH 40-3 and susceptibility to the inhibition compounds mainly was observed in SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b) and '*K. piscis*' SH 19-2(b).

Another aim of this study was to isolate lytic bacteriophages against the *Kaistella* species that may be pathogenic to fish or may cause food spoilage by using a two-fold agar overlay in a plaque experiment. Thirty-four phage isolates were obtained from the sewage and fishpond water samples. Strains SH 11-3(a) and SH 11-3(b) showed sensitivity toward a few phage isolates while '*K. piscis*' SH 19-2(a) displayed the greatest resistance towards phage infection. Phage strains 11-3(b)-S2 showed a broader host spectrum than other phage isolates because they displayed lytic activity against 5/12 *Kaistella* isolates. Most of the isolated phages were identified by transmission electron microscopy as members of the *Corticoviridae, Plasmaviridae, Microviridae, Siphoviridae and Tectiviridae* families.

The three new members of the genus *Kaistella* were accurately classified, described, and named. The role of the six *Kaistella* species in virulence was determined. Some isolated phages can potentially prevent, eliminate, or reduce *Kaistella* infections in fish.

Keywords: *Kaistella*, *Chryseobacterium* taxonomy, potential pathogenicity, antimicrobial resistance, biocontrol strategies, biofilm formation, bacteriophage isolation

CHAPTER 1

INTRODUCTION

The genus *Kaistella*, a member of the family *Weeksellaceae* within the phylum *Bacteroidota,* was first described by Kim and colleagues in 2004. The description of the genus has been successively revised by Nicholson et al. (2020). The genus *Kaistella* currently contains 16 validly published species, together with some (*Kaistella anthropi, Kaistella daneshvariae, Kaistella treverensis*) of clinical importance (Kämpfer et al. 2009, Yassin et al. 2010, Nicholson et al. 2020), with *Kaistella koreensis* as the type species (Kim et al. 2004). *Kaistella chaponensis* has been established to be pathogenic to specific fish species (Kämpfer et al. 2011). *Kaistella haifensis* strains are also found in psychrotolerant and proteolytic bacterial communities (Hantsis-Zacharov and Halpern 2007). Some *Kaistella* species that were once categorized as belonging to the genus *Chryseobacterium* were later reclassified due to a whole genome sequencing (WGS) analysis study (Nicholson et al. 2020).

In a study by Engelbrecht (1992) at the University of the Free State, Bloemfontein, South Africa, several yellow-pigmented bacteria were isolated from Cape marine fish. *Merluccius capensis* is a highly valued shallow water fish that is harvested commercially off the coasts of South Africa (Reed et al. 2018). Many of these bacteria were identified as members of the *Flavobacteriaceae* family (Engelbrecht et al. 1996a), and some were identified as new species of the genus *Chryseobacterium.* However, several remained unidentified. Some of these unidentified isolates were further investigated (Gavu 2019). Six isolates clustered closely using 16S rRNA sequencing and belonged to the genus *Chryseobacterium*, at that time (Gavu 2019). However, research by Garcia-López et al. (2019) and Nicholson et al. (2020) resulted in these isolates being identified as *Kaistella* strains. However, these six isolates could not be associated with any validly published species of *Kaistella* (Gavu 2019).

A crucial task in microbiology is accurately identifying bacterial isolates (Tang et al. 1998, Bochner 2009). For the most prolonged period, the classification of microbes relied on traditional methods based on the cultivation of microorganisms and the evaluation of the phenotypic traits (Vandamme et al. 1996, Emerson et al. 2008, Donelli et al. 2013, Franco-Duarte et al. 2019). However, these techniques could have been more time-consuming, laborious, and frequently insufficient for classifying phenotypically similar species (Woese 1994, Tang et al. 1998, Bochner 2009). Microbial taxonomy was revolutionized by polyphasic taxonomy, which incorporates all available phenotypic and genotypic data into a consensus taxonomy type and frames it in a broad phylogeny obtained from 16S rRNA sequence analysis (Vandamme et al. 1996, Donelli et al. 2013, Hugenholtz et al. 2021).

The emergence of genotypic classification has offered an alternative to phenotypic techniques (Donelli et al. 2013, Winand et al. 2013, Buszewski et al. 2017). Genetic identification provides a robust and diverse data output with taxonomically relevant molecular information on individual strains and entire populations (Emerson et al. 2008). The essential foundation for developing bacterial classification schemes has been provided by molecular systematics (Hugenholtz et al. 2021). Advancements in the next generation of sequencing technologies have led to sequence-based approaches that are more accessible and affordable at all stages of microbial characterization and identification (Emerson et al. 2008, Winand et al. 2019). Using whole-genome comparisons between related species, for instance, describes a species at the genomic level as having 95% average nucleotide identity between two strains, which equates to an estimate of at least 70% reassociation for DNA-DNA hybridization, which has been the classical standard for distinguishing whether a species is a novel (Vandamme et al. 1996, Goris et al. 2007).

Virulent and spoilage bacteria are frequently discovered living in sessile communities known as biofilms, which are responsible for the degradation of products in the food, dairy, and food processing industries and can also spread diseases (Galié et al. 2018, Carrascosa et al. 2021, Olanbiwoninu and Popoola 2022). The ability of an organism to infect the host and spread disease is referred to as virulence (Peterson 1996, Casadevall and Pirofski 1999, Casadevall and Pirofski 2001, Sharma et al. 2017). The molecules, known as virulence factors, aid the bacterium in the cellular colonization of the host (Sharma et al. 2017). These elements have a secretory, membrane-related, or cytosolic character. No research has been done on the significance of *Kaistella* species in food sources. Therefore, it is essential to look into whether these species can function as pathogens or as food spoilage bacteria by producing virulence factors, e.g., siderophore production, enzyme production, antibiotic resistance, and biofilm production.

Gram-negative bacteria have developed a noticeable increase in antibiotic resistance due to several mechanisms, including mutation of the drug target, antibiotic degradation, and alteration of permeability across the bacterial membrane (Munita and Arias 2016, Reygaert 2018, Alomari et al. 2021). These mechanisms have restricted the development of new antibiotics. Bacteriophages are recommended as potential antibiotic alternatives for treating bacterial infections in humans and animals and are currently being explored to mitigate pathogen concentrations in food products of animal and plant origin (Lin et al. 2017, Alomari et al. 2021). Studies on using bacteriophages to control bacterial infections, or to prevent the growth of spoilage organisms on fish, especially relating to *Kaistella* species, are limited to non-existing.

Purpose:

Several unidentified *Kaistella* isolates belonging to the genus *Kaistella* were previously isolated from fish in a study in the Food Science section of the University of the Free State, Bloemfontein, South Africa (Engelbrecht 1992). However, they were not identified at a species level, and their pathogenicity and/or spoilage capabilities have yet to be discovered since studies on the importance of *Kaistella* species in food sources are uncommon. There was, therefore, a need to investigate the ability of these species to act as pathogens and/or food spoilage organisms.

Virulence factors and determination of spoilage potentials, such as enzyme production, siderophore production, antibiotic resistance patterns, and biofilm production, will be used to determine these characteristics. Bacteriophages, as a biocontrol method for bacterial infections and as sanitizing agents, have recently received much attention and will be investigated against *Kaistella* isolates evaluated in this study. Therefore, this study will provide essential information on the virulence and spoilage capacity of these bacterial isolates, their effect on the quality of food, specifically fish products, and whether it will be possible to use bacteriophages to control spoilage or infection by these isolates.

Objectives:

i. To subject four *Kaistella* strains isolated from fish in a study by Engelbrecht (1992) to the latest taxonomic techniques for accurate characterisation and classification.
ii. To describe and name any new species.
iii. To evaluate the potential of six *Kaistella* fish isolates for pathogenicity by determining their virulence factors, which included siderophore production, enzyme production, and resistance to antimicrobials.
iv. To evaluate the potential of the six *Kaistella* fish isolates to produce biofilms.
v. To isolate and characterize bacteriophages against the six *Kaistella* fish isolates, use them as potential biocontrol agents against the *Kaistella* fish isolates.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The genus *Kaistella* was initially described in 2004 by Kim and co-workers, and the genus description has been subsequently amended by Nicholson and co-workers (2020). The genus has recently been moved from the *Flavobacteriaceae* family into the novel *Weeksellaceae* family (García-López et al. 2019). Novel species of *Kaistella* are being isolated regularly, and these novel species need to undergo taxonomic studies in order to assign them to the correct genus. Although the members of this genus have been isolated from various habitats, this study is particularly interested in the food sources from which some species have already been isolated, e.g., milk, fish, and meat.

To the author's knowledge, the significance of the *Kaistella* species in the food sources has not yet been investigated, therefore, there is a need to investigate the ability of these species to act as pathogens and/or food spoilage organisms. Methods used to assess these traits include virulence factor and spoilage potential determination, such as enzyme production, siderophore production, and biofilm formation.

Bacteriophage therapy has recently received much attention as a replacement for antibiotic treatment to control food spoilage or pathogens. Phage therapy, or the application of bacteriophages for treating bacterial infections, has existed for decades but has gained attention over the past two decades as a probable alternative treatment method because of the rise of antibiotic-resistant bacterial strains (Housby and Mann 2009). The increased interest has been enhanced by the fact that bacteriophages are effective against a wide variety of pathogenic bacteria because they are inexpensive and easy to isolate, and do not interfere with the average human microbiota or the environment (Mahadevan et al. 2009, Loc-Carillo and Abedon 2011, Nilsson 2014).

This literature review aims to provide a brief historical overview of the taxonomy of the genus *Kaistella* and the family *Weeksellaceae*; to indicate the ecology of this genus with a focus on food sources; to discuss taxonomic methods used to classify and identify bacterial organisms; to provide a brief discussion on virulence factors to indicate possible pathogenicity and/or spoilage characteristics; and finally, to discuss the function of bacteriophages in limiting bacterial growth. As a result, the findings of this study will contribute to a greater understanding of the possible role of *Kaistella* species in the microbial ecology of food spoilage and/or pathogenicity.

2.2. The Genus *Kaistella*

2.2.1. Historical Overview

The genus *Kaistella* was described initially by Kim et al. (2004), with *Kaistella koreensis* regarded as the type species of the genus. *Kaistella* was originally classified in the family *Flavobacteriaceae.* However, the name of the genus was contested by Kämpfer et al. (2009), who believed that *Kaistella koreensis* belonged to the genus *Chryseobacterium* as *Chryseobacterium koreense*.

However, from its initial description, after an extensive polyphasic study, it was placed in the family *Weeksellaceae* (García-López et al. 2019), order *Flavobacteriales*, class *Flavobacteriia*, phylum *Bacteroidota* (Parte et al. 2020). García-López et al. (2019) also recommended that old names for genera and species could be revived to accommodate the members of the *Weeksellaceae*. Shortly after this publication, the research by Nicholson et al. (2020) indeed supported this recommendation, and some genera, e.g., *Kaistella* and *Sejongia*, were revived to accommodate some of the previously described *Chryseobacterium* species, as indicated in Table 2.1.

The family *Weeksellaceae* currently consists of the following 22 genera: *Algoriella, Apibacter, Bergeyella, Chishuiella, Chryseobacterium, Cloacibacterium, Cruoricaptor, Elizabethkingia, Empedobacter, Epilithonimonas, Frigoriflavimonas, Halpernia, Kaistella, Moheibacter, Ornithobacterium, Planobacterium, Riemerella, Sejongia, Soonwooa,* Spongiimonas*, Wautersiella,* and *Weeksella* (type genus) (Parte et al. 2020). The genus *Kaistella* currently consists of 16 validly published species (Parte et al. 2020), indicated in Table 2.1.

2.2.2. The description of *Kaistella* species

Kaistella (Ka.is.tel'la. L. dim. suff. -ella; N.L. fem. n. *Kaistella* arbitrary name after KAIST, Korea Advanced Institute of Science and Technology). Cells are Gram-staining-negative, non-sporulating rods. All strains grow aerobically. All strains grow at room temperature. Carotenoid pigments are usually produced. Colonies may be opaque, translucent, or transparent. Catalase and oxidase are generally positive but not always, and arginine dihydrolase and β-galactosidase are usually negative but not consistently. Aesculin hydrolysis is present in some species. Nitrite is not reduced, and most species do not reduce nitrate. It does not grow on cetrimide agar. The acid production of mannitol, trehalose, and xylose is negative; acid production of glucose varies between species. The DNA base composition ranges from 31.3 to 41.6 mol% G+C. The major isoprenoid quinone is MK-6. The type species is *Kaistella koreensis* (Kim et al. 2004). The differential phenotypic and biochemical characteristics of the 15 species are indicated in Table 2.2.

Table 2.1: Validly published *Kaistella* species (Parte et al. 2020).

Validly published name	Previous or synonym name	Isolation source	References
Kaistella antarctica	*Sejongia antarctica, Chryseobacterium antarcticum*	Soil samples from penguin habitats in Antarctica	Yi et al. 2005, Kämpfer et al. 2009, Nicholson et al. 2020
Kaistella anthropi	*Chryseobacterium anthropi*	Blood from a hospitalized patient	Kämpfer et al. 2009, Nicholson et al. 2020
Kaistella carnis	*Chryseobacterium carnis*	Raw beef carcass from an unspecified abattoir	Holmes et al. 2013, Nicholson et al. 2020
Kaistella chaponensis	*Chryseobacterium chaponense*	Diseased farmed Atlantic salmon (*Salmo salar*)	Kämpfer et al. 2011, Nicholson et al. 2020
Kaistella daneshvariae	-	The peritoneal cavity of a patient	Nicholson et al. 2020
Kaistella flava	-	Soil from King George Island, Marine Antarctica	Peng et al. 2021
Kaistella gelatinilytica	-	Soil collected in the Antarctic	Ren et al. 2021
Kaistella haifensis	*Chryseobacterium haifense*	Raw cow milk	Hantsis-Zacharov and Halpern, 2007; Nicholson et al. 2020
Kaistella jeonii	*Sejongia jeonii, Chryseobacterium jeonii*	Moss near penguin habitat	Yi et al., 2005; Nicholson et al. 2020
Kaistella koreensis	-	Natural mineral water	Kim et al. 2004
Kaistella montana	*Chryseobacterium montanum*	Mountain soil	Guo et al. 2016, Nicholson et al. 2020
Kaistella palustris	*Chryseobacterium palustre*	Industrially contaminated soil	Pires et al. 2010, Nicholson et al. 2020
Kaistella solincola	*Chryseobacterium solincola*	Hydrocarbon-polluted soil	Benmalek et al. 2010, Nicholson et al. 2020
Kaistella soli	-	oil-contaminated soil	Chaudhary et al. 2022
Kaistella treverensis	*Chryseobacterium treverense*	Clinical (human blood)	Yassin et al. 2010, Nicholson et al. 2020
Kaistella yonginensis	*Chryseobacterium yonginense*	Mesotrophic artificial lake	Joung and Joh 2011, Nicholson et al. 2020

Table 2.2: Differential characteristics of the 16 *Kaistella* species currently validly published. +, positive; -, negative; ND, not determined; V, variable; w, weakly positive; d, delayed reaction.

Genus and species	*Kaistella antarctica*	*Kaistella anthropi*	*Kaistella carnis*	*Kaistella chaponensis*	*Kaistella haifensis*	*Kaistella jeonii*	*Kaistella koreensis*	*Kaistella montana*	*Kaistella palustris*	*Kaistella solincola*	*Kaistela soli*	*Kaistella treverensis*	*Kaistella yonginensis*	*Kaistella daneshvariae*	*Kaistella gelatinilytica*	*Kaistella flava*
Production of yellow to orange (+), flexirubin (F), and / or carotenoid (C) type pigments	+, not F	–	+	+	+, F (w), C	+, not F	+	+, C	+	+, C	+, C	+, F	+, F, C	+	+, C	–
Growth on																
MacConkey agar	–	–	+	–	+	–	–	ND	ND	–	ND	–	–	–	–	–
Growth with 3% NaCl	+	V	ND	–	–	V	+	+	+	+	–	+	–	ND	+	+
Growth at (°C)																
5	+	+	d	+	+	+	–	+	–	w	–	+	+	ND	+	+
30	d	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
37	–	+	+	+	+	–	+	+	+	w	–	+	–	+	–	+
42	–	–	–	–	–	–	d	+	–	–	–	–	–	–	–	–

Degradation of																
Esculin	V	–	–	–	V	+	V	–	+	–	+	+	+	–	+	+
DNA	+	+	+	ND	ND	+	ND	ND	ND	–	ND	–	–	ND	–	w
Starch	+	+	+	–	+	V	w	+	+	–	ND	+	ND	ND	–	+
Tween 80	+	V	–	ND	–	+	+	–	–	–	ND	–	ND	ND	–	–
L-Tyrosine	w	+	–	–	ND	V	w	ND	ND	ND	ND	w	ND	ND	ND	ND
Urea	–	–	–	V	–	–	d	–	–	+	–	–	–	–	–	+
Production of																
Hydrogen sulphide	–	–	–	–	–	–	–	–	ND	–	ND	–	ND	+	–	ND
Indole	V	V	+	–	+	V	+	+	–	–	ND	–	+	+	+	+
β-Galactosi-dase	–	–	–	–	+	–	–	–	–	–	–	–	–	ND	–	–
Reduction of																
Nitrite	–	ND	–	ND	ND	–	ND	ND	–	ND	ND	ND	ND	d	ND	ND
Acid production from																
L-Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	w	ND	+	+
Cellobiose	–	–	–	ND	–	–	–	+	–	–	ND	–	–	ND	+	–
Ethanol	ND	ND	–	ND	ND	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-Fructose	–	–	–	ND	+	–	–	–	–	–	ND	ND	ND	ND	+	ND
D-Glucose	+	+	+	–	+	+	V	+	+	+	+	–	w	d	+	+
Glycerol	–	ND	–	ND	ND	–	ND	ND	–	ND	ND	ND	ND	ND	+	ND
Lactose	–	–	–	–	+	–	–	+	–	–	ND	–	ND	–	+	–
Maltose	+	+	+	–	+	+	+	+	+	–	+	–	ND	d	+	+
D-Mannitol	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	+	–
Salicin	–	ND	–	–	ND	–	ND	ND	–	–	ND	ND	+	ND	ND	–
Sucrose	–	–	–	–	V	–	–	–	–	–	ND	–	+	–	+	+

2.2.3. Ecology

Kaistella species have been isolated from clinical, environmental, industrial, and food sources (Table 2.1). However, for this study, the emphasis will be on food sources. According to Hantsis-Zacharov and Halpern (2007), Kämpfer et al. (2011), Holmes et al. (2013) and Nicholson et al. (2020), members of the genus *Kaistella* are found in food sources such as seafood (*K. chaponensis*), meat (*K. carnis*) and milk (*K. haifense*).

Kaistella chaponensis was recovered from diseased Atlantic salmon farmed in Lake Chapo (Kämpfer et al. 2011). These bacterial strains were identified as fish pathogens after being isolated from the exterior lesions, gills, and fins of diseased fish in mixed cultures with the infamous salmonid pathogen, *Flavobacterium psychrophilum* (Kämpfer et al. 2011).

A psychrotolerant bacterium, *Kaistella haifensis*, formerly *Chryseobacterium haifense,* was isolated from raw milk (Hantsis-Zacharov and Halpern 2007). The organism was reported to have a wide range of proteolytic and lipolytic abilities during an investigation of the psychrotolerant bacterial community in raw milk (Hantsis-Zacharov and Halpern 2007). The ability of microbes to degrade food has been associated with lipolytic and proteolytic activities (Signorini et al. 2003).

Another species of *Kaistella* related to food, is *K. carnis*, which was isolated from beef (Holmes et al. 2013). Its ability to produce indole from the breakdown of tryptophan (Table 2.2) may be a virulence factor because indole is involved in many aspects of bacterial life, including virulence induction, cell cycle regulation, acid resistance, and, most importantly, signalling biofilm formation (Hu et al. 2010).

2.3. Taxonomic techniques for the description of novel species

For the longest time, microbial systematics was based on analysing physical and biochemical properties until researchers saw a need for a more pragmatic approach. The discovery of nucleic acids, the hypothesis that DNA encodes genetic information, and Watson and Crick's description of the DNA molecule structure laid the groundwork for the emerging field of molecular biology (Watson and Crick 1953). Modern molecular approaches have revealed an extraordinary diversity of microorganisms (Agrawal et al. 2015).

Bacterial taxonomy development led to a polyphasic approach, a term invented by Colwell in 1970, which employed several methods to characterise microorganisms completely. Polyphasic taxonomy has been the gold standard for bacteria delineation since it integrates data and information from microorganisms' phenotypic, genotypic, and phylogenetic aspects.

2.3.1. Genotypic methods

Pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), plasmid DNA profiling, and RFLP derivative techniques were among the traditional genotypic techniques first employed to identify bacteria (Vandamme et al. 1996). All these techniques were based on agarose gel electrophoresis to separate DNA based on size and then use the separated DNA fragments to confirm the identity of a bacterial isolate. Developments in molecular biology gave rise to more advanced molecular techniques, such as 16S rRNA gene sequencing, DNA-DNA hybridization (DDH), whole genome sequencing, and guanine and cytosine ratio (G+C Ratio) (Brenner et al. 1969, Wayne et al. 1987, Helene et al. 2022).

2.3.1.1. 16S rRNA Gene Sequencing

Studies of the regular associations between prokaryotes and their application-transformed classification have been made possible by 16S rRNA gene sequence analysis (Kämpfer 2010). The 16S rRNA gene in bacteria has the advantages of being broadly distributed, highly conserved, essential to protein synthesis, unable to horizontal transfer, and evolving at a rate that characterizes an appropriate amount of organismal diversity (Clarridge 2004). According to Tindall et al. (2010), organisms of the same species have a 16S rRNA gene sequence similarity percentage of 97% or greater. However, it is required to compare several species isolates, since sequence similarities might vary by up to 5% (Bernardet et al. 2002). For species classification, higher 16S rRNA similarity estimates of 98.7–99.0% (Stackebrandt and Ebers 2006) and 98.2–99.0% (Meier-Kolthoff et al. 2013) have been suggested. However, the 16S rRNA gene must be used with other additional techniques, such as average nucleotide identities (ANI), amino acid indices (AAI), and DNA-DNA reassociations for accurate species classification (Stackebrandt and Goebel 1994, Gillis et al. 2001, Konstantinidis and Tiedje 2005, Goris et al. 2007, Richter and Rosselló-Mora 2009, Figueras et al. 2014).

There is a need for phylogenetic tree construction after 16S rRNA sequencing because the 16S rRNA is often considered the best tool to infer prokaryotic phylogeny (Woese 1987). The phylogenetic tree is essential in classification because it identifies the genus to which the strain belongs and its closest relatives (Prakash 2007). It must be constructed using at least two techniques to describe a new species (Bernardet et al. 2002). The maximum likelihood (ML), maximum-parsimony (MP), unweighted pair group (UPGMA), and neighbour-joining (NJ) approaches are available (Sokal 1966, Cavalli-Sforza and Edwards 1967, Fitch 1971, Sneath and Sokal 1973, Felsenstein 1978, Saitou and Nei 1987).

The MP method uses regions that have, at minimum, two distinct nucleotides or amino acids. In contrast, the UPGMA Method acknowledges that the nucleotide and amino acid substitutions rate is similar in all evolutionary trees (Nei and Kumar 2000). The NJ approach is first used to build a tree by the ML method, and then the branch lengths are changed to optimize the likelihood of the data (Nei et al. 1998). Variants are produced using the nearest neighbour interchange (NNI) approach (Zhang 2019). Branch lengths with the highest likelihood are calculated, and only variants with the highest probability are retained (Nei and Kumar 2000).

The bootstrap analysis demonstrates the stability of the phylogenetic tree's branching (Bernardet et al. 2002). Only when the internal branch's bootstrap value is 95% or above can the branch's structure be considered accurate (Nei and Kumar 2000). Including all related species in the phylogenetic analysis will increase the significance of the phylogenetic treeing approach that would have been used (Bernardet et al. 2002).

The 16S rRNA gene sequencing method, however, has several limitations, including the inability to classify organisms that have recently branched into different species and the presence of multiple intragenomic copies of this gene which could carry subtle nucleotide substitutions, may overestimate microbial abundance (Johnson et al. 2019). Most bacteria have multiple copies of the 16S rRNA gene, which has both advantages (easier to amplify by PCR) and disadvantages (not all copies are identical). As a result, a more holistic approach, such as the whole-genome sequencing method, has gained dominance (Land et al. 2015).

2.3.1.2. Whole-Genome Sequencing (WGS)

Whole-genome sequence studies have revolutionised bacterial isolates' identification and taxonomic categorization (Coenye et al. 2005, Nicholson et al. 2020). The main objective of genomic taxonomy is to obtain taxonomic data from WGS that can be used to build a robust framework for identifying and categorizing prokaryotes (Fitz-Gibbon and House 1999, Thompson 2013).

More research has revealed that genomic sequences can provide information on taxonomic factors, such as chromosomal gene order, metabolic pathways, comparisons of orthologous genes, and indels or single nucleotide polymorphisms (SNPs) in conserved genes (Gupta 2001). Studies created by comparing 16S rRNA genes and phylogenetic analyses based on orthologous genes' comparisons and the presence or absence of genes further demonstrated their strong similarities (Zhi et al. 2012).

Gene content-based methods for bacterial taxonomy are becoming more promising thanks to the application of WGS. Additionally, it exposes many distinct genes only found in specific genomes and can be used for taxonomic classification (Gupta and Sharma 2015).

The number of prokaryotic genomes that have been sequenced has expanded rapidly since the development of genome sequencing methods. Since they use reliable and repeatable data, genomic sequences are frequently recommended for taxonomic studies (Ramasamy et al. 2014). Genome sequencing was not initially well suited to routine use because it was time-consuming and expensive. However, next-generation sequencing methods have become widely used and have made it possible to sequence thousands of genomes due to their low cost and high throughput (Soon et al. 2013). Over the past decade, sequencing technologies have become affordable for routine microbial identification (Nakamura et al. 2008, Nakamura et al. 2011). Consequently, bacterial species can be identified using their genetic signature (Thompson et al. 2009).

The data generated from whole genome sequences allows for the determination of the digital DNA-DNA hybridization, average nucleotide identity (ANI) and average amino acid indenty (AAI) values, which are widely used and compulsory for the description of novel species (Konstantinidis and Stackebrandt 2005, Gosselin et al. 2022).

2.3.1.3. DNA-DNA Hybridization

Whole-genome sequencing was not available until recent years, and even then, it was limited to a small proportion of organisms. Therefore, other parameters were required to classify organisms into practical categories (Goris et al. 2007). DNA-DNA hybridization (DDH) has been used to ascertain the relatedness of bacteria since the 1960s. They formed part of a few methods that could provide genome-wide comparisons between species (Goris et al. 2007). DNA-DNA hybridization, or DNA-DNA reassociation, is another approach used in microbial taxonomy to classify microorganisms. Wayne and colleagues (1987) suggested that most bacterial species include strains with a 70% or higher DNA-DNA reassociation value. DNA association is determined by enabling single-stranded DNA from one strain to reassociate with single-stranded DNA from another, forming a double-stranded DNA molecule (Baron 1996).

The main drawbacks of DNA-DNA reassociation include the time-consuming nature of pairs cross-hybridizations, the need to use isotopic elements, and the difficulty in creating a central database (Cho and Tiedje 2001). Another drawback is that the method only determines the relative proportion of similarity and not the precise identity of the sequence (Prakash et al. 2007).

However, since the advent of WGS, it is now possible to calculate the digital DNA-DNA hybridization (dDDH) information directly from the WGS data. Different application tools, such

as the Genome-Genome Distance Calculator (http://ggdc.dsmz.de/), are used to calculate dDDH (Chun et al. 2018).

2.3.1.4. Average Nucleotide Identity (ANI)

Average nucleotide identity (ANI) is the latest example of genome-derived measures of genetic relatedness that have been applied to species classifications (Thompson et al. 2015). The concept of using ANI to assess the genetic similarity of bacterial strains was proposed nearly 17 years ago (Konstantinidis and Tiedje 2005). It was determined that values of about 95% matched the cut-off value of 70% for DNA-DNA hybridization, which is frequently used to distinguish between bacterial species (Konstantinidis and Tiedje 2005, Goris et al. 2007, Richter and Rosselló-Mora 2009). The ANI values can also be extracted from genomic data and applied for taxonomic objectives (Konstantinidis and Tiedje 2005, Thompson et al. 2013, Gosselin et al. 2022). Average nucleotide identity can be calculated using the Kostas lab ANI calculator (http://enve-omics.ce.gatech.edu/ani/., JSpeciesWS (https://jspecies.ribohost.com/jspeciesws/), ezbiocloud ANI calculator (Ezbiocloud.net), etc.

2.3.1.5. Amino Acid Identity (AAI)

Another method used in novel species identification, resulting from developments in molecular biology, is the amino acid identity (AAI) determination (Rosselló-Mora 2005). As in the case of ANI, it can be calculated from two genomic datasets of proteins using computer-based calculation tools, such as the Kostas lab AAI calculator (Rodriguez-R and Konstantinidis 2014), which indicates the average AAI using both best hits (one-way AAI) and reciprocal best hits (two-way AAI). It assesses genome-wide identity between distant organisms and is strongly suggested for more distantly related populations, as resolution gradually decreases at the nucleotide level due to nucleotide sequences changing more quickly than amino acid sequences (Konstantinidis and Tiedje 2005, Rosselló-Mora 2005, Rodriguez-R and Konstantinidis 2014). It was discovered that 70% DNA-DNA hybridization threshold corresponds to ~95 to 96% AAI.

2.3.1.6. Guanine and Cytosine Ratio (G+C Ratio)

Analysis of the DNA G+C ratio is one of the conventional genotypic methods in taxonomy (Prakash et al. 2007). One of the most widely used taxonomic criteria in microbiology is the ratio of guanines and cytosines to all other nucleotides in a genome (Rosselló-Mora and Amann 2001). A 15–75% G+C range can be found in bacterial DNA. However, this proportion is precise and, to some extent, non-exclusive (Lightfield et al. 2011). One species may or may not be represented by two isolates with the same G+C composition (Baron 1996). If the G+C contents differ significantly, the strains cannot belong to the same species. The G+C composition of the strains usually varies by less than three mol% in novel species (Mesbah et

al. 1989). However, when an organism's complete genome sequencing data are available, the G+C content may be determined from a high-quality genome sequence, eliminating the need for conventional assessment techniques (Hahnke et al. 2016).

2.3.2. Chemotaxonomic techniques

Chemotaxonomy provides essential and consistent data on chemical characteristics for classification and identification purposes (Velázquez et al. 2006). The most commonly used chemotaxonomic indicators include cell wall and membrane components, such as peptidoglycans. However, the peptidoglycan type of Gram-negative bacteria is relatively uniform and offers limited data (Goodfellow and O'Donnell 1994, Vandamme et al. 1996). However, polar lipids, fatty acids (both composition and relative ratio), isoprenoid quinones, and polyamines may be used to differentiate between species (Suzuki et al. 1993; Vandamme et al. 1996).

Fatty acids are the major constituents of lipids and lipopolysaccharides and have been used extensively for taxonomic purposes (Vandamme et al. 1996, de Carvalho and Caramujo 2016). The cellular fatty acid methyl ester content is a stable parameter, provided that highly standardized culture conditions are used. The method is cheap, rapid, and has reached a high degree of automation (Vandamme et al. 1996). Fatty acid composition of strains can differentiate between bacterial genus-level groups through fatty acid methyl ester analysis (FAME) (Austin and Priest 1986).

A range of lipids are present in bacterial cells. Polar lipids are the main components of the lipid bilayer of bacterial membranes and have been studied regularly for classification and identification purposes (Vandamme et al. 1996, Sohlenkamp and Geiger 2016).

The terpenoid lipids, known as respiratory quinones, are crucial chemotaxonomic indicators and components of bacterial plasma membranes. Menaquinone (MK)-6 is the only or predominant respiratory quinone in species of the *Weeksellaceae* family (Kim et al. 2004, Nicholson et al. 2020), while MK-7 is prevalent in species of related families (Hanzawa et al. 1995, Bernardet et al. 2002). This method can identify bacteria down to the genus level in other bacterial groups (Vandamme et al. 1996, Prakash et al. 2007).

Chemotaxonomic methods were compulsory for describing novel species up to the recent past. However, the event of whole genome sequencing, from which data such as fatty acids may be deducted, and the fact that chemotaxonomic markers within a genus and species stay relatively constant, has driven the decision by the International Journal of Systematic and Evolutionary Microbiology Committee to make the inclusion of these techniques in species descriptions, optional (IJSEM Board meeting, 8 September 2022). However, for novel genus

and higher taxa descriptions, these methods still need to be included (IJSEM Instructions for authors https://www.microbiologyresearch.org/content/journal/ijsem).

2.3.3. Phenotypic Tests

Traditional microbiological techniques have been used for bacterial identification for a long time and are still a crucial component of all microbiological laboratories today (Morgan et al. 2009). Basic growth parameters, like growth at various temperatures, growth under aerobic or anaerobic conditions, the requirement for particular nutrients, the capacity to produce specific metabolites, and the production of enzymes, all provide crucial information on an organism's identity, as well as its potential for spoilage or having pathogenic characteristics (Banwart 1989). Nevertheless, it has become evident that classifying microorganisms using these methods does not correlate well with evolutionary relationships (Lane et al. 1985).

2.3.3.1 Morphological and Biochemical Methods

The structure of the bacterial cell and colony and the metabolic processes within the bacterium represent the phenotypic characteristics of bacteria (Bisen et al. 2012). Morphological traits include shape, elevation, edge, optical properties, consistency, colony surface, and colouration (Abiola and Oyetao 2016). Production of enzymes (such as catalase and oxidase) and the metabolism and oxidation of various carbohydrates are biochemical properties (Prakash et al. 2007, Bisen et al. 2012, Aguilera-Arreola 2015).

Several cell treatment methods, such as Gram or Ziehl-Neelsen stains, which aid in the microscopic identification of the morphology of the bacterial cells, the presence or absence of spores, and motility, can be applied after a pure culture has been established (Aguilera-Arreola 2015). For the isolation and identification of bacteria, suitable atmospheric conditions (e.g., aerobic, or anaerobic) are necessary (Bisen et al. 2012). Incubation temperature, pH, number of nutrients required for growth, and antibiotic resistance, are other crucial features that must be determined (Bisen et al. 2012).

Most genera in the family *Weeksellaceae* produce yellow to bright orange yellow-pigmented colonies on agar media, and this primarily depends on the medium and incubation temperature or time (Hugo et al. 2019).

2.3.3.2. Automated BIOLOG™ Omnilog System

The phenotypic identification of bacteria and fungi (filamentous and yeast) uses the BIOLOG™ Omnilog identification approach (Sandle et al. 2013). The BIOLOG™ method uses tetrazolium-based colour changes that enable colourimetric quantification of the increased respiration when microbial cells oxidize a carbon source (Miller et al. 1993).

All the wells in a 96-well microtiter plate are initially colourless, but when a chemical is oxidized, a spurt of aerobic respiration induces the reduction of the tetrazolium dye in the well, establishing a purple colour. This colour indicates that the tetrazolium-based reagent has been irreversibly reduced to a soluble, purple compound called formazan (Miller et al.1993, Sandle et al. 2013). Numerous carbon sources, including carbohydrates, amino acids, hexose acids, carboxylic acids, esters, fatty acids, etc., are present in the 96-wells and various chemical susceptibility tests (NaCl, acidic pH, lactic acid, reducing power, etc.).

Various microorganisms can be classified and distinguished using the BIOLOG™ Omnilog system. Within the 96-well microplate, microorganisms form a unique pattern from discrete test reactions (Biolog 2008). An OmniLog™ incubator reads the microplates in a completely automated system. Metabolic fingerprints form quickly in this method and frequently produce results within ± 4 h. The data can be automatically read and recorded in a matter of seconds. Sophisticated software automatically optimizes for varying turbidity/colour intensities, doing away with subjective visual analysis. For final identification, the patterns are then matched to an extensive database. There are currently 2900 microorganisms in the database.

2.4. Virulence Factors

The native beneficial bacteria (naturally present in the body) are harmless and play beneficial roles in human health (Linares et al. 2016). However, bacteria can colonize, invade, and cause damage to different tissues and are categorized as pathogens (Cepas and Soto 2020). Pathogenic bacteria use a variety of mechanisms including virulence factors to enhance their ability to cause disease in their hosts (Peterson 1996, Wilson et al. 2002). Virulence factors are molecules, often glycoproteins or glycolipids, derived from bacterial genes that assist the bacterium in colonizing the host at the cellular level (Sharma et al. 2017). Examples of virulence factors are given in Table 2.3. Microbes use virulence factors to kill phagocytic cells, block phagocytosis, evade fusion with lysosomes, block killing within phagocytes, and enhance phagocyte replication (Zachary 2017).

However, bacterial pathogenicity is a complex and multifactorial process that depends on both host and bacterial factors. The virulence mechanisms bacteria use to cause infection include adhesins, invasins, type three secretion systems, outer membrane proteins, toxins, capsules, iron acquisition systems, and biofilm formation, among others (Peterson 1996, Cepas and Soto 2020). Moreover, determining the presence or absence of virulence factors is crucial because these factors determine whether a microbe can cause disease (Edberg et al. 1996). This study will focus only on the enzyme production by bacteria as virulence factors.

Table 2.3: Examples of virulence factors and their function.

Virulence factor	Function	Reference
Adhesins	Help the pathogen to attach to host cells and tissues and facilitate entry into the cell by endocytosis/phagocytosis	Klemm and Schembri 2000, Zachary 2017
Invasins	Cell dysfunction and lysis	Zachary 2017
Capsules	Provides bacteria protection from the host immune response and antibiotics	Wilson et al. 2002
Endotoxins	Stimulate macrophages and endothelial cells to secrete pro-inflammatory cytokines and nitric oxide, multiple organ dysfunction, and lysis	Wang and Quinn 2010, Zachary 2017
Exotoxins	Inhibit biochemical pathways within a cell	Zachary 2017
Hemolysins	Resistance to the host defence mechanism, tissue damage	Elliot et al.1998
Enzymes		
Lipases	Breaks down lipids	Edberg et al. 1996
Proteases	Degrades immunoglobulins and components of the complement system and facilitates propagation of micro-organisms	Maeda and Yamamoto 1996
Hyaluronidase	Breaks down hyaluronan	Zachary 2017
Collagenase	Breaks down collagen fibres	Zachary 2017
Coagulase	Promotes fibrin clotting, helps protect bacteria in host tissues	Madigan et al. 2000
Elastase	Dissolves cellular glue/elastin	Edberg et al. 1996
DNase	Destroys the nucleic acid of cells	Edberg et al. 1996
Kinase	Digest fibrin and prevent clotting of the blood needed to wall off bacteria	Zachary 2017
Gelatinase	Hydrolyses/degrades gelatine	Edberg et al. 1996
Lecithinase	Breaks down phospholipids in cell membranes	Madigan et al. 2000

2.4.1. Enzyme Production

Several enzymes have been associated with microbial virulence. Enzymes measured as virulence factors are usually active against host components and contribute to virulence by damaging host tissues (Casadevall and Pirofski 2009). Tissue damage makes the host susceptible to microbial infection. It is evident from Table 2.3 that the enzymes produced by bacteria can be divided into two groups. The first group consists of proteases that assist the pathogen in invading the host by degrading proteins, acquiring required amino acids, and moving through the hosts' tissues, e.g., elastase and hyaluronidase, which weaken connective tissues. The second group of enzymes provides the pathogen with what it needs for survival, e.g., lipases, which break down fats into more readily utilizable compounds (Madigan et al. 2000, Casadevall and Pirofski 2009). Other enzymes, such as urease, contribute to virulence by facilitating survival inside phagocytic cells (Cox et al. 2000).

2.4.2. Siderophore production

Iron is essential for many processes necessary for life, including DNA replication and electron transfer, due to its ability to take on multiple oxidative states (Crichton 2009, Haldon and Bachman 2015). Each oxidation state brings about challenges to the cell. Ferrous iron (Fe^{2+}) is extremely toxic in its free form, whereas ferric iron (Fe^{3+}) is insoluble at physiological pH and not readily bioavailable (Haldon and Bachman 2015). As a result, free iron levels are deficient, and most of the iron in the human body is bound by storage-, transport-, and metabolic proteins. Pathogens must then compete to acquire iron the host tightly regulates because bacteria require iron for replication during colonization and infection. To outcompete tight iron binding by host molecules, Gram-negative bacteria, Gram-positive bacteria, and some fungi secrete small iron-sequestering molecules called siderophores (Fischbach et al. 2006, Correnti and Strong 2012, Haldon and Bachman 2015).

Siderophores are low molecular weight chelators of iron and/or surface receptor proteins that bind transferrin, lactoferrin, ferritin, haemoglobin, ferrous iron transporters, heme or haptoglobin–haemoglobin complexes (Casadevall and Pirofski 2009, Crumbliss and Harrington 2009). Overall, bacteria that can survive either within or outside of a host use siderophores to obtain iron, whereas species-specific organisms that do not survive in the environment acquire iron from the host through surface receptors for transferrin, lactoferrin, and other iron-containing molecules (Genco et al. 1991, Casadevall and Pirofski 2009). Siderophores are significant virulence factors, predominantly in pathogens that code for multiple siderophores due to the acquisition of siderophore synthesis systems by horizontal gene transfer (Vokes et al. 1999).

Bacterial strains that can over-produce siderophores are considered hyper-virulent, while strains incapable of secreting siderophores have reduced virulence and fitness during infection and colonization (Haldon and Bachman 2015).

In addition to promoting virulence, siderophores also play a counteractive role in controlling disease in fish by restricting the amount of iron required for virulence and bacterial interactions (Li and Chi 2004). The pathogenic bacterium infects the fish host in two different ways: (i) by producing toxic enzymes, such as proteases and cholesterol acyl transferase, to thwart the host's defensive mechanisms, and (ii) by producing transferrin to compete with the host for iron and stunt its growth (Yano 1996). According to Gram and co-workers (1999), the main component of the biocontrol process is the rivalry between the siderophore produced by biocontrol agents and the transferrin produced by pathogens in complexes with iron. Fish farming uses siderophore-producing bacteria (*Pseudomonas fluorescens*) as probiotics because they can reduce the growth of several fish pathogens, such as *Vibrio anguillarum*, *V. ordalii*, *Aeromonas salmonicida*, *Lactococcus garvieae*, *Streptococcus iniae* and *Flavobacterium psychrophilum* (Gram et al. 2001, Brunt et al. 2007, Dimitroglou et al. 2011).

Since iron is vital to several cellular processes, its chelation by siderophores could significantly affect host cellular homeostasis (Holden and Bachman 2015, Wilson et al. 2016, Khasheii et al. 2021). Depending on the cellular response, siderophores can act as immunomodulators or toxins, causing cell death. Using siderophores to obtain some degree of host iron during infection allows bacteria to compete successfully for replicative niches in the intestines and the nasal cavity, allowing bacterial colonization (Holden and Bachman 2015, Wilson et al. 2016). The production of siderophores can affect the anatomic site and pattern of infection. The ability of siderophores to induce host cell hypoxic responses can alter the outcome of infection; therefore, disruption of host cell homeostasis by siderophores can be fatal. Still, it can also function as a dangerous signal that triggers a protective response to infection (Holden and Bachman 2015, Wilson et al. 2016).

The significance of siderophores as virulence factors during colonization and infection is still being investigated today. Individual siderophores can serve specific roles, such as evading Lipocalin-2, a constituent of neutrophil secondary granules expressed by macrophages and the epithelium in response to inflammation. It is one of the proteins that sequester ferric siderophore complexes away from bacterial receptors. It binds non-iron heavy metals, initiates, or inhibits host cellular pathways, and determines the replicative niche during infection (Clifton et al. 2009, Holden and Bachman 2015).

2.4.3. Antibiotic Resistance

An antibiotic is a compound a microbe produces that kills other microbes (Waksman et al. 1947, Waksman et al. 2010). The use of antibiotic-producing microbes to prevent disease dates back many years (Hutchings et al. 2019), but the innovation of anti-bacterial drugs and the basic principle of chemotherapy is mainly ascribed to Paul Ehrlich, who established the artificial arsenic-based drug salvarsan (salvation arsenic) and neo-salvarsan over a century ago, to treat syphilis, a sexually transmitted disease (STD), caused by *Treponema pallidum* (Gelpi et al. 2015).

Infectious diseases used to be the second principal cause of death in the world prior to the invention of antibiotics (Kaplan and Laing 2004). The introduction of antibiotics offered the medical community the essential tools for combating these diseases. Antibiotics were extensively used, following their application in innovative therapeutic practices, such as transplantations or aggressive surgical procedures (Vranakis et al. 2013). The antibiotic era revolutionized the treatment of infectious diseases worldwide, with much success in developed countries (Adedeji 2016).

Sulphonamides were the first highly viable antimicrobials that acted against an extensive range of disease-causing bacteria in clinical use, and they are still used today. Still, they were rendered obsolete by the emergence of penicillin, discovered in 1928 on a contaminated Petri dish by Alexander Fleming (Fleming 1929). Selman Waksman began a comprehensive investigation of microbial species as producers of antimicrobial compounds after discovering penicillin, tyrocidine and various reports of antimicrobial compound production by microorganisms (Hutchings et al. 2019).

The bacterial cell wall is the target of several different types of antibiotics, including β-lactams (on their own or in conjunction with β-lactamase inhibitors), glycopeptides (vancomycin, teicoplanin), lipoglycopeptides (telavancin, oritavancin), cyclic peptides (daptomycin), D-cycloserine, fosfomycin, and bacitracin (Table 2.4). Antibiotics that disrupt the cell wall of bacteria employ techniques that block the activity of enzymes essential for the formation, maturation, and development of the peptidoglycan layer (Vranakis et al. 2014). Antibiotics that inhibit protein synthesis include tetracyclines, aminoglycosides, macrolides, and chloramphenicol (Leviton 1999). Quinolones inhibit the crucial bacterial enzymes DNA gyrase and DNA topoisomerase IV, whereas rifampicin inhibits RNA synthesis (Drlica et al. 2009, Vranakis et al. 2014).

Table 2.4: Classification of antimicrobials based on their mechanism of action (Dowling et al. 2017, Ullah et al. 2017).

Antimicrobial family	Mechanism of action	Resistance mechanism
β Lactams Cefepime Cephalosporins Penicillins	Interfere with the synthesis of the cell wall	Beta-lactamase production, primarily *bla* genes
Aminoglycosides Amikacin Gentamicin Streptomycin Neomycin	Inhibition of protein synthesis	Phosphorylation stops them binding
Quinolones Ciprofloxacin Levofloxacin Moxifloxacin Gatifloxacin	Blockage of bacterial replication	Efflux pumps
Polymyxin Colistin Polymyxin B	Disruption of the cell membrane	Membrane permeability
Sulphonamides Sulfamerazine Bactrim Sulfadiazine Trimethoprim	Inhibition of folate synthesis in the initial stages	Resistant dihydrofolate reductase (*dfr* gene).
Macrolides Roxithromycin Azithromycin Clarithromycin Erythromycin	Protein synthesis inhibition	Blocking the binding of DNA gyrase

Resistance to antimicrobial agents has become the primary concern in human health and veterinary medicine. Antimicrobial agents are used in several countries in veterinary practice for the treatment and prevention of infectious diseases and growth promotion in food animals

(Koga et al. 2015). However, the unselective use of antimicrobial agents can result in bacterial selection pressure on the intestinal microbiota of animals (Argudín et al. 2012).

Antimicrobial resistance can occur in one of three ways:

i. Innate resistance occurs when genes encoding inherent antibiotic resistance in the bacteria confer natural resistance. Antibiotics exert selective pressure on bacteria, quickening evolution and concurrent adjustments in genetics and metabolism to produce multidrug-resistant phenotypes (Vranakis et al. 2013).
ii. Acquiring resistance is due to selective antibiotic pressure from the environment and, therefore, shows that a threshold concentration of antibiotics is necessary to induce and sustain resistance genotypes (Kester and Fortune 2013).
iii. Adaptive resistance reflects the ecological niche of the bacteria and includes environmentally induced genetic changes (Schroeder et al. 2017).

Antimicrobial resistance mechanisms can be divided into four major groups: (i) reducing drug uptake; (ii) altering drug targets; (iii) inactivating drugs; and (iv) active drug efflux (Fig. 2.1). Enzymatic alteration of the antibiotic is a typical method of antibiotic resistance (Wright 2005). The four main enzymes that alter antibiotics are hydrolases, group transferases, redox enzymes, and lyases. Many of these are termed exotoxins and boost virulence while triggering antibiotic resistance simultaneously (Schroeder et al. 2017).

Hydrolases cleave hydrolytically susceptible chemical bonds such as esters and amides, often essential for antibiotic biological activity (Wright 2005). It is an overall strategy to engender antibiotic resistance and virulence. These water-soluble enzymes can be easily excreted from bacteria as a prophylactic countermeasure, intercepting antibiotic molecules before contact with bacteria (Wright 2005, Worthington and Melander 2013). The group transferases represent a broad and well-characterized class of bacterial enzymes that impose covalent modifications on antibiotics to inhibit antibiotic target binding. Redox enzymes do not represent a widely recognized strategy for antibiotic modification (Shaw et al. 1993). Lyases cleave carbon-carbon, carbon-oxygen, carbon-nitrogen, and carbon-sulfur bonds by a mechanism independent of either hydrolytic or oxidative cleavage (Mukhtar et al. 2001).

Among bacteria, antibiotic resistance is rising in both community and hospital settings, associated with increased mortality and morbidity (Cepas and Soto 2020). The emergence of resistance in bacteria threatens the ability to treat common infectious diseases, leading to prolonged illness, disability, and death (Cepas and Soto 2020).

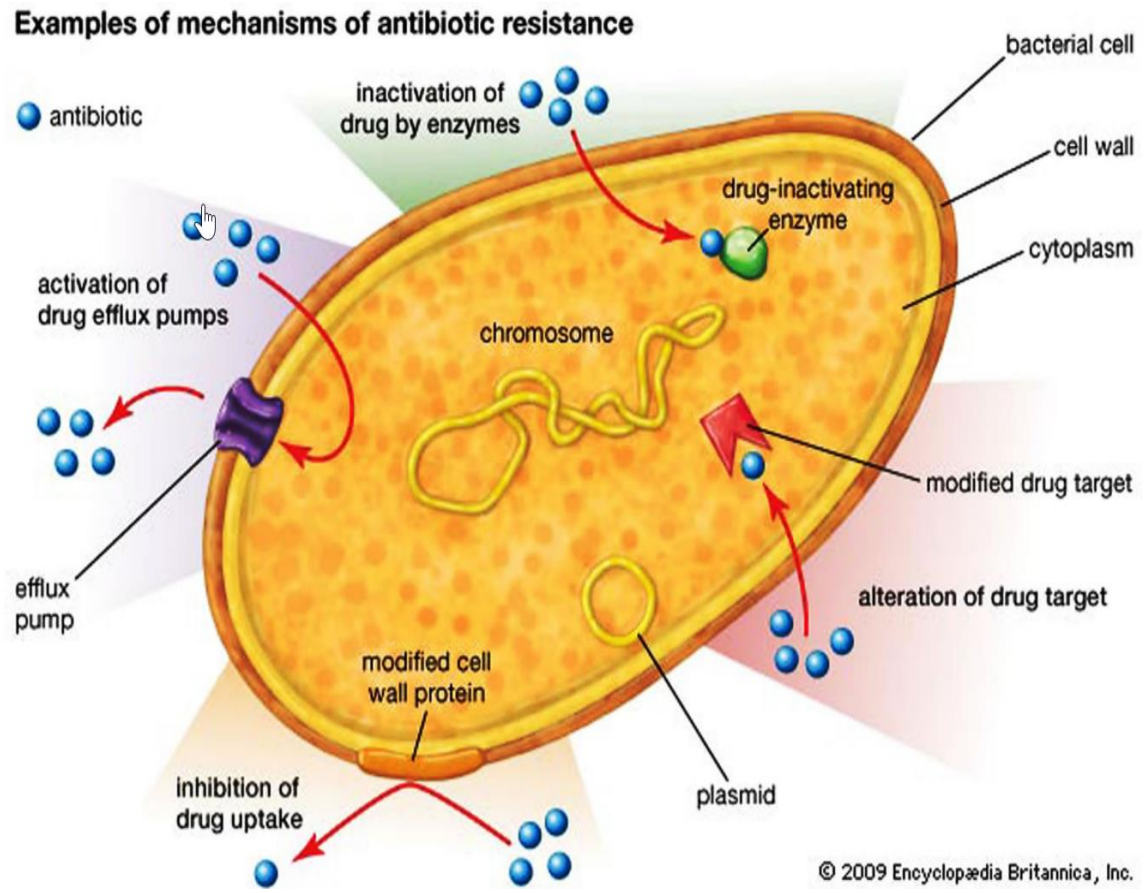

Figure 2.1: Antimicrobial resistance mechanisms in bacteria. https://www.britannica.com/science/antibiotic-resistance#/media/1/1027479/129670.

In a study conducted by Peng and colleagues (2021), genes associated with resistance to antibiotics and toxic compounds, including multiple antibiotic resistance (the MAR locus), copper homeostasis, cobalt–zinc–cadmium resistance, resistance to fluoroquinolones, copper tolerance, β-lactamase, zinc resistance and resistance to chromium compounds were identified in *Kaistella flava*.

Antimicrobial susceptibility data on members in the genus *Kaistella* remain very limited since these organisms are rarely isolated from clinical specimens. However, since most species were initially classified as belonging to the genus *Chryseobacterium*, antimicrobial resistance in the genus *Chryseobacterium* is expected to be substantially similar.

Chryseobacterium species are known to exhibit resistance to aminoglycosides, tetracyclines, chloramphenicol, erythromycin, clindamycin, and teicoplanin and are variably susceptible to

piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, minocycline, tigecycline, ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazol (Aber et al. 1978, Bloch et al. 1997, Hsueh et al. 1997, Kirby et al. 2004, Lin et al. 2010, Chou et al. 2011, Chen et al. 2013, Lo and Chang 2014).

In a study conducted in 2014 by Lo and Chang, it was reported that *C. gleum* isolates showed multi-drug resistance features, with trimethoprim/sulfamethoxazole and minocycline being the most potent drugs. A comparative study between C. *gleum* and C. i*ndologenes* found that *C. gleum* was significantly more susceptible to piperacillin, piperacillin-tazobactam, ceftazidime, tigecycline, and levofloxacin than *C. indologenes* (Lin et al. 2019).

Chryseobacterium indologenes showed resistance to aminoglycosides, first-generation cephalosporins, aminopenicillins, aztreonam and carbapenems (Chang et al. 2015, Arif et al. 2019, Izaguirre-Anariba and Sivapalan 2020). In a recent study, *C. indologenes* and *C. joostei* showed resistance to 80% of the antimicrobials evaluated (Mwanza et al. 2022).

It can, therefore, be hypothesized that the *Kaistella* species will have similar antibiotic susceptibilities/resistance since they are closely related to the genus *Chryseobacterium.*

2.4.4. Biofilm formation

Persistent infections have become a global challenge due to their ability to withstand antibiotics, host defence systems, and other external stresses (Costerton et al. 1999, Chen and Wen 2011, Sharma et al. 2019). Bacteria can adhere to almost every surface, forming architecturally complex communities termed biofilms (López et al. 2010). Bacterial biofilms are clusters of bacteria attached to a surface and/or to each other and embedded in a self-produced matrix (Vestby et al. 2020). The biofilm matrix comprises fibrin, alginate, and eDNA (Vestby et al. 2020). There are numerous benefits that a bacterial community might obtain from the formation of biofilms. In addition to the protection offered by the matrix, biofilms provide resistance to many antimicrobials and protection from protozoan grazing and bacteria in biofilms can use various survival strategies to overcome the host defence mechanisms (Matz and Kjelleberg 2005, López et al. 2010, Vestby et al. 2020). Biofilm formation is a two-stage process that involves the up-and down-regulation of several genes, that starts with the adherence of bacteria to a substrate surface, extracellular matrix secretion, and detachment in response to different environmental conditions (Chen and Wen 2011, Oliveira et al. 2020).

The diffusion of nutrients, vitamins, or cofactors is slow in biofilms, resulting in a bacterial community in which some cells are metabolically inactive (López et al. 2010). Moreover, the bacterial growth rate is enhanced because cells within a biofilm are confined to limited space (Stewart and Franklin 2008). This condition is similar to the stationary phase created under

laboratory conditions. Therefore, biofilm formation, in a way, represents the natural stationary phase of bacterial growth. During the stationary phase, bacteria profoundly change their physiology by increasing the production of secondary metabolites such as antibiotics, pigments, and other small molecules (Martin and Liras 1989). These secondary metabolites also function as signalling molecules to initiate the process of biofilm formation or to inhibit biofilm formation by other organisms that inhabit the same habitat (Lopez and Kolter 2009).

Quorum sensing, adhesion, and signalling are processes involved in microbial pathogenicity associated with biofilm development (Casadevall and Pirofski 2009). However, the organism, and the method used to evaluate virulence, seem to impact how essential biofilm formation is to the pathogenic process (Casadevall and Pirofski 2009). Some methods used to detect biofilm production include the tissue culture plate (TCP) method, the tube method (TM), the Congo red agar method (CRA), the bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination (Hassan et al. 2011).

2.5. The use of bacteriophages as a bacterial growth control measure

Antibiotic treatment is one of many strategies to control human and animal diseases. The application of antibiotics, however, has led to an increase in the number of antimicrobial-resistant bacteria and drug residues in food, which has become one of the main issues driving researchers to look for alternative, risk-free solutions. A potential substitute for antibiotics to inactivate bacteria is phage treatment, which focuses on using lytic bacteriophages (O'Flynn et al. 2004, Soliman et al. 2019). Bacteriophages are small viruses that can infect bacteria and have an enormous impact on the environment, as they play an essential role in maintaining the microbial balance (Zhan et al. 2015, El-Shibiny and El-Sahhar 2017). Phages are abundant – they can be isolated in all natural habitats, including aquatic and terrestrial systems, where their bacterial hosts are present (El-Shibiny and El-Sahhar, 2017). Phages can exhibit a virulent or temperate life cycle within their bacterial host. All phages comprise a nucleic acid genome (DNA or RNA) enclosed within a capsid (Sillankorva et al. 2012, El-Shibiny and El-Sahhar 2017, Stone et al. 2019, Cristobal-Cueto et al. 2021).

2.5.1. History

The history of bacteriophage discovery has been the subject of lengthy debates, including a controversy over priority claims (Sulakvelidze et al. 2001). Ernest Hankin, in 1896 reported on the presence of marked antibacterial activity (against *Vibrio cholerae*), which was observed in the waters of the Ganges and Jumna rivers in India and suggested that an unidentified substance (which passed through fine porcelain filters and was heat labile), was responsible for this phenomenon and for limiting the spread of cholera epidemics (Sulakvelidze et al. 2001). Two years later, Gamaleya, a Russian bacteriologist, saw a similar phenomenon while

working with *Bacillus subtilis*, and the observations of several other investigators are also thought to have been related to the bacteriophage phenomenon (Van Helvoort 1992). However, none of these investigators further explored their discoveries until Frederick Twort, a bacteriologist from England, investigated the topic almost two decades after Hankin's observation. Felix d'Herelle, a French-Canadian microbiologist at the Pasteur Institute in Paris, made the "official" discovery of bacteriophages two years later. Nevertheless, the discovery was not explored.

The nature of the bacteriophages was disputed until the early 1940s when the electronic microscope was invented. d'Hérelle immediately used his discovery to treat patients suffering from bacterial infections and reported great success (Summers 1993, Brives and Pourraz, 2020).

The development of phage therapy and its active application continued to increase within the Soviet Union and Eastern Europe, where it was well supported, until the fall of the Soviet Union (Abedon et al. 2011). Consequently, phage therapy was 'rediscovered' in the English literature beginning with the work of Smith and Huggins in the 1980s (Smith et al. 1987). However, this regeneration of Western phage therapy received momentum only in the 1990s, as access was increasingly gained to the rich trove of Soviet and Polish work. The field finally began maturing, starting approximately in the year 2000, a progression coupled with an explosion of genomics and broad ecology-based phage research, with this latest era of phage therapy research and application continuing to this day (Abedon et al. 2011).

2.5.2. Isolation and Taxonomy

A bacteriophage's host range can determine the phage's utility for phage therapy. Some phages will infect only a few strains (narrower host range), others will infect many strains of the same species (broader host range), and a few phages can infect more than one species (polyvalent host range) (Hayman 2019). Standard isolation protocols do not seem to select any host range. Host range, instead, is usually characterized after the phage is isolated, with isolation typically accomplished on a single strain of bacteria. However, some groups favour using a mix of multiple host strains for phage isolation (Van Twest and Kropinski 2009, Carvalho et al. 2010).

The basic approach to phage isolation has remained unchanged for decades. A phage water sample mixed with a culture of susceptible bacteria and incubated (Hymann 2019). During or after incubation, the phage-cell mixture is monitored for lysis (d'Hérelle and Smith 1929). Various ways of isolating bacteriophages have been developed and depend on the type of bacteria, whether it can be grown on solid media or only in broth, and whether simple killing or more information on the infection process is needed (Hymann 2019).

Most of these methods are also used when establishing host range using multiple potential host strains.

Virus classification is based on characteristics such as morphology, type of nucleic acid, replication mode, host organism, and kind of disease. The International Committee on Taxonomy of Viruses (ICTV) has produced an ordered system for classifying viruses (https://ictv.global/taxonomy). The ICTV classifies viruses based on the collection and comparison of various characters that describe the virus and can then be used to distinguish one virus from another. Characters can consist of any property or feature of the virus, including the genome's molecular composition, the virus capsid's structure and whether or not it is enveloped, the gene expression program used to produce virus proteins, host range, pathogenicity and sequence similarity.

Phage genomes are composed of either DNA or RNA, which may be double-stranded or single-stranded. This genetic material is packaged into a capsid that can be polyhedral (*Microviridae*, *Corticoviridae*, *Tectiviridae*, *Leviviridae,* and *Cystoviridae*), filamentous (*Inoviridae*), pleomorphic (*Plasmaviridae*), or connected to a tail (*Caudovirales*) (Ackerman 2009). The characteristics of bacterial virus families are summarized in Table 2.5 and Figure 2.2. It is suggested that about 96% of the described phages are tailed and belong to the order *Caudovirale* (Ackerman 2009).

2.5.3. Life cycles

Bacteriophages are highly specific and can only infect bacterial cells that present cell surface receptors matching those of the phage (similar to a lock and key mechanism) (Lindberg 1973). Without the matching receptors, phages cannot multiply and can quickly be degraded in the environment (Lindberg 1973). Phages can reproduce via the lytic or lysogenic cycles (virulent phages) or temperate phages. Multiplication is established by the actions taken once their nucleic acid has been injected into the host cell. The lytic cycle of virulent phages always releases phage progeny when the host cell ruptures (Engelkirk and Burton 2006). The life cycle of temperate phages may be two-fold – by the lytic cycle. They can create a long-lasting relationship with the host whereby their genome integrates into the bacterial chromosome and replicates alongside the host DNA instead of their lytic genes being expressed (Little 2005).

Lytic phages infect bacterial cells, causing inhibition of host metabolism and subverting it to the production of phage progeny. The lytic cycle results in the bacterium's lysis and the release of multiple phage particles. The new progeny phages produced by the host bacterium spread to infect other cells. The time for the whole cycle is usually within 1–2 h, and the number of phages produced depends on the phage type (Guttman et al. 2005).

Table 2.5: Overview of bacterial viruses and their basic properties according to the International Committee on Taxonomy of Viruses (https://ictv.global/taxonomy).

Family	Order	Morphology	Genome type	Additional features
Chaseviridae	*Caudovirales*	Head-tail	Linear dsDNA	Contractile tail
Corticoviridae	*Vinavirales*	Icosahedral	Circular dsDNA	Internal membrane
Herelleviridae	*Caudovirales*	Head-tail	Linear dsDNA	Contractile tail
Plasmaviridae	No higher-rank classification	Pleomorphic	Circular dsDNA	Cell membrane
Podoviridae	*Caudovirales*	Head-tail	Linear dsDNA	Non-contractile tail
Siphoviridae	*Caudovirales*	Head-tail	Linear dsDNA	Non-contractile tail
Tectiviridae	*Kalamavirales*	Non-enveloped	Linear dsDNA	Internal membrane
Finnlakeviridae	No higher-rank classification	Icosahedral	Circular ssDNA	Internal membrane
Inoviridae	*Tubulavirales*	Filamentous	Circular ssDNA	Long flexible filaments
Microviridae	*Petitvirales*	Icosahedral	Circular ssDNA	Non-enveloped
Cystoviridae	*Mindivirales*	Icosahedral	Linear segmented dsRNA	Enveloped, multilayered
Picobirnaviridae	*Durnavirales*	Spherical	Linear bisegmented dsRNA	Small, non-enveloped
Leviviridae	*Levivirales*	Icosahedral	Linear ssRNA	Polio-virus like

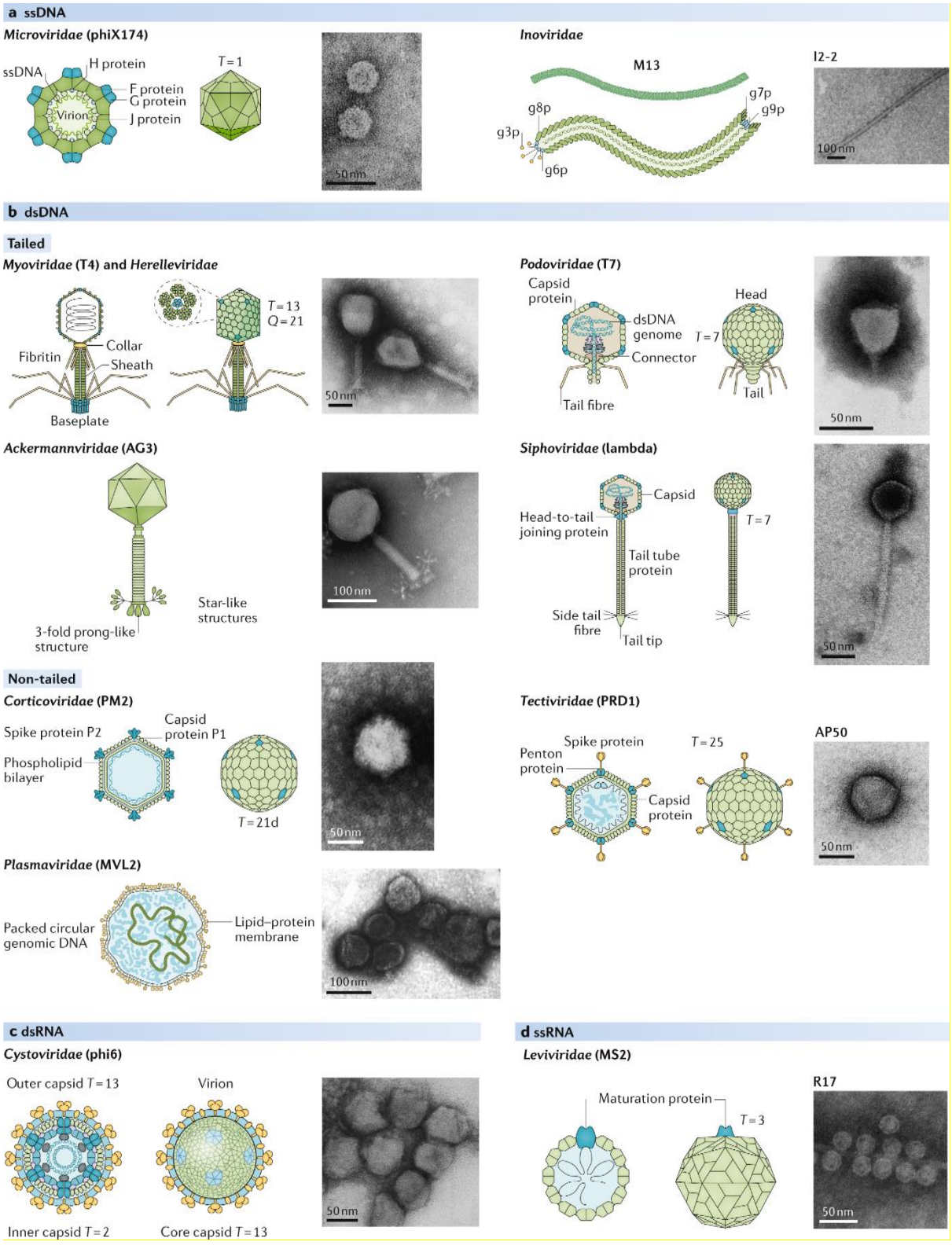

Figure 2.2: Phage classification based on morphology and genome type (Dion et al. 2020).

Lysogenic phages invade cells and integrate their genetic material into the host cell's genome. They can also exist as an episomal element, resulting in a long-term prophage relationship with the cell and its offspring. Phages do not generate virions or lyse bacteria during lysogeny. A temperate phage's genome finds a haven in the lysogenic interaction with its host bacteria, which also inhibits the replication of non-virulent homologous phages and may change the phenotypic characteristics of the host cell (Gill and Abedon 2003).

2.5.4 Phage therapy

Phage therapy aims to maximize the number of phages that reach and infect as many bacteria as possible. These phage infections eventually result in clinically insignificant bacteria levels without causing unwanted side effects (Nilsson 2019). To accomplish this, the titer of phages and the bacteria must be sufficiently high at the site of infection, and the phage titer must pass the 'inundation threshold', where the phage replication outruns the bacterial replication (Abedon 2011). This can be achieved either through a single phage infection cycle or the following cycles of phage infection and reproduction (i.e., active, or productive phage infection), but also by repeated administration of phages (Nilsson 2019).

When penicillin was first used in the 1940s, it was considered a ‘wonder drug’. However, delirious excitement and high expectations from physicians led to it being overused and abused before people understood the consequences and the development of antibiotic-resistant bacteria (Singh and Barrett 2006). As the antibiotic resistance crisis escalated (Neu 1992), Western scientists increasingly regained interest in phage therapy (Alisky et al. 1998). Bacteriophages are very specific to bacterial species, and most known phages only infect and replicate in a limited number of host strains (Hyman and Abedon 2010). This specificity makes bacteriophages harder to apply as blanket antibacterial agents. However, phage therapy offers its own set of advantages.

Phages recognize and bind to their hosts taking advantage of bacterial cell surface receptors; therefore, the interactions between phages and bacteria tend to be very specific. Some are so specific that they may target only a sub-group of a species rather than the whole species (Koskella and Meaden 2013). This characteristic of the phage, called narrow-host range, can be a double-edged sword. As mentioned previously, the benefits of specific phage-bacteria interactions mean that the beneficial bacterial microbiota of the body is minimally disturbed (Wu et al. 2013). In infections and diseases, a healthy and unaltered human microbiome imparts a robust immunity to the host, which may reduce the incidence of preventable diseases associated with disrupted microbiota (Pflughoeft and Versalovic 2012).

Awareness of the numerous potential applications of bacteriophages in the food industry has increased in recent years (García et al. 2008). They have been suggested as alternatives to antibiotics in animal health, as bio-preservatives in the food industry, and as tools for detecting pathogenic bacteria throughout the food chain (García et al. 2008). Bacteriophages and their derivatives have emerged as new, practical, and harmless options for inhibiting, treating, and/or eradicating contaminants in various foods and food processing environments (Endersen et al. 2014). Traditional antimicrobial methods can reduce microbial populations in foods to a certain extent. However, they also have significant disadvantages, possibly the most outstanding being that they kill indiscriminately, including often beneficial bacteria naturally present in foods (Moye et al. 2018). One promising technique that addresses several shortcomings is bacteriophage biocontrol (Moye et al. 2018).

The concept of phage biocontrol is to apply lytic bacteriophages, with strong lytic potency against one or more foodborne bacterial pathogens, to foods at high risk of contamination (Vikram et al. 2021). The phages can lyse the targeted contaminating bacteria and significantly reduce (or eradicate) the foodborne pathogen(s), thus making foods safe for consumption (Vikram et al. 2021). Phage biocontrol is increasingly accepted as a natural and green technology that effectively targets bacterial pathogens in various foods. Members of the *Weeksellaceae* family have been associated with clinical, fish, food and environmental sources, however to the author's knowledge, no research has been performed yet on the bacteriophages active against the members of the *Weeksellaceae* family.

Research on the use of phages against foodborne pathogens of animal origin has mainly focused on optimising preharvest interventions, where the phage administration routes and delivery processes have received the most attention, and on optimising the optimization of postharvest strategies (Sillankorva et al. 2012). The usage of phages as a preharvest strategy is made directly by administering phages to livestock to prevent animal illness and/or minimize the pathogen carriage in the gastrointestinal tract, thereby preventing pathogen entry into the food supply (Sillankorva et al. 2012). Post-harvest strategies use phages directly on animal carcasses to sanitize the carcasses.

Although the biological properties of lytic bacteriophages provide advantages in improving food safety, these properties also lead to some of the limitations and drawbacks of phage biocontrol. Bacteriophages are highly specific, and as a result, they are only effective against the bacterium of interest. Still, if foods are contaminated with multiple bacteria, a combination of phage biocontrol products could target more than one bacterium (Vikram et al. 2021).

Bacteriophages are microorganisms; generally, disinfectants or chemicals could inactivate them. Therefore, their use must be carefully coordinated within the processing line (Vikram et al. 2021).

2.6. Conclusions

The genus *Kaistella* is a member of the family *Weeksellaceae*, phylum *Bacteroidota*, and currently consists of 15 species, with *Kaistella koreensis* as the type species. Eleven species from *Chryseobacterium* were recently transferred to the genus *Kaistella* based on amino acid identity (AAI) values from whole-genome sequencing. Members of the genus *Kaistella* are aerobic, rod-shaped and Gram-stain-negative and widely distributed in clinical, environmental, industrial, and food sources.

The taxonomy method integrates the physiological and genetic traits required to classify and characterize novel and existing species. Microbiologists may now properly categorize bacteria down to the species level based on the genotypic characteristics of many closely related strains because of the development of molecular tools in microbiology. However, it should be noted that standardising procedures in describing phenotypic traits are essential to achieving provisional identification. Phenotypic characterisation is equally vital in species identification and should not be disregarded.

Virulence factors are the molecules that assist the bacterium to colonize the host at the cellular level. Virulence factors discussed in this study included enzyme, siderophore, and biofilm production. These factors enhance the ability of a bacterium to withstand destruction resulting in being able to act as a pathogen or spoilage bacterium.

The spread of pathogens has been hindered by the discovery and widespread use of antimicrobial agents; however, antimicrobial resistance has increased globally. The use of bacteriophages is one of the therapeutic approaches that can be used to counteract antimicrobial resistance and prevent any bacterial infection since they have had numerous applications in the food industry in recent years.

CHAPTER 3

TAXONOMIC STUDY OF *Kaistella* SPECIES ISOLATED FROM FISH

Abstract

Strains SH 11-4(b), SH 19-2(b), SH 20-4, and SH 40-3 are rod-shaped, Gram-stain-negative, non-motile, and aerobic bacteria isolated in a previous study (Engelbrecht 1992) from fresh Cape marine fish in South Africa intended for human consumption. These strains were subjected to phylogenetic classification, whole genome sequencing and phenotypic characterization. This study's 16S rRNA sequencing results supported the affiliation of strains SH 11-4(b), SH 19-2(b), SH 20-4 and SH 40-3 to the genus *Kaistella*. The ANI, AAI, and dDDH values of strains SH 11-4(b), SH 19-2(b), SH 20-4 and SH 40-3 were 81.94%, 85.55%, 25.40%, 81.83%, 85.83%, 25.40%; 79.19%, 81.07%, 22.30%; 81.93%, 85.30%, 25.40%, respectively, with *K. antarctica,* and 82.32%, 85.34%, 27.70%; 82.17%, 75.24%, 26.00%; 97.84%, 96.98%, 79.70%; 82.15%, 84.3%, 26.00%, respectively, with *K. carnis*. It indicated that strains SH 11-4(b), SH 19-2(b) and SH 40-3 were members of three novel species for which the names *K. merluccii, K. piscis* and *K. frigidipiscis* were proposed. The ANI, AAI and dDDH results indicated that strain SH 20-4 could be considered another member of *K. carnis*.

3.1. Introduction

Since Aristotle, a philosopher, originally introduced taxonomy, other taxonomists, such as Carl Linnaeus, have contributed to its advancement. Numerous technological advances have been made throughout the history of prokaryotic systematics to fill the theoretical gap and establish a more accurate description of species (Gevers et al. 2006). The characterization and identification of organisms are greatly aided by taxonomy (Prakash et al. 2007). It is based on species identification, categorization, and nomenclature (Bisen 2012). Previously, the classification of bacteria focused more on morphological and physiological traits, but nowadays, genetic information has been more prevalent (Gevers et al. 2006).

The polyphasic approach was a significant development in microbial taxonomy, which offered a legitimate and spontaneous system for classifying bacteria (Prakash et al. 2007). Colwell first used the word in 1970 to describe the combination of a microbe's genetic, chemotypes, and physiological characteristics to classify the organism accurately. Based on morphological and biochemical data and data from molecular approaches, it is used to differentiate between species of bacteria (Prakash et al. 2007).

More advanced molecular techniques, such as 16S rRNA gene sequencing DNA-DNA hybridization and G+C determinations, have emerged due to advances in molecular biology (Wayne et al. 1987, Stackebrandt 2002). Stackebrandt and Goebel (1994) considered DNA-DNA hybridization to be the gold standard for prokaryotic species delineation; however, average nucleotide identity (ANI) and average amino acid identity (AAI) are nowadays accompanying methods to DNA-DNA-hybridization for species descriptions (Richter and Rosselló-Móra 2009, Thompson et al. 2013, Figueras et al. 2014).

Whole-genome sequence studies have revolutionized bacterial isolates' identification and taxonomic categorization (Didelot et al. 2012, Lasken and McLean 2014, Pightling et al. 2018, Nicholson et al. 2020). For example, the genus *Kaistella* was previously regarded to belong to the family *Flavobacteriaceae;* however, the latest findings from a whole-genome sequence (WGS) analysis of 1000 type strain genomes from the phylum *Bacteroidota* demonstrated that they belong to the family *Weeksellaceae* (Garca-López et al. 2019). *Kaistella koreensis* was identified as the type species of the genus when it was first described by Kim et al. (2004), but the taxonomic status of the genus has changed since then. For example, in a recent study, 11 species that previously belonged to the genus, *Chryseobacterium* were reclassified to the genus *Kaistella* due to discontinuities in the classification system (amino acid identity values) (Nicholson et al. 2020). These species included [*Chryseobacterium] anthropi*, [*Chryseobacterium] antarcticum, [Chryseobacterium] carnis, [Chryseobacterium] chaponense, [Chryseobacterium] haifense, [Chryseobacterium] jeonii, [Chryseobacterium] montanum, [Chryseobacterium] palustre, [Chryseobacterium] solincola, [Chryseobacterium] treverense*, and [*Chryseobacterium] yonginense*.

This study aimed to conduct a polyphasic study on four novel fish-isolated bacterial strains, SH 11-4(b), SH 19-2(b), SH 20-4, and SH 40-3. Conventional techniques were used to identify the phenotypic traits of the organism. Whole-genome sequencing was then performed as part of genotypic studies. The genomes of the strains were assembled and annotated using the PATRIC (Pathosystems Resource Integration Center) and RAST (Rapid Annotation with Subsystem Technology) algorithms. The results were combined to provide comprehensive descriptions of these strains.

3.2. Materials and Methods

3.2.1 Cultures used and their maintenance

The strains used in this study and their reference strains are provided in Tables 3.1 and 3.2, respectively. The unidentified test strains used in this study were isolated from fresh Cape hake (*Merluccius capensis*) obtained from commercial fish retailers in Cape Town, Western

Cape Province, South Africa, in a previous study (Engelbrecht 1992). The six *Kaistella* species used as reference strains in this study were selected because the results of phylogenetic analysis, based on 16S rRNA gene sequences, indicated that they are closely related to the test isolates. *Kaistella koreensis* was used because it is the type strain of the genus *Kaistella.* Since the test isolates were initially classified as belonging to the genus *Chryseobacterium, C. gleum* was used because it is the type strain for the genus *Chryseobacterium.*

All strains had been preserved in a freeze-dried state in glass ampoules. For shorter-term maintenance, the isolates were freeze-dried on 6 mm diameter filter paper discs (FLAS321260, Lasec SA (Pty) Ltd, Bloemfontein, South Africa) and stored in sterile Wasserman tubes (AXI07TCTT, Axiology Labs (Pty) Ltd, Vereeniging, South Africa) at -20 °C. Before use, strains were reactivated in 10 ml of nutrient broth (NB; Oxoid CM67). Purity was checked by streaking on nutrient agar (NA; Oxoid CM0003) and Gram-staining. Incubation was at 25 °C for 48 h for the *Chryseobacterium* and *Kaistella* reference strains. The pure cultures were streaked on NA slants, incubated at 25 °C for 24 h, stored at 4 °C for short-term maintenance and re-streaked every 4–6 weeks.

Table 3.1: The four unidentified aerobic Gram-stain-negative bacterial strains isolated from fresh South African marine fish (Engelbrecht 1992) were used in this study.

Isolate	**Source**	**Place of isolation**	**Date of isolation**
SH 11-4(b)	Fresh Marine Fish (*Merluccius capensis*)	Cape Town – Atlantic Ocean	1990
SH 19-2(b)	Fresh Marine Fish (*Merluccius capensis*)	Cape Town – Atlantic Ocean	1990
SH 20-4	Fresh Marine Fish (*Merluccius capensis*)	Cape Town – Atlantic Ocean	1990
SH 40-3	Fresh Marine Fish (*Merluccius capensis*)	Cape Town – Atlantic Ocean	1990

Table 3.2: Reference strains used for this study's automated and conventional phenotypic methods. NCTC, National Collection of Type Cultures (United Kingdom); CCUG, Culture Collection, University of Göteborg (Sweden); KCTC, Korean Culture for Type Collections (Korea); LMG, Laboratorium voor Microbiologie, (Belgium).

Genus and species	Culture collection	Source of collection	References
Chryseobacterium gleum	NCTC 11432^T	Vaginal swab	Vandamme et al. 1994
Kaistella koreensis	KCTC 12107^T	Natural mineral water	Kim et al. 2004
Kaistella carnis	CCUG 60559^T	Raw beef carcass from an unspecified abattoir	Holmes et al. 2013, Nicholson et al. 2020
Kaistella chaponensis	CCUG 58959^T	Diseased farmed Atlantic salmon (*Salmo salar*)	Kämpfer et al. 2011, Nicholson et al. 2020
Kaistella antarctica	LMG 24720^T	Soil samples from penguin habitats in Antarctica	Yi et al. 2005, Kämpfer et al. 2009, Nicholson et al. 2020
Kaistella yonginensis	KCTC 22744^T	Mesotrophic artificial lake	Joung and Joh 2011, Nicholson et al. 2020

3.2.2. Phenotypic Characterisation

3.2.2.1. Morphological tests

The strains were streaked on NA plates and incubated for 48 h at 25 °C aerobically to observe the morphology of the colonies.

The presence or absence of a fruity odour and colour was noted. Gram-staining was determined according to the method of MacFaddin (1980).

For scanning electron microscopy (SEM), strains were grown on NA for 48 h at 25 °C, after which the cell material was scraped and placed in an Eppendorf tube containing 0.1 M (pH 7.0) sodium phosphate-buffered glutardialdehyde (3%) for at least three h to fix the cells, followed by one-hour fixation in a similarly buffered osmium tetroxide (1%) solution. The material was collected on 0.2 µm polycarbonate membrane filters and dehydrated in a graded ethanol series (50%, 70% and 95%, for 20 min in each phase, followed by two changes in 100% ethanol for one hour in each phase).

The material was dried using a critical point dryer (Tousimis, Maryland, U.S.A.). After drying, the material was mounted on stubs (Cambridge pin type, 10 mm) using double-sided carbon tape and gold coated (± 60 nm) with a Bio-Rad sputter coater (United Kingdom). The samples were examined and analysed with a JSM-7800F Extreme-resolution analytical field emission SEM (FE-SEM).

3.2.2.2. Biochemical tests

A range of phenotypic tests that were selected based on being able to differentiate between members of the genus *Kaistella* were used. The tests used were according to the minimal standards for the description of new taxa in the family *Weeksellaceae* (García-López et al. 2019), and the biochemical tests were performed according to Cowan (1974) and MacFaddin (1980).

The production of oxidase, catalase, lipolytic, and proteolytic enzymes was determined according to MacFaddin (1980). Motility was determined by simultaneous stab inoculation in NA tubes and growth in NB at 22 °C for 16–24 h and by noting motility with phase-contrast microscopy (Nikon). The determination of flexirubin-type pigment production was carried out by flooding a mass of cells, smeared on a glass slide, with 20% (w/v) potassium hydroxide (KOH) (Bernardet et al. 2002). If flexirubin-type pigment was present, colonies turned from yellow to red, purple, or brown. An acidic solution (1 N HCl) was used to remove excess KOH and restore the cells to their initial colour (Reichenbach 1989).

The strains were revived and cultivated in 10 ml of NB to standardize the inoculum concentration for the rest of the phenotypic tests. After that, the cell culture was transferred and cultured in 100 ml of NB in 500 ml Erlenmeyer flasks at 25 °C for 48 h. The cells were then centrifuged at 1,087 x *g* for 10 min with an Eppendorf 5430 R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was discarded, and the cell pellets were washed with phosphate buffer (0.1 M, pH 7). Centrifugation was repeated as previously indicated. Cell pellets were resuspended in 10 ml of phosphate buffer (0.1 M, pH 7) and standardized compared to a McFarland number 2 density standard (Difco 0691326). This served as the inoculum for the different biochemical tests.

The following range of phenotypic tests was carried out according to Cowan (1974) and MacFaddin (1980), unless otherwise indicated: growth in 0 to 10% (w/v) sodium chloride, with 0.5% increments; growth at 4, 10, 15, 25, 30, 37 and 42 °C; growth on NA, MacConkey agar (Oxoid CM0007), trypticase soy agar (Oxoid CM0131), R2A agar (Oxoid CM0906), cetrimide agar (Oxoid CM0579), β-hydroxybutyrate and standard plate count agar (Oxoid CM0463); production of hydrogen sulphide (Kliger iron agar; Oxoid CM0033), indole (Kovac's reagent,

Merck 1.09293), urease on Christensen's urea agar (Richard and Kiredjian 1995; Hugo 1997), 3-ketolactose, phenylalanine deaminase, phosphatase and DNase (Oxoid CM321); hydrolysis of casein, aesculin (Yabuuchi et al. 1990), tyrosine (Barrow and Feltham 1993), tween 20 and 80 (West and Colwell 1984), gelatine (plate method), and lecithin (egg yolk media); oxidative or fermentative metabolism of glucose; methyl red and Voges Proskauer reactions; gluconate oxidation; potassium cyanide tolerance, malonate utilization; and citrate utilization on Simmons citrate agar and Christensen's citrate agar (Holmes et al. 1975); reduction of 0.4% selenite (Holmes et al. 1975); nitrate and nitrite reduction and decarboxylation of lysine, ornithine, and arginine.

3.2.3. Automated Phenotypic Tests

3.2.3.1. BIOLOG Omnilog Gen III Identification System

The *Kaistella* test strains and reference strains were grown on NA plates for 24 h at 25 °C and profiled using the BIOLOG Omnilog Gen III identification system (BIOLOG Inc., Hayward, CA, USA) according to the manufacturer's recommendations. Following incubation, a sterile swab was used to collect a single colony from the previously incubated plate. The colony was suspended in an inoculating fluid (IF-A), used for organisms that usually grow at the recommended cell density (BIOLOG). A 100 µl of the cell suspension was then inoculated into each of the 96 GEN III MicroPlate wells, which were further incubated in the BIOLOG incubator at 25 °C for 24 h to allow the phenotypic fingerprint to form. RetroSpect 2.0 software was used to view the data, which was further analysed using Microsoft Excel.

3.2.4 Phylogenetic analysis

The sequences of *Kaistella* species from EzTaxon (http://www.ezbiocloud.net/) and the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) were compared with the 16S rRNA gene sequences of strains SH 11-4(b), SH 19-2(b), SH 20-4 and SH 40-3 obtained by whole-genome sequencing. MEGA version 7 was used to build the phylogenetic trees (Tamura et al. 2016). Neighbour-joining (Saitou and Nei 1987), maximum likelihood (Kumar et al. 2016), and the parameter distance measure, Kimura two (KP2), were applied. The confidence intervals at 1000 replicates were calculated using the bootstrap technique (Felsenstein 1985).

3.2.5. Genomic Methods

3.2.4.1. Whole-genome sequencing

Whole-genome sequencing of strains SH 11-4(b), SH 19-2(b), SH 20-4, and SH 40-3 was carried out at the National Institute for Communicable Diseases (NICD) Unit in Johannesburg, South Africa.

Genomes were sequenced using an Illumina HiSeq sequencer, and assembly was carried out using the PATRIC database (https://www.patricbrc.org/), with SPAdes 3.10.0 as the assembly method.

The whole-genome sequences were annotated by uploading to the RAST (Rapid Annotation with Subsystems Technology) database (http://rast.nmpdr.org). RAST was used to obtain genomic data, such as gene number, genome size, G+C content, coverage, N50 value, number of contigs, and complete 16S rRNA sequence. The algorithms listed in Table 3.3 were used to obtain the average nucleotide identity (ANI), amino acid identity (AAI), digital DNA-DNA hybridisation (dDDH), DNA G+C content data, and the Venn diagrams with shared and unique genes.

Table 3.3: Algorithms and tools used to calculate the overall genome relatedness indices (OGRI) and Venn diagram creation.

OGRI	Tool	URL link	References
ANI	OrthoANIu	https://www.ezbiocloud.net/tools/ani	Yoon et al. 2017
AAI	Newman Lab ROSA Calculator	http://lycofs01.lycoming.edu/~newman/ROSA.html	
dDDH	Genome-Genome Distance Calculator (GGDC 3.0, formula 2)	http://ggdc.dsmz.de/	Meier-Kolthoff et al. 2014, Meier-Kolthoff et al. 2022)
Venn diagram	Venn Diagram Data Generator V 2.1	http://lycofs01.lycoming.edu/~newman/CurrentResearch.html	

3.2.4.2. Average Nucleotide Identity (ANI)

The Kostas lab ANI calculator at http://enve-omics.ce.gatech.edu/ani/ was used to calculate the average nucleotide identity (ANI), which assesses the similarity of two genome sequences.

3.2.4.3. Average Amino Acid Identity (AAI)

The average amino acid identities were determined using the Newman Lab AAI and BBH Calculator (http://lycofs01.lycoming.edu/~newman/ROSA/OrthologyScore.htm).

This application uses a calculator to create matrices of AAI and BBH (Bidirectional Best Hit) values from a collection of 11 files that contain the contigs of the query strain's closest relatives, using each genome as a reference.

3.2.4.4. Digital DNA- DNA Hybridization (dDDH)

The Genome-to-Genome Distance Calculator (GGDC) of the German Culture Collection DSMZ (http://ggdc.dsmz.de/) (Meier-Kolthoff et al. 2014, Meier-Kolthoff et al. 2022) was used to calculate the estimated DNA-DNA hybridization values. The assembled genome sequences and the reference genomes were uploaded to the GGDC website's distance calculation form to calculate the intergenomic distances.

3.2.4.5. Venn Diagram

The RAST SeedViewer tool (Overbreek et al. 2005, Overbreek et al. 2014) was used to extract and compare the annotated genome sequences of the query and reference strains. The Venn diagram was constructed using the Newman Lab Venn diagram data generator tool (http://lycofs01.lycoming.edu/~newman/CurrentResearch.html) to compare the number of genes present in every genome, as well as the unique and shared genes.

3.3. Results and Discussion

3.3.1. Colony and Cell Morphology

All the colonies of the test and reference strains on TSA were circular, convex, entire, smooth, and up to 2 mm in diameter. Only *C. gleum* produced deep yellow colonies by a non-diffusible, nonfluorescent, flexirubin-type pigment. Conversely, all the *Kaistella* fish test isolates and the *Kaistella* reference strains had very pale yellow or creamy-white colonies (Fig. 3.1).

The cell morphology of strains SH 11-4(b), SH 19-2(b), SH 20-4, SH 40-3, and *C. gleum* can be seen in Figures 3.2–3.6, respectively. These micrographs demonstrated a rod-shaped morphology, with no flagella or spore formation, by members of the genera *Chryseobacterium* and *Kaistella.* All isolates had smooth surface and varied slightly from each other in terms of size.

The cells of strains SH 11-4(b) (0.35-0.50 µm wide and 1.3-2.5 long), SH 19-2(b) (0.30-0.49 µm wide and 1-3 µm long) and SH 40-3 (0.30-0.50 µm wide and 1-2.3 µm long) can be differentiated from those of strain SH 20-4 (0.33-50 µm wide and 0.87-1.8 µm long) and *C. gleum* (0.29-0.48 µm wide and 0.92-1.4 µm long) by their slightly longer structure. The cells of strain SH 20-4 resemble those of *C. gleum* more concerning their surface texture and apparent shorter length.

Fig. 3.1: Colony morphology and colour of *Kaistella* strain SH 40-3, *Chryseobacterium gleum* and *Kaistella koreensis* from left to right.

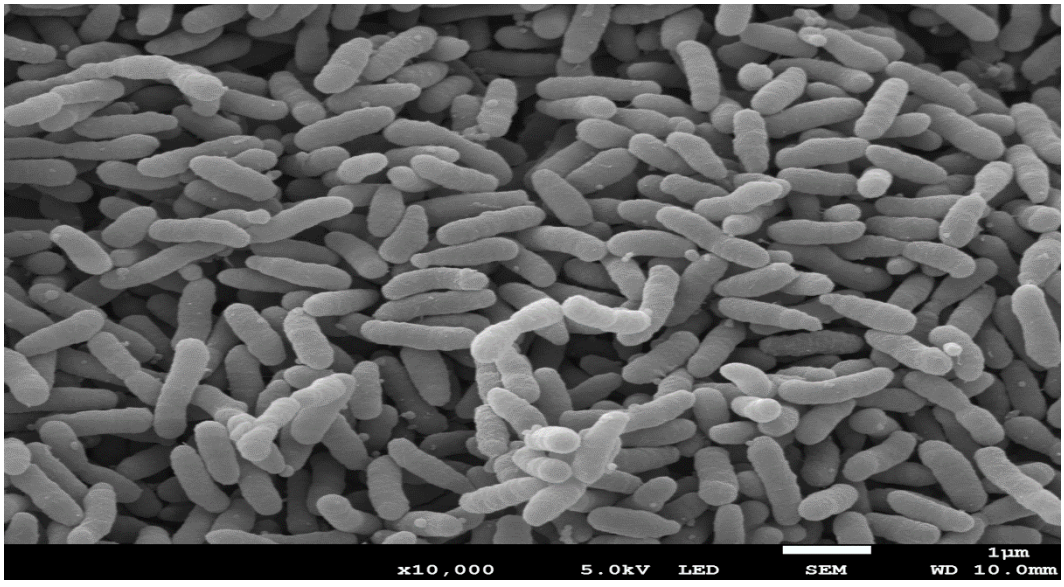

Fig. 3.2: Scanning electron micrograph of strain SH 11-4(b) grown on nutrient agar at 25 °C for 48 h; Bar, 1 µm.

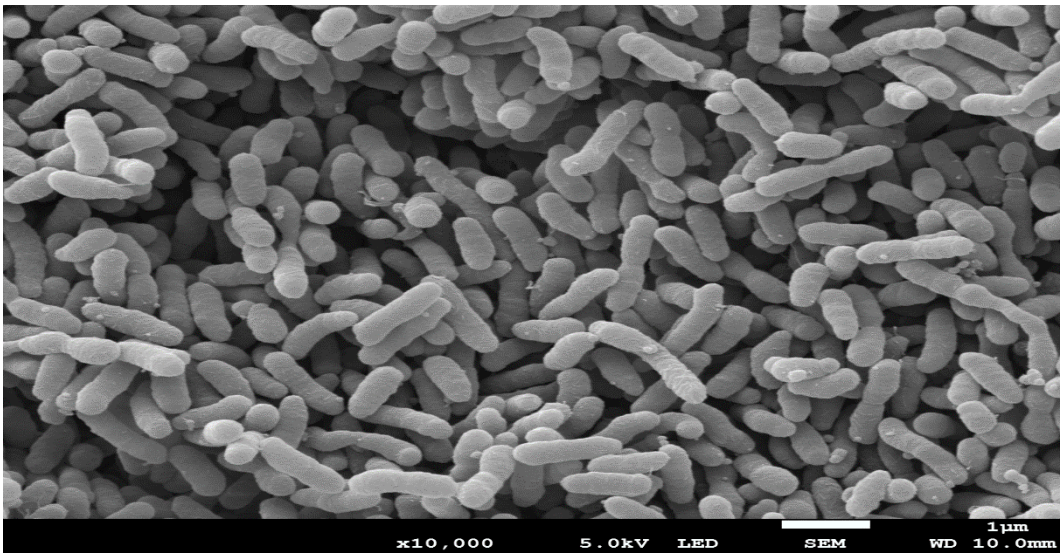

Fig. 3.3: Scanning electron micrograph of strain SH 19-2(b) grown on nutrient agar at 25 °C for 48 h; Bar, 1 µm.

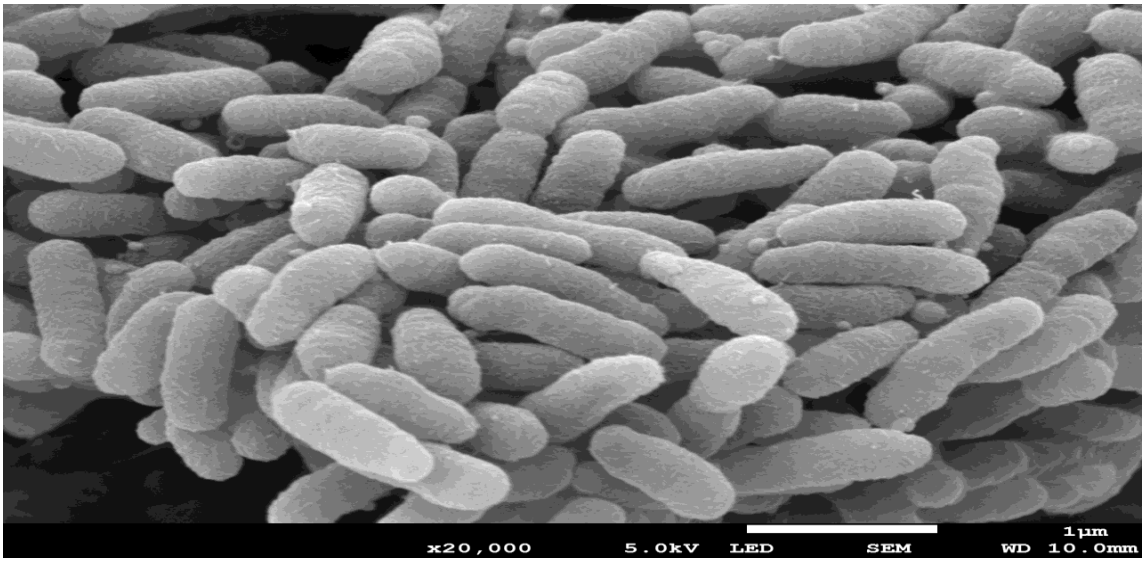

Fig. 3.4: Scanning electron micrograph of strain SH 20-4 grown on nutrient agar at 25 °C for 48 h; Bar, 1 µm.

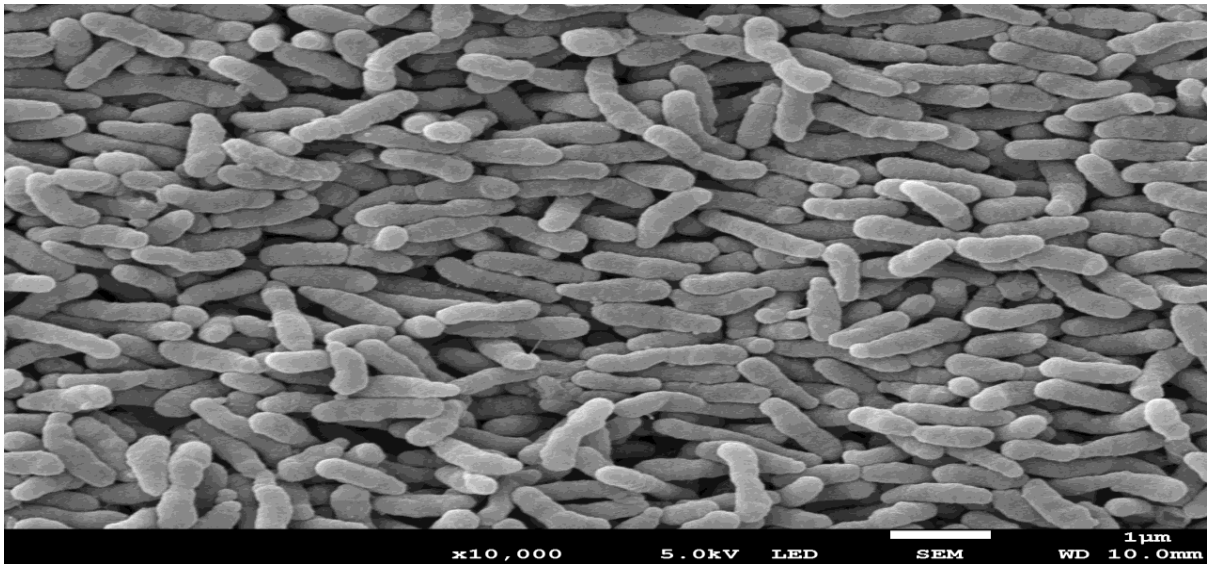

Fig. 3.5: Scanning electron micrograph of strain SH 40-3 grown on nutrient agar at 25 °C for 48 h; Bar, 1 µm.

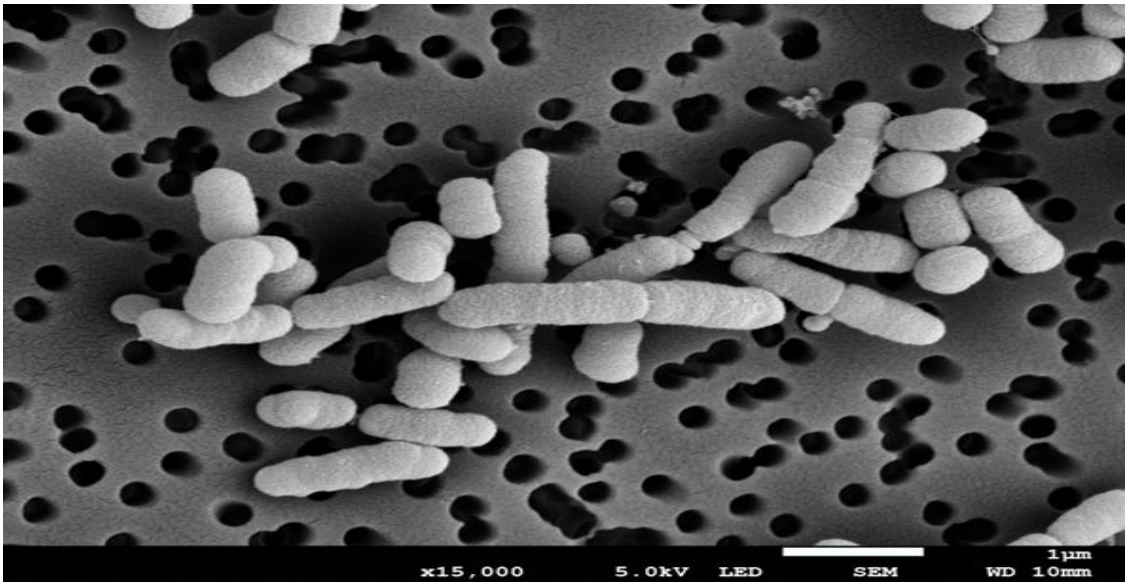

Fig. 3.6. Scanning electron micrograph of *Chryseobacterium gleum* grown on nutrient agar at 25 °C for 48 h; Bar, 1 µm.

3.3.2. Conventional Phenotypic Characterisation

All strains grew well between 4–30 °C, at 0.5–3% NaCl inclusion levels, on TSA agar, R2A agar, NA agar. Cells were strictly aerobic, non-motile Gram-stain-negative rods with a fruity/spoiled odour. All strains were positive for the following tests: production of catalase, oxidase, caseinase, DNase, gelatinase, urease, and phosphatase; tyrosine hydrolysis and brown pigment production. Oxidative metabolism of glucose in the O/F test; arginine, lysine, and ornithine decarboxylase production; methyl-red reaction and Vogues-Proskauer reaction.

All strains were negative for the following tests: fermentative metabolism of glucose in the O/F test; motility; phenylalanine deaminase; hydrogen sulphide production; 3-ketolactose production; 0.4 % selenite, nitrite reduction; gluconate oxidation; growth on cetrimide agar and Tween 80 hydrolysis.

The differential phenotypic traits of each of the four test isolates and the six reference strains used in this study are listed in Table 3.4. Strain SH 11-4(b) was the only test isolate able to grow at 42 °C, and it shared this trait with *C. gleum* and *K. antarctica*. Strain 11-4(b), together with *K. antarctica,* can be differentiated from all the other organisms by their inability to grow on MacConkey agar. Growth was observed with 0–3 % NaCl in most strains and reference strains. The only strains that could grow at 4% NaCl inclusion level were SH 11-4(b), SH 19-2(b), *C. gleum,* and *K. antarctica*.

Strains SH 19-2(b) and SH 20-4 could be compared with *K. carnis* and *K. chaponensis* due to their inability to produce indole, while strain SH 20-4 was also unable to degrade both aesculin and lecithin and shared this trait with *K. carnis* and *K. chaponensis*. Most strains' ability to produce lipolytic and proteolytic enzymes affiliated them with the genus *Kaistella* since *K. haifensis* and *K. koreensis* have been linked to hydrolytic enzyme production (Kim et al. 2004, Hantsis-Zacharov and Halpern 2007). The presence of specific enzymes is an essential feature for identifying and characterising bacteria and the discrimination of related organisms (Busse et al. 1996).

3.3.3. Automated BIOLOG^{TM} Omnilog Gen III Phenotypic Profiling

The Biolog Omnilog plates contain tests for carbohydrates, amino acids, hexose acids, carboxylic acids, esters, fatty acids, and inhibitory substances. The results are indicated in Table 3.5 and were obtained from the mean of the experiments carried out in triplicate for the four fish isolates and *C. gleum* as the reference strain.

All four strains were positive for the following: growth at pH 6 and 1% NaCl; oxidation of gelatin, L-aspartic acid, L-glutamic acid, tetrazolium violet, tetrazolium blue, K-tellurite, tween 40, acetoacetic acid, acetic acid, propionic acid, Sodium butyrate, and aztreonam. All four strains were negative for: stachyose, pH 5, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, 4 and 8% NaCl, D and L-fucose, L-rhamnose, fusidic acid, D-sorbitol, D-mannitol, D-arabitol, myoinositol, D-aspartic acid, glycerol, D-fructose-6-PO_4, troleandomycin, rifamycin SV, vancomycin, niaproof 4, L-galacturonic acid lactone, p-hydroxy-phenylacetic acid, methyl pyruvate, bromo-succinic acid, g-amino-butyric acid, nalidixic acid, α-hydroxy-butyric acid, β-hydroxy-D, L-butyric acid and α-keto-butyric acid.

Table 3.4: Differential characteristics of strains SH 11-4(b), SH 19-2(b), SH 20-4, SH 40-3, and related members of the genus *Chryseobacterium* (*C. gleum*) and *Kaistella* (*K. koreensis, K. carnis, K. chaponensis, K. antarctica, K. yonginensis*). +, positive; -, negative; w, weakly positive.

Characteristics	**SH 11-4(b)**	**SH 19-2(b)**	**SH 20-4**	**SH 40-3**	*C. gleum*	*K. koreensis*	*K. carnis*	*K. chaponensis*	*K. antarctica*	*K. yonginensis*
Growth in NaCl%										
3	+	+	+	+	+	w	+	w	+	w
4	+	w	-	-	w	-	-	-	+	-
5	w	-	-	-	-	-	-	-	+	-
Growth at (°C):										
30	+	+	+	+	+	+	w	w	+	+
37	+	+	+	+	+	+	w	w	+	+
42	+	-	-	-	+	-	-	-	+	w
Growth on:										
MacConkey agar	-	+	+	+	w	w	-	-	+	w
β-hydroxybutyrate	+	w	w	+	+	+	w	+	+	w
Production of:										
Indole	+	-	-	+	+	+	-	-	+	+
Phosphatase	+	w	w	-	+	+	+	-	+	w
Degradation of:										
Aesculin	+	+	-	+	+	+	-	-	+	w
Lecithin	+	+	-	+	+	+	-	-	+	-
Tween 20	+	+	-	-	+	+	-	-	+	+
Reduction of:										
Nitrate	+	+	-	-	+	+	-	-	+	+
Selenite	-	+	+	-	+	-	-	+	+	-

Table 3.5: Biolog™ Omnilog Gen III system biochemical tests showing the differential characteristics of four *Kaistella* isolates and *Chryseobacterium gleum* as reference strain. +, positive; -, negative; w, weakly positive.

Substrate	SH 11-4(b)	SH 19-2(b)	SH 20-4	SH 40-3	*C. gleum*
Carbohydrates					
D-maltose	+	+	w	-	-
D-trehalose	+	+	-	-	-
D-cellobiose	-	w	-	-	w
Gentiobiose	+	+	-	+	+
Sucrose	-	+	-	-	-
α-D-glucose	+	+	w	w	+
D-mannose	+	+	-	+	+
D-fructose	+	+	-	-	-
D-galactose	+	-	-	-	-
D-arabitol	+	-	-	-	-
D-glucose-6-PO_4	+	-	+	+	+
Amino acids					
Glycyl-L-proline	+	+	-	-	-
L-alanine	w	-	-	-	w
L-arginine	+	w	-	-	w
L-histidine	+	+	-	-	-
L-pyroglutamic acid	-	-	+	+	-
Guanidine HCl	+	-	-	-	+
D-serine	+	-	-	-	+
L-serine	+	w	-	-	-
Carboxylic acids					
Citric acid	+	-	-	-	-
Formic acid	+	-	w	w	-
Polymers					
Dextrin	+	+	-	+	+
Pectin	w	+	-	+	-
Inhibitory Substances					
Minocycline	-	-	-	+	-
Lincomycin	-	-	-	-	+
Lithium chloride	-	-	-	+	-
Sodium butyrate	w	-	-	-	-

The carbohydrates were used in varying degrees by specific isolates. Gentibiose, D-mannose and glucose were oxidised in almost all isolates. Only strain SH 20-4 was unable to oxidise gentibiose and mannose. The SH 19-2 (b) strain could be differentiated from all organisms by its inability to oxidise glucose. The substrates oxidised by only a few isolates were D-cellobiose, sucrose, D-galactose, and D-arabitol. Strain SH 11-4(b) could be differentiated from the rest of the strains because it was the only isolate able to oxidise D-galactose and D-arabitol, while strain SH 19-2(b) was the only isolate able to oxidise sucrose.

In the amino acid category, strains SH 11-4(b) and SH 19-2(b) could be differentiated from strains SH 20-4, SH 40-3 and *C. gleum* by their ability to oxidise glycyl-L-proline, L-histidine and L-serine. Both strains SH 20-4 and 40-3 could be differentiated from the other organisms by their ability to oxidise L-pyroglutamic acid. Strain SH 11-4(b) was also able to oxidise guanidine HCl and shared this trait with *C. gleum*.

All isolates, except strain SH 11-4(b), could not metabolise citric acid. Strain SH 11-4(b) could also oxidise formic acid, while strains SH 20-4 and SH 40-3 could only weakly metabolise this carboxylic acid.

Most isolates could metabolize dextrin, except strain SH 20-4. Strains SH 19-2(b) and SH 40-3 could be differentiated from strains SH 20-4 and *C. gleum* by their ability to degrade pectin.

In the category of inhibitory substances, strain SH 40-3 could be differentiated from all the organisms by its ability to grow in the presence of the antibiotic minocycline and the presence of the toxic compound lithium chloride, while *C. gleum* could be differentiated from all test isolates by its ability to grow in the presence of the antibiotic lincomycin. Only strain SH 11-4(b) could grow weakly in the presence of the inhibitory compound sodium butyrate.

3.3.4. Phylogenetic analysis

The four test strains used in this study were compared with the type strains of the currently recognized *Kaistella* type species in Figure 3.7 using a neighbour joining (NJ) phylogenetic analysis. The MEGA version 7 software program was used to construct another phylogenetic tree using the maximum likelihood (ML) method (Kumar et al. 2016) (Figure 3.8). According to both trees, all four test strains belonged to the genus *Kaistella* due to clustering with the type species of the genus, *Kaistella koreensis.* The four test isolates were closely related to *K. carnis* G0081^{T}*,* isolated from human clinical sources (Holmes et al. 2013). However, strain SH 20-4 clustered tightly with *K. carnis*, which was supported by a high bootstrap value of 100% (Fig. 3.7). The stability of this phylogeny was also supported by the ML tree (Fig. 3.8).

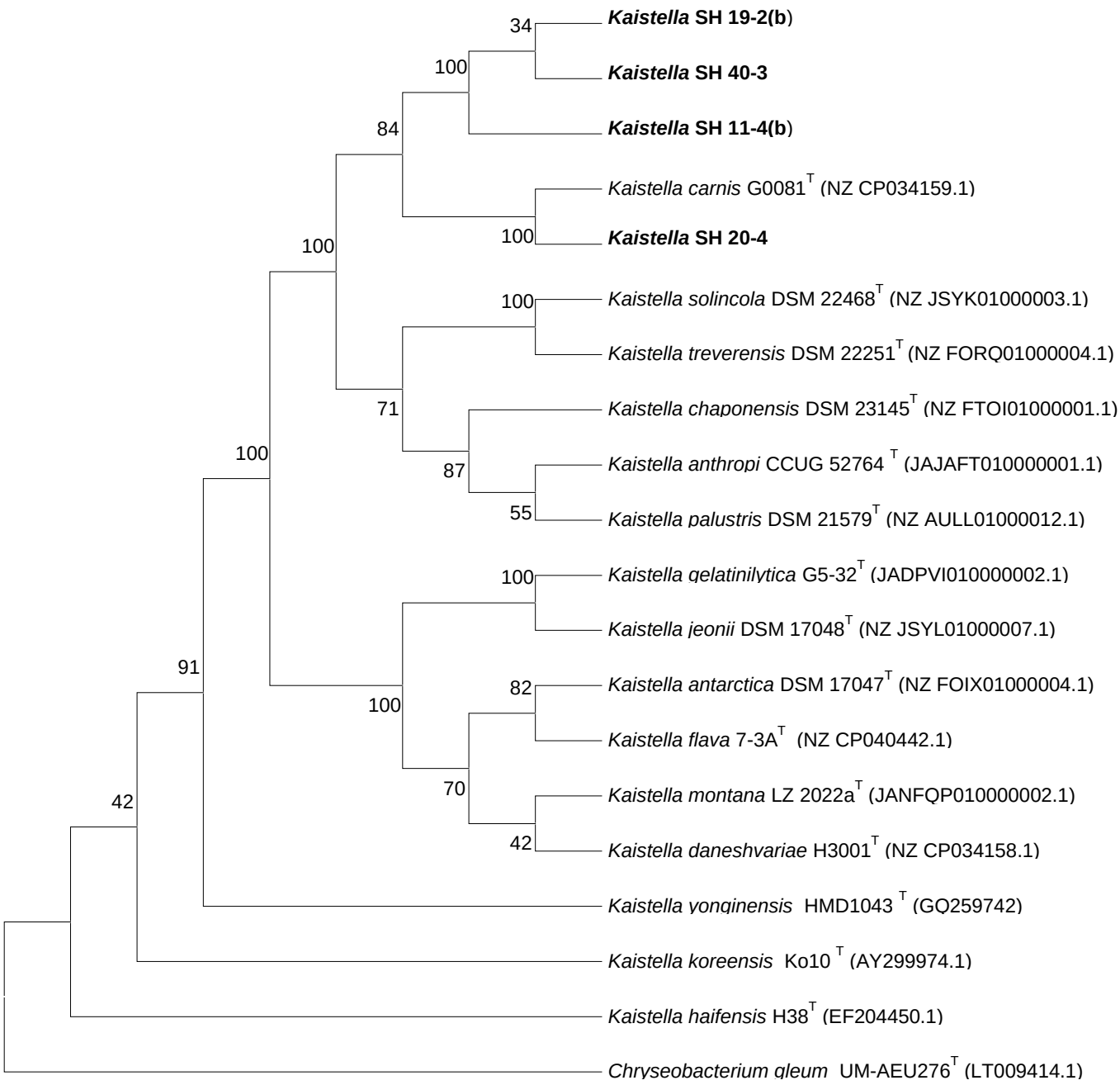

Fig. 3.7: Phylogenetic analysis of the four *Kaistella* strains isolated from fish, nearest *Kaistella* type species and outgroup (*Chryseobacterium gleum*) based on 16S rRNA gene sequences available from the GenBank database (accession numbers are given in parentheses). Multiple alignments were performed, and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the neighbour-joining method in the MEGA version 7 software package (Tamura et al. 2016). Bootstrap values >30%, based on 1000 replications, are given as percentages at branching points. Bar, 0.050 substitutions per nucleotide position.

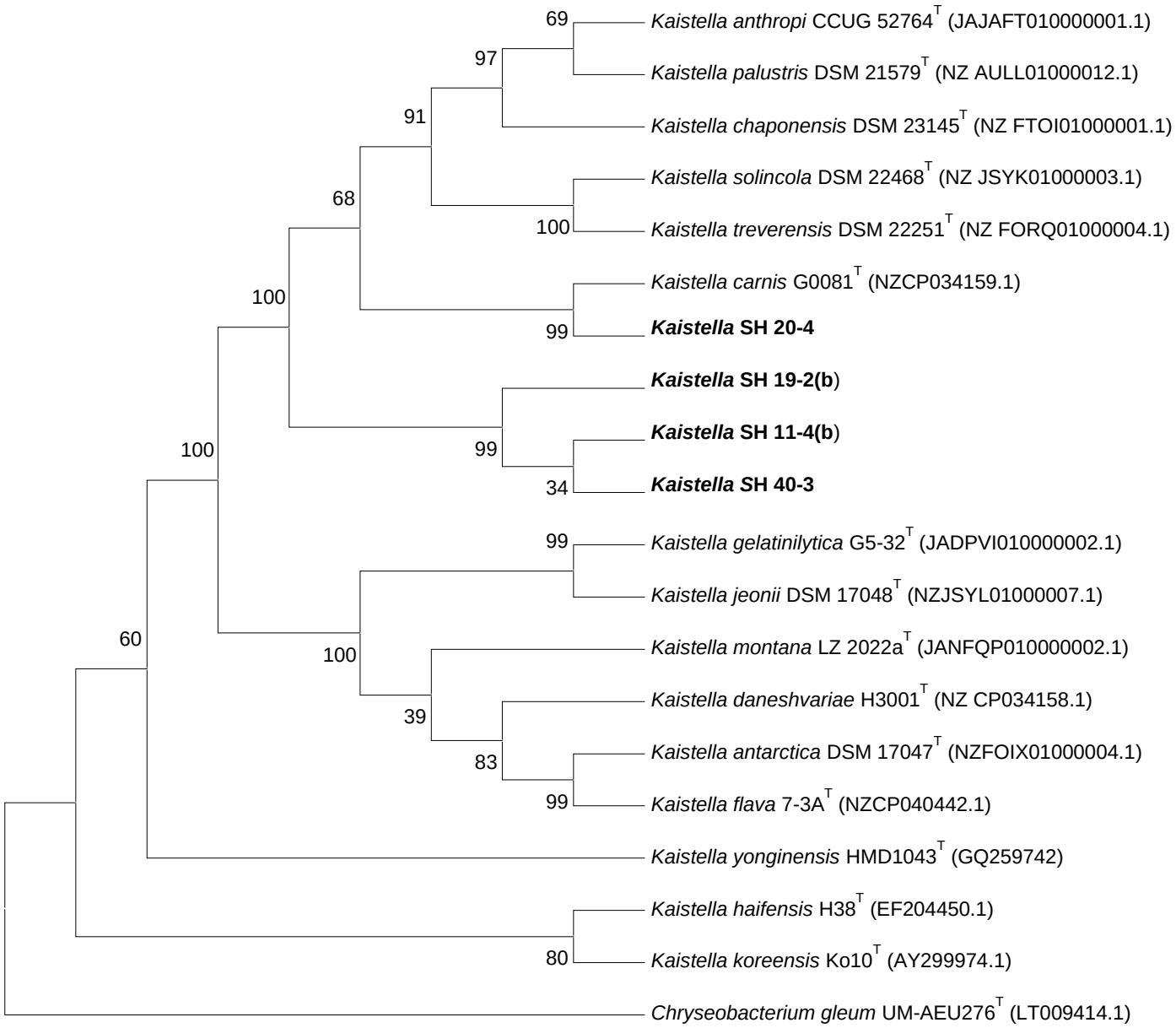

Fig. 3.8. Phylogenetic analysis of the four *Kaistella* strains isolated from fish, nearest *Kaistella* type species and outgroup (*Chryseobacterium gleum*) based on 16S rRNA gene sequences available from the EzBioCloud database (accession numbers are given in parentheses). Multiple alignments were performed, and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the maximum likelihood method in the MEGA version 7 software package (Tamura et al. 2016). Bootstrap values >30%, based on 1000 replications, are given as percentages at branching points. Bar, 0.050 substitutions per nucleotide position.

All organisms shared the same closest relatives with the same 16S rRNA sequence similarity values in that they were all more closely related to the type strains of *K. antarctica* (97.01%) and *K. carnis* (97.41%), except SH 20-4, which had different 16S rRNA sequence similarity values for its closest relatives, *K. antarctica* (97.71%) and *K. carnis* (99.30%) (Tables 3.6–3.9). In both NJ (Fig. 3.7) and ML (Fig. 3.8) methods, strains SH 11-4(b), SH 19-2(b), and SH 40-3 formed separate lineages from the other *Kaistella* species. These results indicated that these three strains could be considered three novel species since the 16S rRNA sequence similarities were less than the 98.7% recommended for species delineation. However, they could not be differentiated from each other. They were further investigated using whole-genome analysis, which will also made way for the evaluation of DDH, ANI, and AAI, which are widely used and compulsory for classifying and describing novel species (Konstantinidis and Stackebrandt 2005, Gosselin et al. 2022). The phylogenetic analysis indicated that strain SH 20-4 could be regarded as another strain of *K. carnis.*

3.3.5. Whole-genome sequencing

Bacterial whole-genome sequencing (WGS) is becoming a widely used technique in research because it allows high-resolution characterization of bacterial species (Gautam et al. 2019). The taxonomic classification of four phenotypically similar fish isolates was evaluated using WGS analysis. A summary of the overall genome relatedness index (OGRI) data, consisting of 16S rRNA similarity values, digital DNA-DNA hybridization (dDDH), average nucleotide identity (ANI) and average amino acid identity (AAI) values, between strains SH 11-4(b), SH 19-2(b), SH 20-4, SH 40-3, and their closest reference strains, can be seen in Tables 3.6–3.9, respectively.

A bacterial species is defined as novel when DNA-DNA hybridization (DDH) values are below 70%, and data from the 16S rRNA gene sequence analysis show more than 3% sequence divergence, provided that all the phenotypic and chemotaxonomic features agree with the above definition (Wayne et al. 1987, Prakash et al. 2007). However, it has been proven that this is not always the case, in that organisms may have 16S rRNA gene sequences over 99% similar or identical and still belong to different species. DDH assays proved challenging to implement, and as genome sequences became available, an average nucleotide identity (ANI) of ~95–96% was suggested to serve as a proxy for 70% DDH (Konstantinidis and Tiedje 2005). An early comparative study between DDH and 16S rRNA gene sequence similarity revealed that 97% 16S rRNA gene sequence similarity corresponded to 70% DDH (Stackebrandt and Goebel 1994). However, higher 16S rRNA gene sequence thresholds of 98.7–99.0% have also been used (Stackebrandt and Ebers 2006, Kim et al. 2014).

The 16S rRNA gene sequence similarities values of strains SH 11-4(b), SH 19-2(b) and SH40-3, with their three most closely related neighbours, *K. antarctica, K. carnis* and *K. chaponensis*, ranged from 97.01 to 97.41% (Tables 3.6, 3.7 and 3.8). The digital DDH values of the *K. antarctica, K. carnis,* and *K. chaponensis* reference strains used in this study, 25.40%, 27.70%, 21.00% for SH 11(b), 25.40%, 26.00%, 21.00% for strain SH 19-2(b), and 25.40%, 26.00%, 20.30% for strain SH 40-3, were below 70%, which confirmed the classification of strains SH 11-4(b), SH 19-2(a) and SH 40-3 as three novel species of the genus *Kaistella*.

The average nucleotide identity (ANI) values of strains SH 11-4(b), SH 19-2(b) and SH 40-3 closest relatives (Tables 3.6, 3.7, 3.8) fell below the cut-off value of 95% for an organism to belong to the same species (Konstantinidis and Tiedje 2005, Richter and Rosello-Mora 2009). This was further supported by amino acid identity (AAI) values below the accepted cut-off value of 95% for the same species classification (Konstantinidis and Tiedje 2005). ANI and AAI results also confirmed that strains SH 11-4(b), SH 19-2(b), and SH 40-3 were novel species of *Kaistella.*

Table 3.6: 16S rRNA similarity values and OGRIs of strain SH 11-4(b) and its nine closest *Kaistella* relatives. dDDH, digital DNA-DNA hybridization; ANI, average nucleotide identity; AAI, average amino acid identity; ND, not determined. The reference strains used for this study are indicated in bold.

Strain	**16S rRNA (EzBioCloud) (%)**	**dDDH (%)**	**ANI (%)**	**AAI (%)**	**G+C content (%)**
K. antarctica	**97.01**	**25.40**	**81.94**	**85.55**	**36.10**
K. carnis	**97.41**	**27.70**	**82.32**	**85.34**	**36.30**
K. chaponensis	**97.36**	**21.00**	**77.96**	**79.12**	**35.30**
K. daneshvariae	96.04	19.90	76.27	79.18	40.00
K. flava	97.36	29.20	84.84	84.82	34.54
K. gelatinilytica	97.01	20.30	78.18	76.08	35.70
K. jeonii	96.94	23.30	78.40	80.22	34.90
K. palustris	96.11	21.20	74.62	77.06	41.50
K. solincola	95.97	19.30	76.46	80.46	39.50

Table 3.7: 16S rRNA similarity values and OGRIs of strain SH 19-2(b) and its nine closest *Kaistella* relatives. dDDH, digital DNA-DNA hybridization; ANI, average nucleotide identity; AAI, average amino acid identity; ND, not determined. The reference strains used for this study are indicated in bold.

Strain	16S rRNA (EzBioCloud) (%)	dDDH (%)	ANI (%)	AAI (%)	G+C content (%)
K. antarctica	**97.01**	**25.40**	**81.83**	**85.52**	**36.10**
K. carnis	**97.41**	**26.00**	**82.17**	**75.24**	**36.30**
K. chaponensis	**97.36**	**21.00**	**78.06**	**79.12**	**35.30**
K. daneshvariae	96.04	19.90	76.33	79.18	40.00
K. flava	97.36	29.10	84.87	84.30	34.54
K. gelatinilytica	97.01	20.30	78.20	79.90	35.70
K. jeonii	96.94	23.10	78.39	76.03	34.90
K. palustris	96.11	21.20	74.63	76.93	41.50
K. solincola	95.97	19.30	76.52	78.30	39.50

Table 3.8: 16S rRNA similarity values and OGRIs of strain SH 40-3 and its nine closest *Kaistella* relatives. dDDH, digital DNA-DNA hybridization; ANI, average nucleotide identity; AAI, average amino acid identity; ND, not determined. The reference strains used for this study are indicated in bold.

Strain	16S rRNA (EzBioCloud) (%)	dDDH (%)	ANI (%)	AAI (%)	G+C content (%)
K. antarctica	**97.01**	**25.40**	**81.93**	**85.30**	**36.10**
K. carnis	**97.41**	**26.00**	**82.15**	**84.30**	**36.30**
K. chaponensis	**97.36**	**20.30**	**77.92**	**79.17**	**35.30**
K. daneshvariae	96.04	19.90	77.33	79.18	40.00
K. flava	97.36	29.20	84.80	84.33	34.54
K. gelatinilytica	97.01	20.30	78.33	82.44	35.70
K. jeonii	96.94	23.10	78.33	80.00	34.90
K. palustris	96.11	21.20	74.59	76.92	41.50
K. solincola	95.97	19.30	76.56	77.1	39.50

The G+C content of strains SH 11-4(b) (35.10%), SH 19-2(b) (35.00%), SH 20-4 (36.29%) and SH 40-3 (35.00%) classified them into the genus *Kaistella* since the DNA G+C content in the genus *Kaistella* ranges from 31.3 to 41.6 mol% (Kim et al. 2004; Nicholson et al. 2020).

The 16S rRNA gene sequence values (99.30%), dDDH (79.70%), ANI (97.84%) and AAI (96.98%) values of strain SH 20-4, compared to *K. carnis,* were higher than the threshold values for species delineation (Table 3.9). Therefore, it was concluded that strain SH 20-4 was not a novel species of the genus *Kaistella* but could instead be another strain of *Kaistella carnis.*

Table 3.9: 16S rRNA similarity values and OGRIs of strain SH 20-4 and its nine closest *Kaistella* relatives. dDDH, digital DNA-DNA hybridization; ANI, average nucleotide identity; AAI, average amino acid identity; ND, not determined. The reference strains used for this study are indicated in bold.

Strain	16S rRNA (EzBioCloud) (%)	dDDH (%)	ANI (%)	AAI (%)	G+C content (%)
K. antarctica	**97.71**	**22.30**	**79.19**	**81.07**	**36.10**
K. carnis	**99.30**	**79.70**	**97.84**	**96.98**	**36.30**
K. chaponensis	**97.98**	**20.90**	**77.24**	**79.15**	**35.30**
K. daneshvariae	97.50	19.80	76.46	80.46	40.00
K. flava	96.79	24.40	81.01	100.00	34.54
K. gelatinilytica	97.43	21.10	77.51	82.32	35.70
K. jeonii	97.78	25.30	77.92	80.08	34.90
K. palustris	97.22	20.90	74.48	77.76	41.50
K. solincola	95.97	19.30	76.56	77.1	95.97

According to a Venn diagram generated with the coding sequences with bidirectional best hits, strain SH 11-4(b) contained 607 distinct genes (Fig. 3.9). Genes associated with hydrolytic activity, such as amidases (nicotinamidases), *S41* peptidases and phosphatases; defence mechanism genes, including rhodanese, photolyase, acid resistance locus, chaperone protein *htpg,* and *Phd* antitoxin, an antitoxin protein encoded by the plasmid addiction operon of the P1 plasmid (which is the plasmid prophage of bacteriophage P1) that prevent host death by binding and neutralizing toxin (Smith and Magnuson 2004); and virulence genes, such as *hopPmaJ*, peptide deformylase and multidrug resistance proteins (*MRP*), which confer resistance to natural product drugs when overexpressed in cultured cells (Deeley and Cole 1997) were identified. Strain SH 11-4(b) and its three closest relatives, including the type strain

Kaistella koreensis, shared 1628 genes. *Kaistella carnis* and strain SH 11-4(b) shared the most genes (114). This is consistent with the 16S rRNA gene sequence analysis since *K. carnis* is closely related to strain SH 11-4 (b). The Venn diagrams in Figures 3.9 - 3.11 show the unique genes of strains SH 11-4(b), SH 19-2(b), and SH 40-3, as well as the genes shared with their closest relatives.

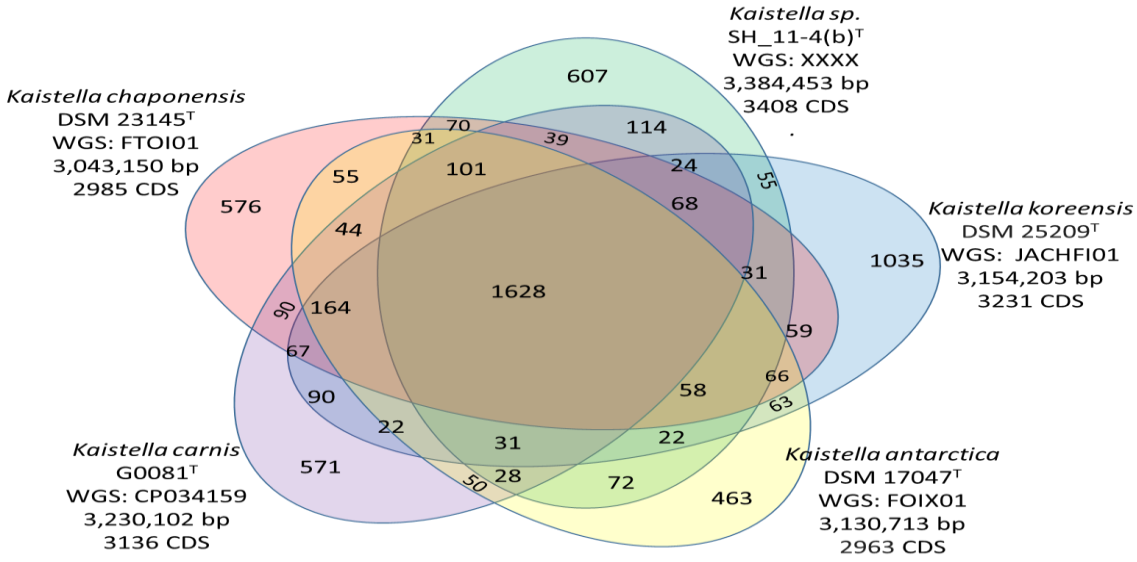

Fig. 3.9: Venn diagram illustrating the unique and shared genes of strain SH 11-4(b), its three closest relatives, *K. carnis, K. antarctica* and *K. chaponensis*, and *K. koreensis*, the type species for the genus *Kaistella*.

Strain SH 19-2(b) shared 1643 genes with its three closest relatives and the *K. koreensis* type strain of the genus (Fig. 3.10). The number of its unique genes was 499, and it shared most genes with *K. chaponensis* (74). Chitinase A was among the hydrolytic class of genes identified. Chitinases are enzymes that hydrolyse chitin, a major structural component in fungi, crustaceans, and insects (Koch et al. 2015). Catalytic genes identified included SOUL heme-binding protein and biotin synthase. Other genes identified in strain SH 19-2(b) had a phage protein, autolysis response regulator protein *LytR,* metallopeptidase, and lycopene cyclase, which plays a crucial role in the synthesis of β-carotene, a significant and most effective vitamin A precursor among carotenoids (Zeng et al. 2015).

Strain SH 40-3 shared a total of 1651 genes with its closest relatives, had 470 unique genes, which was the second lowest compared to its closest relatives and the type species of the genus and also shared most genes with *K. carnis* (70) (Fig. 3.11). Organic hydroperoxide resistance protein, β-lactamase and penicillin-binding protein, *TonB*-dependent transporters (TBDT), outer membrane proteins that bind and transport ferric chelates called siderophores

(Noinaj et al. 2010) and o-antigen acetylase, were among the defence and virulence genes identified in strain SH 40-3.

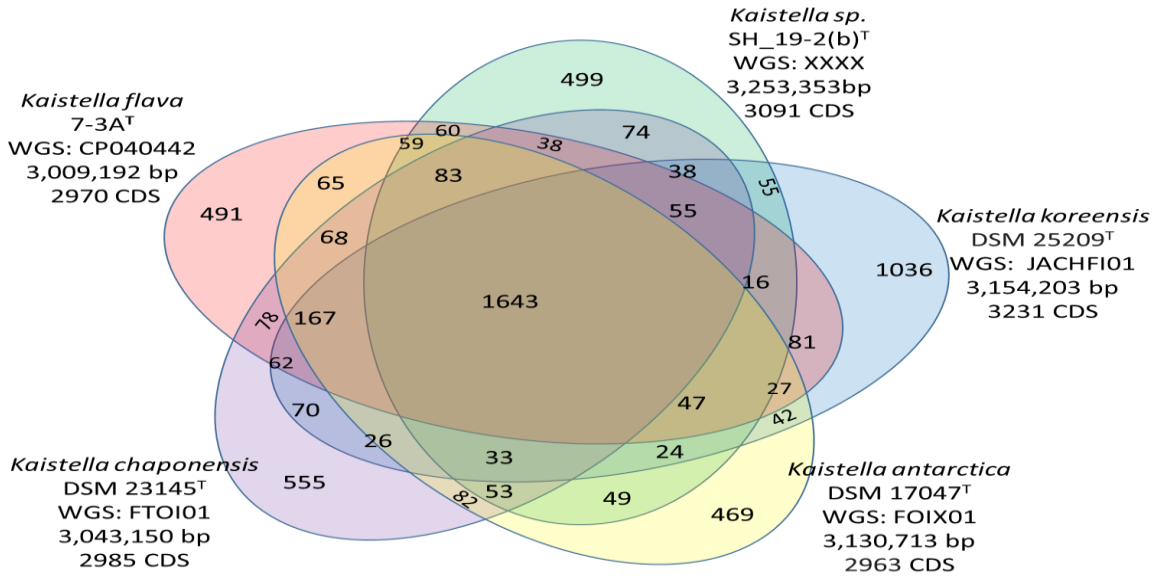

Fig. 3.10: Venn diagram illustrating the unique and shared genes of strain SH 19-2(b), its three closest relatives, *K. carnis, K. antarctica* and *K. chaponensis*, and *K. koreensis*, the type species for the genus *Kaistella*.

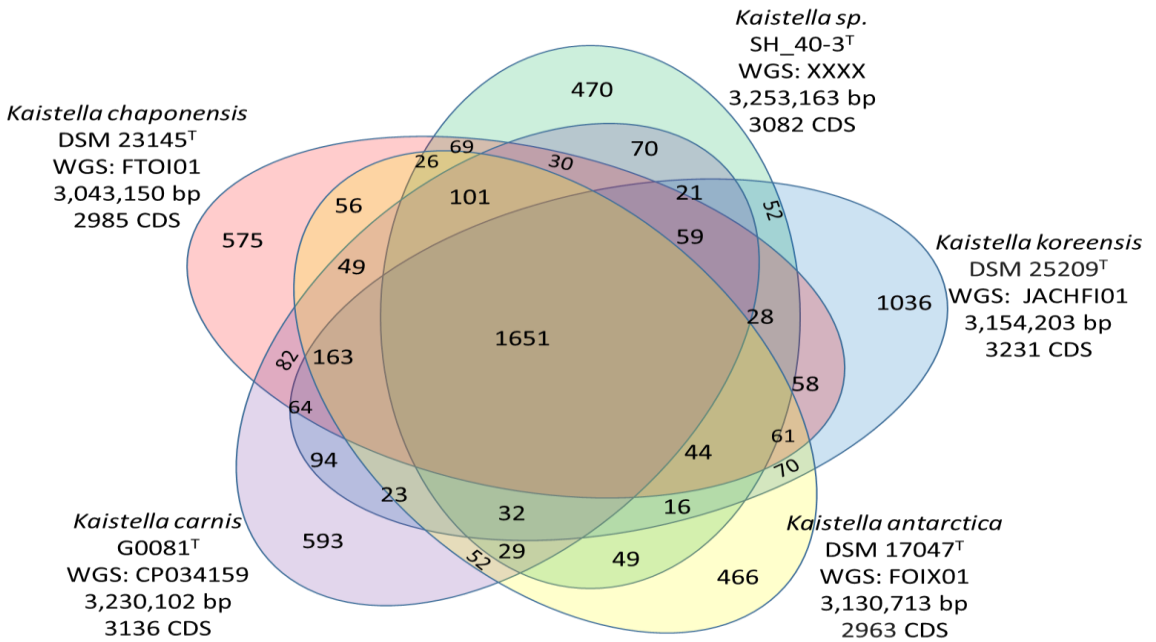

Fig. 3.11: Venn diagram illustrating the unique and shared genes of strain SH 40-3, its three closest relatives, *K. carnis, K. antarctica* and *K. chaponensis*, and *K. koreensis*, the type species for the genus *Kaistella*.

The results of the phenotypic, phylogenetic, and genomic studies concluded that strains SH 11-4(b), SH 19-2(b) and SH 40-3 were three novel species, and the names *K. merluccii, K. piscis* and *K. frigidipiscis* were proposed for the three novel type species. The GenBank identification information for these strains is indicated in Table 3.10.

Table 3.10: GenBank identification information for *Kaistella* strains SH 11-4(b), SH 19-2(b), and SH 40-3.

Identification Information	**Strain SH 11-4(b)**	**Strain SH 19-2(b)**	**Strain SH 40-3**
Accession	JAQYUR000000000	JAQYUQ000000000	JAQYUS000000000
Genome submission	SUB12110890	SUB12167843	SUB12167815
BioProject	PRJN886649	PRJNA891483	PRJN891482
BioSample	SAMN31136170	SAMN31329401	SAMN31329399
GenBank assembly accession	Awaiting	Awaiting	Awaiting
Assembly name	SH11_4_assembly.fasta	SH19_2b_assembly.fasta	SH40_3_assembly.fasta
WGS project	Awaiting	Awaiting	Awaiting
Taxid	2983602	2986944	2986943

3.3.6. Description of Novel Species

3.3.6.1. Description of *Kaistella merlucci* sp. nov.

Kaistella merluccii (mer.luc'ci.i. N.L. gen. n. *merluccii*, of the Cape hake *Merluccius*)

Cells are aerobic, Gram-staining-negative, nonfluorescent, non-flagellated, nonmotile, nonsporulating rods (0.35–0.50 µm wide and 1.3–2.5 long). Colonies are circular, entire, low-convex, smooth, opaque, and creamy white. Flexirubin is not produced. Growth at 4, 20, 25, 30, 37 and 42 °C (optimum at 25 °C) and in 1–4% (w/v) NaCl (optimum at 0%). Grows on nutrient agar, trypticase soy agar, R2A and β-hydroxybutyrate agar, but not on MacConkey agar or cetrimide agar.

Catalase, oxidase, and phosphatase were positive. Indole, urease and DNase are produced, but not hydrogen sulphide, 3-ketolactose or phenylalanine deaminase. Positive for arginine, lysine, and ornithine decarboxylase production. Casein, aesculin, tyrosine, gelatine, and lecithin are degraded, but not tween 80. Nitrate is reduced but not nitrite or selenite. Positive for the methyl red and Voges-Proskauer tests. Oxidative metabolism of glucose in the O/F test.

In the Biolog system, positive for oxidation of D-maltose, D-trehalose, gentibiose, α-D-glucose, D-mannose, D-fructose, D-galactose, D-arabitol, D-glucose-6-PO_4, dextrin, glycyl-L-proline, L-alanine, L-arginine, L-histidine, guanidine HCl, D-serine, L-serine, pH 6, 1% NaCl, gelatin, pectin, L-aspartic acid, L-glutamic acid, tetrazolium violet, tetrazolium blue, K-tellurite, tween 40, acetoacetic acid, acetic acid, citric acid, formic acid, propionic acid, Sodium butyrate, aztreonam and sodium butyrate.

In the Biolog system, negative for oxidation of D-cellobiose, sucrose, stachyose, pH 5, D-raffinose, α-D-lactose, D-Melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D- galactosamine, N-acetyl neuraminic acid, 4 and 8% NaCl, D and L-fucose, L-rhamnose, fusidic acid, D-sorbitol, D-mannitol, D-arabitol, myoinositol, D-aspartic acid, glycerol, D-fructose-6-PO_4, L-pyroglutamic acid, lithium chloride-troleandomycin, rifamycin SV, linocycline, lincomycin, vancomycin, niaproof 4, L-galacturonic acid lactone, p-hydroxy-phenylacetic acid, methyl pyruvate, bromo-succinic acid, g-amino-butyric acid, nalidixic acid, α-hydroxy-butyric acid, β-hydroxy-D, L-butyric acid and α-keto-butyric acid.

The type strain is SH 11-4(b)^T (still needs to be deposited in two international culture collections), isolated from Cape hake (*Merluccius capensis*) that was caught in the Atlantic Ocean off the Cape coast, South Africa.

General features of the genome assembly are as follows: genome size, approximately 3384453 bp; the number of contigs, 301; coding sequences, 3408; N50 value, 343707; coverage, 25.0×. The DNA G+C content is 35.1 mol%.

3.3.5.2. Description of *Kaistella piscis* sp. nov.

Kaistella piscis (pis'cis. L. gen. n. *piscis*, of a fish)

Cells are aerobic, Gram-staining-negative, nonfluorescent, non-flagellated, nonmotile, nonsporulating rods, 0.30−0.49 μm wide and 1–3 μm long. Colonies are circular, entire, low-convex, smooth, opaque, and creamy white. Flexirubin is not produced. Growth at 4, 20, 25, 30, and 37 ^oC (optimum at 25 ^oC) and in 1–4% (w/v) NaCl (optimum at 0%).

Grows on nutrient agar, trypticase soy agar, R2A and β-hydroxybutyrate agar, and MacConkey agar, but not on cetrimide agar.

Catalase, oxidase and phosphatase positive, urease and DNase are produced, but not indole, hydrogen sulphide, 3-ketolactose or phenylalanine deaminase. Positive for arginine, lysine, and ornithine decarboxylase production. Casein, aesculin, tyrosine, gelatine, lecithin, and tween 20 are degraded, but not tween 80. Nitrate and selenite are reduced but not nitrite. Positive for the methyl red and Voges-Proskauer tests and oxidative glucose metabolism in the O/F test.

In the Biolog system, positive for the oxidation of D-maltose, D-trehalose, D-cellobiose, gentibiose, sucrose, α-D-glucose, D-mannose, D-fructose, dextrin, glycyl-L-proline, L-arginine, L-histidine, L-serine, pH 6, 1% NaCl, gelatin, pectin, L-aspartic acid, L-glutamic acid, tetrazolium violet, tetrazolium blue, K-tellurite, tween 40, acetoacetic acid, acetic acid, propionic acid, sodium butyrate, and aztreonam.

In the Biolog system, negative for oxidation of stachyose, pH 5, D-raffinose, α-D-lactose, D-Melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, 4 and 8% NaCl, D- and L-fucose, L-rhamnose, fusidic acid, D-sorbitol, D-mannitol, D-arabitol, myoinositol, D-galactose, D-arabitol, D-glucose-6-PO_4, L-pyroglutamic acid, guanidine HCl, D-serine, D-aspartic acid, glycerol, D-fructose-6-PO_4, troleandomycin, rifamycin SV, vancomycin, minocycline, lincomycin, lithium chloride, niaproof 4, citric acid, formic acid, L-galacturonic acid lactone, p-hydroxy-phenylacetic acid, methyl pyruvate, bromo-succinic acid, g-amino-butyric acid, nalidixic acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, α-keto-butyric acid and sodium butyrate.

The type strain is SH 19-2(b)^T (still needs to be deposited in two international culture collections), isolated from Cape hake (*Merluccius capensis*), which was caught in the Atlantic Ocean off the Cape coast of South Africa.

General features of the genome assembly are as follows: genome size, approximately 3253353 bp; the number of contigs, 49; coding sequences, 3091; N50 value, 343707; coverage, 25.0×. The DNA G+C content is 35.0 mol%.

3.3.5.3. Description of *Kaistella frigidipiscis* sp. nov.

Kaistella frigidipiscis (fri.gi.di.pis'cis. L. masc. adj. *frigidus*, cold; L. masc. n. *piscis*, fish; N.L. gen. n. *frigidipiscis*, of cold fish)

Cells are aerobic, Gram-staining-negative, nonfluorescent, non-flagellated, nonmotile, nonsporulating rods, 0.30–0.50 µm and 1–2.3 µm long. Colonies are circular, entire, low-convex, smooth, opaque, and creamy white. Flexirubin is not produced. Growth at 4, 20, 25, 30, 37 °C (optimum at 25 °C) and in 1–3% (w/v) NaCl (optimum at 0%). Grows on nutrient agar, trypticase soy agar, R2A and β-hydroxybutyrate agar, and MacConkey agar, but not on cetrimide agar.

Catalase, oxidase, and phosphatase were positive. Indole, urease and DNase are produced, but not hydrogen sulphide, 3-ketolactose, or phenylalanine deaminase. Positive for arginine, lysine, and ornithine decarboxylase production. Casein, tyrosine, gelatine, aesculin, and lecithin are degraded, but not tween 20 and tween 80. Nitrate is reduced but not nitrite or selenite. Positive for the methyl red and Voges-Proskauer tests and oxidative glucose metabolism in the O/F test.

In the Biolog system, positive for oxidation of gentibiose, D-mannose, dextrin, pH 6, 1 % NaCl, gelatin, pectin, formic acid, L-aspartic acid, L-glutamic acid, tetrazolium violet, tetrazolium blue, K-tellurite, tween 40, acetoacetic acid, acetic acid, propionic acid, minocycline, lithium chloride, aztreonam and sodium butyrate.

In the Biolog system, negative for oxidation of D-maltose, D-trehalose, D-cellobiose, sucrose, D-fructose, D-galactose, stachyose, pH 5, D-raffinose, α-D-lactose, D-Melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, 4 and 8% NaCl, D- and L-fucose, L-rhamnose, fusidic acid, D-sorbitol, D-mannitol, D-arabitol, myoinositol, D-aspartic acid, glycerol, D-fructose-6-PO_4, glycyl-L-proline, L-alanine, L-arginine, L-histidine, troleandomycin, rifamycin SV, lincomycin, vancomycin, niaproof 4, citric acid, L-galacturonic acid lactone, p-hydroxy-phenylacetic acid, methyl pyruvate, bromo-succinic acid, g-amino-butyric acid, nalidixic acid, α-hydroxy-butyric acid, β-hydroxy-D, L-butyric acid, α-keto-butyric acid and sodium butyrate.

The type strain is SH 40-3^T (still needs to be deposited in two international culture collections), isolated from Cape hake (*Merluccius capensis*), which was caught in the Atlantic Ocean off the Cape Coast, South Africa.

General features of the genome assembly are as follows: genome size, approximately 3253163 bp; the number of contigs, 51; coding sequences, 3082; N50 value, 240780; coverage, 25.0×. The DNA G+C content is 35.0 mol%.

3.4. Conclusions

A taxonomic study was conducted that considered all available phenotypic and genotypic data and integrated them into a consensus type of classification to determine the taxonomic positions of the four strains of the genus *Kaistella* isolated from fish.

The identification and taxonomic categorization of bacterial isolates have been revolutionized by whole-genome sequence studies in that the genome sequence of a microbial strain is the ultimate information for microbial taxonomy. Overall, it can be concluded that the 16S rRNA gene sequences obtained from the whole-genome analysis were used to infer phylogenetic relationships between the test strains and allowed differentiation between organisms at the genus level.

Based on the prokaryotic classification, strains were considered novel when they had 16S rRNA sequence similarity values of less than 98.7%. Strains SH 11-4(b), SH 19-2(b) and SH 40-3 had 16S rRNA sequence similarity percentages below the cut-off value for species delineation. Furthermore, the isolates had DNA G+C mol content ranging from 35.00 to 36.29, which affiliated them to the genus *Kaistella*.

Among the available genome-relatedness indices, average nucleotide identity (ANI) is one of the most robust measurements of genomic relatedness between strains. It has great potential in the taxonomy of bacteria. An ANI threshold range (95-96%) for species boundary was previously suggested. The results for the ANI and AAI of the closest relatives of strains SH 11-4(b), SH 19-2(b), and SH 40-3 were less than 95%, confirming that these strains were novel species of the genus *Kaistella*. The 16S rRNA gene sequence value (99.30%), dDDH (79.70%), ANI (97.84%) and AAI (96.98%) of strain SH 20-4 compared to *K. carnis* were higher than the threshold values for species delineation. Therefore, it was concluded that strain SH 20-4 was not a novel species of the genus *Kaistella* but could be considered another strain of *Kaistella carnis*.

Therefore, based on the above phenotypic, genotypic, and phylogenetic characterizations, strains SH 11-4(b), SH 19-2(b), and SH 40-3 represented three novel species of the genus *Kaistella*, with the following proposed names: *Kaistella merluccii*, *Kaistella piscis*, and *Kaistella frigidipiscis,* respectively.

CHAPTER 4

VIRULENCE CHARACTERISTICS OF *Kaistella* SPECIES ISOLATED FROM FISH

Abstract

Members of the genus *Kaistella* are isolated from a variety of environments. However, little is known about their pathogenicity and/or spoilage mechanisms. This study tested six Kaistella strains isolated from fresh marine fish for their ability to produce siderophores, haemolytic activity, virulence enzymes, and antimicrobial resistance patterns. Although the temperature had a significant impact on the ability of the organisms to secrete siderophores, all six *Kaistella* strains were able to produce siderophores, even though *Chryseobacterium gleum,* the positive control, had siderophores that were more prominent than the *Kaistella* fish isolates. The organisms used in this study produced a wide range of enzymes, including gelatinase, lipase, proteinase, lecithinase, hyaluronidase, chondroitinase and DNase, with fibrinolysin being the only enzyme that was not produced by any of the test and reference strains. *Chryseobacterium gleum* synthesized 87.5% (7/8) enzymes, and shared this property with *K. antarctica*, which produced 87.5% (7/8) enzymes. '*Kaistella merluccii'* SH 11-4 (b), could also be regarded as another potential pathogen, as it was able to produce 50% (4/8) enzymes evaluated in this study. Together with *C. gleum* and *K. antarctica*, strains SH 11-3(a) and *'K. frigidipiscis*' SH 40-3 showed resistance to 52.9% (9/17) antimicrobials used in this study.

4.1. Introduction

The genus *Kaistella* has been isolated from clinical, environmental, industrial, and food sources. However, their virulence characteristics have not been studied in detail. Bacterial virulence is the relative capacity to overcome defences in the host (Sparling 1983). Virulence is the product of many interacting variables involving the bacterium and the host (Sparling 1983). Bacterial pathogens express an extensive range of molecules that bind host target cells, enabling them to trigger a wide range of host responses (Wilson et al. 2002). The virulence mechanisms bacteria use to cause infection include adhesins, invasins, type three secretion systems, outer membrane proteins, toxins, capsules, iron acquisition systems, and biofilm formation, among others (Cepas and Soto 2020). Several enzymes have also been linked to microbial virulence. Enzymes measured as virulence factors are typically active against host components and contribute to virulence by causing damage to host tissues (Casadevall and Pirofski 2009).

Many bacteria use siderophores to aid ferric iron uptake in the environment (Louden et al. 2011). Siderophores are low molecular weight iron chelators that bind transferrin, lactoferrin, ferritin, haemoglobin, ferrous iron transporters, heme, or haptoglobin-haemoglobin complexes (Casadevall and Pirofski 2009, Crumbliss and Harrington 2009). Because free iron is limited and often tightly bound to many proteins, such as haemoglobin, transferrin, and lactoferrin, siderophore production is essential to a pathogen's ability to cause disease (Miethke and Marahiel 2007). The term "hypervirulent" refers to bacterial strains that may produce excessive amounts of siderophores, whereas "reduced virulence" refers to strains that are less effective at infecting and colonizing host cells (Haldon and Bachman 2015).

Antibiotic resistance has become a significant cause of illness and mortality worldwide. In the early 1900s, when antibiotics were first developed, it was believed that humanity had defeated microbes. However, it soon became clear that the microorganisms could become resistant to any treatment that was being utilized. It seems that most pathogenic bacteria can become resistant to at least some antimicrobial substance. Limiting drug absorption, altering a drug target, inactivating a drug, and active efflux are the basic mechanisms of resistance (Wilson 2002, Reygaert 2018). These mechanisms may be native to the microorganisms or acquired from other microorganisms (Wilson 2002).

Some members of the genus *Kaistella* have also shown resistance toward a few antimicrobials; however, their virulence factors have not been studied in similar detail compared to their taxonomy and nomenclature. As a result, this part of the study aimed to determine the virulence characteristics of six *Kaistella* strains isolated from fresh marine fish three by testing their ability to produce virulence enzymes and siderophores and their resistance to antimicrobials.

4.2. Materials and Methods

All Oxoid and Difco products were sourced from Thermo Fisher Scientific, Johannesburg, South Africa. In contrast, all Sigma and Merck products were sourced from Merck Life Science (Pty) Ltd, Johannesburg, South Africa.

4.2.1. Cultures used and their maintenance

The 16 bacterial strains used in this study are shown in Table 4.1. Four *Kaistella* fish isolates from Chapter 3 and two more *Kaistella* fish isolates were used in this study section. The *Kaistella* type species were used as reference strains because the results of phylogenetic analysis, based on 16S rRNA gene sequences, indicated that they were closely related to the test isolates.

Table 4.1: The six *Kaistella* fish isolates and ten reference strains used in this study. UFSBC, University of the Free State Bacterial Culture Collection (South Africa); NCTC, National Collection of Type Cultures (United Kingdom); KCTC, Korean Collection of Type Cultures (Korea); CCUG, Culture Collection of the University of Göteburg (Sweden); LMG, Belgian Culture Collection of Microorganisms (Belgium); ATCC, American Type Culture Collection (United States of America); DSM, Deutsche Sammlung von Mikroorganismen (Germany); CBSC, Carolina Biological Supply Company (United States of America).

Strains used	Culture Collection	Source of Collection	Reference
SH 11-3(a)	UFSBC 628	Fresh Marine Fish	Engelbrecht 1992
SH 11-3(b)	UFSBC 635	Fresh Marine Fish	Engelbrecht 1992
SH 11-4(b)	UFSBC 629	Fresh Marine Fish	Engelbrecht 1992
SH 19-2(b)	UFSBC 623	Fresh Marine Fish	Engelbrecht 1992
SH 20-4	UFSBC 637	Fresh Marine Fish	Engelbrecht 1992
SH 40-3	UFSBC 631	Fresh Marine Fish	Engelbrecht 1992
Chryseobacterium gleum	NCTC 11432^{T}	Vaginal swab	Vandamme et al. 1994
Kaistella koreensis	KCTC 12107^{T}	Natural mineral water	Kim et al. 2004
Kaistella carnis	CCUG 60559^{T}	Raw beef carcass from an unspecified abattoir	Holmes et al. 2013, Nicholson et al. 2020
Kaistella chaponensis	CCUG 58959^{T}	Diseased farmed Atlantic salmon (*Salmo salar*)	Kämpfer et al. 2011, Nicholson et al. 2020
Kaistella antarctica	LMG 24720^{T}	Soil samples from penguin habitats in Antarctica	Yi et al. 2005, Kämpfer et al. 2009, Nicholson et al. 2020
Kaistella yonginensis	KCTC 22744^{T}	Mesotrophic artificial lake	Joung and Joh, 2011, Nicholson et al. 2020
Bacillus subtilis (Ehrenberg) Cohn	ATCC 6051^{TM}	-	Ehrenberg 1835, Kesaulya et al. 2018
Bacillus megaterium	CBSC 154900	-	Bary 1884, Ferreira et al. 2019
Pseudomonas aeruginosa	ATCC 27853^{TM}	Wound infections of soldiers	Gessard 1882, Peek et al. 2012
Pseudomonas fluorescens	DSM 4358^{T}	Raw milk for cheese production	Trapet et al. 2016

Chryseobacterium gleum was used because it is the type strain for the genus *Chryseobacterium* and because the test isolates were initially classified as belonging to *Chryseobacterium.* *Kaistella koreensis* was used because it is the type strain of the genus *Kaistella.* Bacillus and *Pseudomonas* were used because of their proven ability to produce siderophores (Peek et al. 2012, Trapet et al. 2016, Ferreira et al. 2019) and were only included in the siderophore production part of this study.

All strains had been preserved in a freeze-dried state in glass ampoules. For shorter-term maintenance, the isolates were freeze-dried on 6 mm diameter filter paper discs (FLAS321260, Lasec SA (Pty) Ltd, Bloemfontein, South Africa). They were stored in sterile Wasserman tubes (AXI07TCTT, Axiology Labs (Pty) Ltd, Vereeniging, South Africa) at -20 °C. Before use, strains were reactivated in 10 ml of nutrient broth (NB; Oxoid CM67). Purity was checked by streaking on nutrient agar (NA; Oxoid CM0003) and Gram-staining. Incubation was at 25 °C for 48 h for the *Chryseobacterium* and *Kaistella* reference strains, at 37 °C for 24 h for the *Bacillus* strains and at 32 °C for 48 h for the *Pseudomonas* strains. The pure cultures on NA slants were stored at 4 °C for short-term maintenance and re-streaked every 4–6 weeks.

4.2.2. Siderophore production

The chrome azurol S medium was prepared according to Louden et al. (2011). For the blue dye, three solutions were prepared as follows: Solution 1 was prepared by dissolving 0.06 g of chrome azurol S (CAS, Sigma 199532) in 50 ml of ultra-pure water. Solution 2 was prepared by dissolving 0.0027 g of $FeCl_3.6H_2O$ (Merck 234053) in 10 ml of 10 mM HCl. Solution 3 was prepared by dissolving 0.073 g of hexadecyltrimethylammonium bromide (HDTMA, Sigma H9151) in 40 ml of ultra-pure water. Solution 1 was then mixed with 9 ml of solution 2, the two solutions were mixed with solution 3, and a blue colour formation resulted. The blue-coloured mixture was then autoclaved and stored at 4 °C until use.

Mixture solutions:

The preparation of minimal media 9 (MM9) salt solution stock entailed dissolving 15 g KH_2PO_4, 25 g NaCl, and 50 g NH_4Cl in 500 ml of ultra-pure water. Twenty grams of glucose (Sigma G8270) were added to 100 ml of ultra-pure water to prepare a 20% glucose stock and was filter-sterilized using a 0.20 μm GVS syringe filter (AXIFJ25ASCCA002DL0, Axiology Labs). The sodium hydroxide (NaOH) stock was prepared by dissolving 25 g of NaOH (Merck 1019770) in 150 ml ultra-pure water, pH 12. The preparation of the casamino acids solution included dissolving 3 g of casamino acids (Difco 223050) in 27 ml ultra-pure water, then extracting with 27 ml of 3% 8-hydroxyquinoline (Sigma H6878) in chloroform (Sigma 366927)

to remove any trace iron. This solution was filter-sterilized in a sterile container using a 0.20 μm GVS syringe filter.

CAS agar preparation:

A hundred millilitres of the MM9 solution were added to 750 ml of ultra-pure water, and the pH was adjusted to 6. This was followed by dissolving 32.24 g piperazine-N, N'-bis (2-ethane sulfonic acid) (PIPES, Sigma P6757) in the solution. Fifteen grams of bacteriological agar (Oxoid LP0011) were added to the solution, and the mixture was autoclaved and cooled to 50 °C. Then, 30 ml of the sterile casamino acids solution and 10 ml of the sterile 20% glucose solution were added. A 100 ml blue dye solution was slowly added aseptically along the container wall, with enough agitation to mix thoroughly. Plates were then poured and allowed to be set. The test isolates were streaked on the plates and incubated at 4, 25, 32, and 37 °C for 48 h. Yellow discolouration of the medium was taken as a positive result. This experiment was carried out in triplicate.

4.2.3. Inoculum preparation for enzyme production tests

The inocula of the bacterial cultures were standardized: colonies from a 48 h NA plate culture were suspended in 5 ml of sterile, 0.1 M, pH7 phosphate-buffered solution until a density corresponding to the McFarland 1 standard (Difco 0691326) was reached. Ten microlitres of each standardized culture suspension were spotted on media containing the substrate. Each inoculum was spotted in triplicate on two plates to give six data points. Incubation was at 25 °C for 72 h unless otherwise indicated. Qualitative (present/absent) and quantitative (Z-scores) analyses were performed.

4.2.4. Lecithinase production

Lecithinase activity was performed according to Pavlov et al. (2004). A 450 ml solution of McClung Toabe agar base (Difco 294110) was prepared, autoclaved, and cooled to 50 °C. A 50 ml egg yolk emulsion (Oxoid SR0054C) was added to the 450 ml McClung Toabe agar base. The medium was poured into Petri dishes and allowed to be set. Spot inoculation was performed as indicated in 4.2.3. After inoculation, the incubation was at 25 °C for 72 h. The formation of a white precipitate around or beneath the inoculation spot was taken as a positive result.

4.2.5. Chondroitinase production

Production of the chondroitinase enzyme test was performed according to Smith and Willette (1968), Janda and Bottone (1981), Edberg et al. (1996), and Pavlov et al. (2004). Briefly, the primary medium consisted of 100 ml heart infusion broth (Oxoid CM0375) and 1 g of noble agar (Difco 214230), which was autoclaved and cooled to 50 °C. An aqueous solution of 5%

bovine albumin fraction V (Sigma A1595) and 4 mg/ml chondroitin sulphate A (Sigma C9819) was prepared. Before mixing and adding to the molten and cooled primary medium, the solution was filter-sterilized using a 0.20 μm GVS syringe-filter unit. The poured plates were spot inoculated, as indicated in 4.2.3. and incubated at 25 °C for 48 h. A clear zone around the inoculum spot showed a positive result.

4.2.6. Hyaluronidase production

The medium preparation for this test was according to Smith and Willette (1968), Edberg et al. (1996), and Pavlov et al. (2004). The primary medium consisted of 1 g of noble agar (Difco 214230) and 100 ml of heart infusion broth (Oxoid CM0375), which was autoclaved and cooled to 50 °C. Hyaluronic acid (Sigma H1504), which was dissolved in water at a concentration of 2 mg/ml, and a 5% bovine albumin fraction V (Sigma C9819), were filter-sterilized separately using a 0.20 μm GVS syringe filter and the sterile solutions were added to the primary molten medium. Petri dishes were poured, and spot inoculated as indicated in 4.2.3. The plates were incubated at 25 °C for 48 h. A clear zone surrounding the inoculated spot was a positive result.

4.2.7. Fibrinolysin activity

This procedure was carried out according to Janda and Bottone (1981), Edberg et al. (1996), and Pavlov et al. (2004). A 100 ml solution of NA was autoclaved and cooled to 50 °C before adding 280 mg of fibrinogen type III (Sigma F4129). The Petri dishes were poured, and spot inoculated as indicated in 4.2.3. After inoculation, the plates were incubated at 25 °C for 48 hours. The formation of clear zones was regarded as a positive outcome.

4.2.8. Gelatin hydrolysis

This test included two techniques (MacFaddin 1976). In Method A, 5.5 g of bacteriological agar (Oxoid LP0011) was added to 500 ml of nutrient broth No. 2 (Oxoid CM67), and the mixture was heated until the agar was completely dissolved. Before adding 2 g of gelatin (Merck 2503100 EM), the medium was briefly cooled. After standing for 5 min, the medium was sterilized at 110 °C for 10 min, and the plates were poured and left to set. The plates were spot inoculated and incubated for five days at 25 °C, as indicated in 4.2.3. The plates were then flooded with 5–10 ml of Frazier's reagent (12 g mercuric chloride [NT Laboratory Supplies R1615], 80 ml distilled water and 16 ml concentrated HCl). Clear zones around the test spot indicated a positive result.

Method B involved dissolving 60 g of gelatin in 500 ml of distilled water for 30 min. This was followed by heating the mixture to a boiling point after adding beef extract (1.5 g, Oxoid L29) and peptone (2.5 g, Oxoid L37). The pH was adjusted to 7 after allowing it to cool slightly.

Ten millilitres of the slightly cooled medium were dispensed in test tubes and autoclaved. The tubes were kept at 4 °C until they were used. Stab inoculation was used, and the tubes were incubated at 25 °C for 14 days. Every 24 h, the incubated tubes were frozen for 2 h. If, after 2 h, the medium in the tube was still liquid (not solidified), this was taken as a positive gelatin hydrolysis reaction.

4.2.9. Lipase production

The preparation of the medium was performed according to Janda and Bottone (1981), Edberg et al. (1996), and Pavlov et al. (2004). A 100 ml of trypticase soy agar (Oxoid CM0131) was supplemented with 1 ml of tween 80 (Merck 93781) as a substrate in this test. The medium was autoclaved, cooled, and plates were poured and set. The plates were spot inoculated as indicated in 4.2.3 and incubated at 25 °C for 72 h. The appearance of a turbid halo around the test spot was taken as a positive result.

4.2.10. Proteinase production

This experiment modelled one of Edberg and colleagues (1996). Skim milk powder (Oxoid LP0033) was incorporated into brain heart infusion broth (Oxoid CM0375) with 1.5% agar. The medium was autoclaved, allowed to cool to 50 °C, and poured into Petri dishes. Spot inoculation of the medium was performed as indicated in 4.2.3. After inoculation, the plates were incubated for 72 h at 25 °C. The formation of a clear zone around the colonies was taken as a positive result.

4.2.11. DNase production

The agar plates were prepared according to Janda and Bottone (1981), Edberg et al. (1996), and Pavlov et al. (2004). DNase agar (Oxoid CM321) was added to distilled water and supplemented with 0.01% toluidine blue (Merck CH-9470). The medium was sterilized by autoclaving and dispensing into sterile Petri dishes after cooling to 50 °C. The plates were spot inoculated and incubated for 48 h, as indicated in 4.2.3. The plates were then flooded with 0.1% of a 1 M HCl solution. A pink halo, or a zone of clearance, that developed or appeared around a colony indicated a positive result.

4.2.12. Haemolytic activity

The medium to test for the haemolytic activity of the organisms was prepared according to Pavlov et al. (2004). Briefly, sterile laked horse blood (Oxoid SR0048C) was added to sterile cooled (50 °C) NA. The mixture was poured into Petri dishes, spot inoculated with the test organisms, as indicated in 4.2.3, and incubated at 25 °C for 48 h. Clear zones with sharply defined edges around the colonies indicated β-haemolysis (complete lysis of red blood cells).

Green zones around colonies indicated α-haemolysis. The greenish halo around the colony resulted from reduced haemoglobin to methaemoglobin in red blood cells. γ-Haemolysis was indicated by no haemolysis.

4.2.13. Antibiotic resistance

The Kirby-Bauer disk diffusion method, according to Hudzicki (2009), was used to determine the antimicrobial resistance patterns of the test organisms. Table 4.2 contains a list of the antimicrobials and their concentrations used in this study. The density of a MacFarland 1 standard was used to standardize the bacterial suspensions. The suspensions mentioned above were then streaked on a Mueller-Hinton agar (Oxoid CM337) plate in three different directions, using a sterile cotton swab. Antimicrobial discs were placed on the inoculated plates using sterile forceps. Subsequently, the plates were incubated for 48 h at 25 °C. The sizes of the clearance zones surrounding each disc were measured after incubation and compared with those indicated in Table 4.3. This experiment was carried out in triplicate.

Table 4.2: Antimicrobials used in this study.

Name	Concentration (µg)	Antimicrobial group
Ampicillin	10, 25	Penicillin
Oxacillin	1	Penicillin
Piperacillin	75	Penicillin
Cephalothin	30	β-Lactam
Oxytetracycline	30	Tetracycline
Tetracycline	30	Tetracycline
Gentamicin	10	Aminoglycoside
Kanamycin	30	Aminoglycoside
Streptomycin	10, 25	Aminoglycoside
Cefepime	30	Cephems
Cefotaxime	30	Cephems
Ciprofloxacin	1	Fluoroquinolone
Levofloxacin	5	Fluoroquinolone
Erythromycin	10	Macrolide
Vancomycin	30	Glycopeptide

The criteria for an organism to be considered resistant, intermediate resistant, or susceptible are listed in Table 4.3, which was compiled from the Performance Standards for Antimicrobial Susceptibility Testing, 17th, 30th Informational Supplement (M100, Clinical and Laboratory

Standards Institute 2007, 2020) using the criteria established for *Enterobacteriaceae* to interpret the results, since *Kaistella* does not have specific criteria in the CLSI documents.

Table 4.3: Antimicrobial performance standards measured in zone diameter according to the Institute of Clinical and Laboratory Standards (2007, 2020) - performance standards for antimicrobials were not reported.

Antimicrobial	Concentration (µg)	Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ampicillin	10	≤13	14–16	≥17
Ampicillin	25	-	-	-
Oxacillin	1	≤10	11–12	≥13
Piperacillin	75	≤14	15–19	≥20
Cephalothin	30	≤14	15–17	≥18
Oxytetracycline	30	-	-	-
Tetracycline	30	≤11	12–14	≥15
Gentamicin	10	≤12	13–14	≥15
Kanamycin	30	≤13	14–17	≥18
Streptomycin	10	≤11	12–14	≥15
Streptomycin	25	-	-	-
Cefepime	30	≤18	-	≥25
Cefotaxime	30	≤22	23–25	≥26
Ciprofloxacin	1	-	-	-
Levofloxacin	5	≤16	17–20	≥21
Erythromycin	10	≤13	14–22	≥23
Vancomycin	30	≤14	15–16	≥17

4.2.14. Statistical analysis

Microsoft Excel 2016 spreadsheets were used to record all outcomes. The effect of enzyme production and various antimicrobials on the *Kaistella* fish isolates was examined using an ANOVA (NCSS Statistical Software program, version 11.0.20). Zone (Z) scores were used to standardize the results from the analysis of the enzyme activity and antimicrobial resistance of all organisms used in this study. Z = colony diameter (mm)/ (colony diameter (mm) + zone size (mm)). To find significant variations between the treatment means, the Tukey-Kramer multiple comparison test (= 0.05) was used (NCSS Statistical Software package, version 11.0.20).

4.3. Results and Discussion

The results of the virulence factor analysis are discussed below. Qualitative (absence/presence) analysis was performed on siderophore, lecithinase, chondroitinase, hyaluronidase and fibrinolysin production since it was impossible to measure the zones of these test results. The reasons for being unable to measure the zones were either that only colour production was determined (siderophore production) or that the clearance zones were not measurable due to colony spreading. Quantitative analysis (z-scores) was performed on gelatinase, lipase, proteinase, DNase, and β-haemolysis production.

4.3.1. Siderophore production

An overview of the capacity of the test isolates and the reference strains to produce siderophores at 25, 32, and 37 °C are indicated in Figures 4.1, 4.2, and 4.3, respectively. Microorganisms have developed a distinctive mechanism for the sequestration and transport bound iron by producing iron-chelating molecules known as siderophores. These siderophores are crucial for the colonization of microorganisms in the iron-deficient areas of the host, serving as a critical virulence factor (Kumar et al. 2021).

The general secretion of the siderophores varied according to temperature. Compared to all the other organisms used in this investigation, *C. gleum* showed the highest siderophore activity with the largest clearance zone. The rest of the microorganisms evaluated in this study also produced siderophores. The secretion of siderophores by *C. gleum* was more notable at 25 °C (Fig. 4.1), followed by 32°C (Fig. 4.2), and the least significant at 37 °C (Fig. 4.3), while the secretion of siderophores by the other organisms in this study, was only slightly affected at the three respective temperatures. It was speculated that *C. gleum* would have increased virulence fitness during infection and colonization due to its ability to produce excessive amounts of siderophores, making it more virulent than the other strains evaluated in this study. It is consistent with the fact that *Chryseobacterium* species have been reported as emerging opportunistic bacterial pathogens in healthcare settings, with *C. gleum* being linked to septicaemia, meningitis, wound infections, respiratory tract infections, and urinary tract infections (Holmes et al. 1984, Vandamme et al. 1994, Lo and Chang 2014, Virok et al. 2014, Wahab et al. 2015, Brki et al. 2015, Abdalhamid et al. 2016, Wang et al. 2016, Arouna et al. 2017, Sarmis et al. 2020).

The potential of the test strains to produce siderophores at 4 °C was also evaluated since they were isolated from fish caught in cold water environments; however, none of the organisms used in this study produced siderophores at this temperature.

Temperatures below 20 °C and above 40 °C were found to be unsuitable for siderophore production in a 2009 study by Bendale and colleagues on the impact of environmental conditions on siderophore production. Maximum siderophore production was obtained in *Pseudomonas syringae* strain BAF.1 at 30 °C (Yu et al. 2017). In 2017, Colombowala reported maximum siderophore production by *Pseudomonas aeruginosa* at 37 °C, while Sheng and colleagues reported maximum siderophore yields for *Brevibacillus brevis* GZDF3 at 30 °C to 32 °C.

Sixty-three per cent (10/16) of the bacterial strains tested (*B. subtilis, B. megaterium, C. gleum*, *K. koreensis, K. antarctica*, *P. aeruginosa*, *P. fluorescens*, '*K. merluccii'* SH 11-4(b), '*K. piscis'* SH 19-2(b), '*K. frigidipiscis'* SH 40-3) produced visible siderophores at 25 °C (Fig. 4.1). Only *K. yonginensis* and *K. chaponensis* (12.5%) could not secrete siderophores at 25 °C, while 19% (3/16) (SH 11-3(a), SH 11-3(b), and SH 20-4) could only produce siderophores weakly at this temperature. The two *Bacillus* positive controls had both a blue and a yellow halo around their colonies. Different halo colours can be indicative of the type of siderophores produced. Blue to purple colour changes can be linked to catechols, orange (hydroxamates) and yellow can be carboxylate producer (Louden et al. 2011).

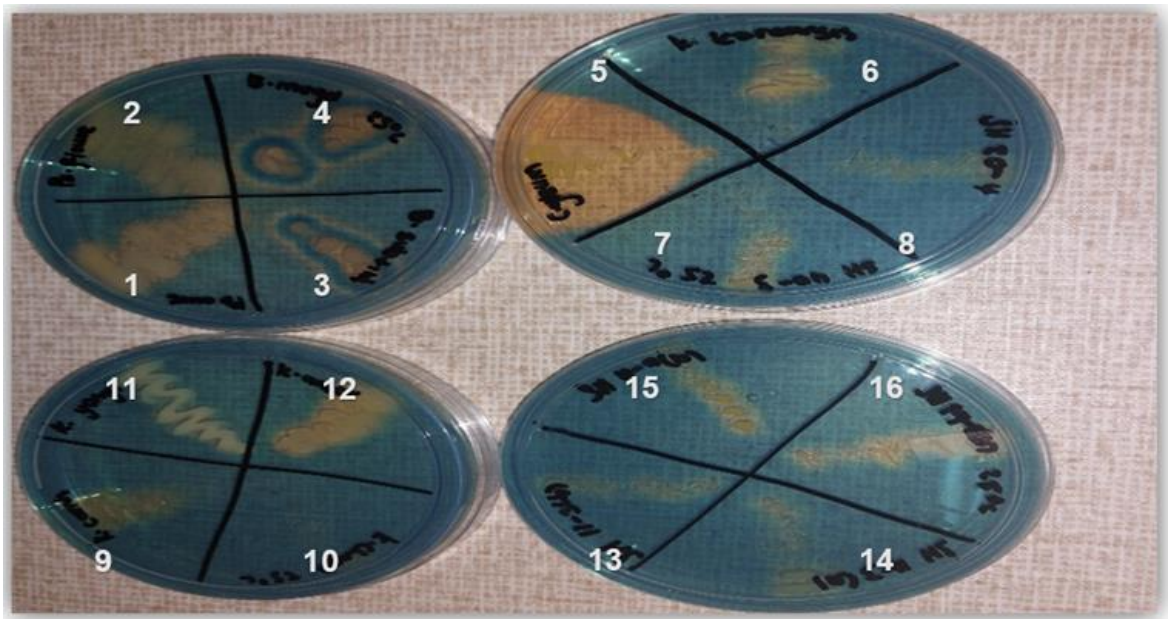

Figure 4.1: Production of siderophores by the six *Kaistella* fish isolates [SH 11-3(a) (14), SH11-3(b) (13), '*K. merluccii'* SH 11-4(b) (15), '*K. piscis*' SH 19-2(b) (16), '*K. frigidipiscis*' SH 20-4 (8) and SH 40-3 (7)] and reference strains [*C. gleum* (5)*, K. koreensis* (6)*, K. carnis* (9), *K. chaponensis* (10), *K. antarctica* (12)*, K. yonginensis* (11)*, B. subtilis* (3)*, B. megaterium* (4)*, P. aeruginosa* (1) and *P. fluorescens* (2)] at 25 °C.

The ability to produce siderophores in all *Kaistella* and *Chryseobacterium* reference strains was compromised at 32 °C (Fig. 4.2) but not in the *Pseudomonas* and *Bacillus* strains, which were the positive control strains for siderophore production.

The potential of the six *Kaistella* test strains to produce siderophores at this temperature increased slightly compared to the results at 25 °C. This indicated that these strains had optimal growth temperatures between 25 and 32 °C. This further demonstrated the importance of considering temperature when assessing virulence factors, as some virulence pathways are inactive below 30 °C (LaBauve and Wargo 2012).

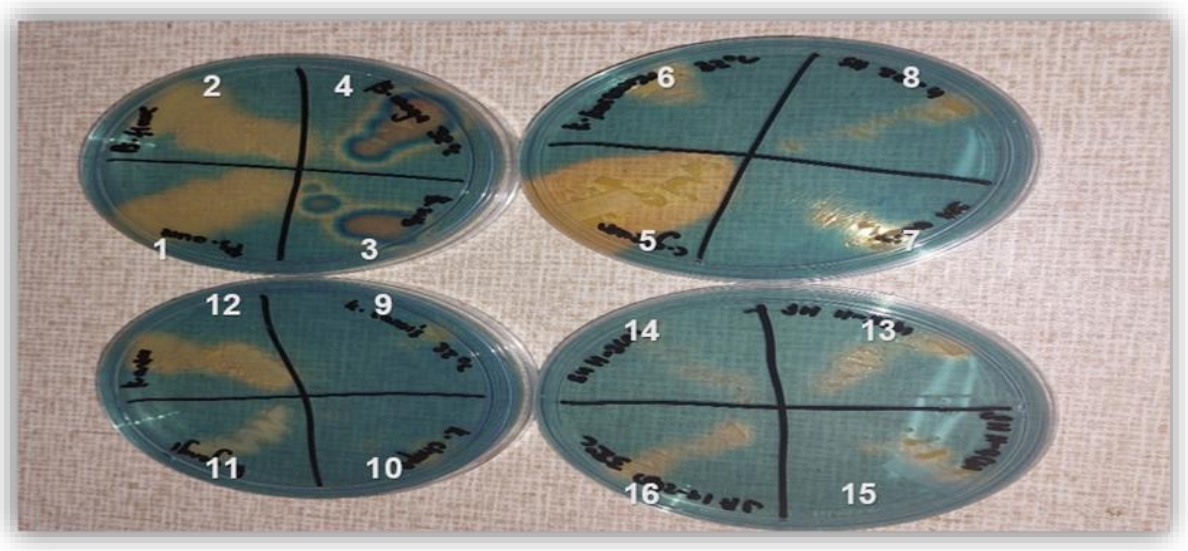

Figure 4.2: Production of siderophores by the six *Kaistella* fish isolates [SH 11-3(a) (14), SH 11-3(b) (13), '*K. merluccii'* SH 11-4(b) (15), '*K. piscis*' SH 19-2(b) (16), '*K. frigidipiscis*' SH 20-4 (8) and SH 40-3 (7)] and reference strains [*C. gleum* (5)*, K. koreensis* (6)*, K. carnis* (9), *K. chaponensis* (10), *K. antarctica* (12)*, K. yonginensis* (11)*, B. subtilis* (3)*, B. megaterium* (4)*, P. aeruginosa* (1) and *P. fluorescens* (2)] at 32 °C.

At 37 °C, all the *Chryseobacterium* and *Kaistella* strains formed visible siderophores at 25 °C [*C. gleum* (strain 5), *K. koreensis* (strain 6), '*K. frigidipiscis*' SH 40-3 (strain 7), *K. antarctica* (strain 12), '*K. merluccii*' SH 11-4(b) (strain 15) and '*K. piscis*' SH 19-2(b) (strain 16)], were adversely affected (Fig. 4.3). The secretion of siderophores from these organisms was less enhanced than at 25 °C, with '*K. frigidipiscis*' SH 40-3 (strain 7) and '*K. merluccii*' SH 11-4(b) (strain 15), being the most affected.

Therefore, it was possible to conclude that 25 °C was the temperature most of the organisms in this study preferred when comparing the total siderophore production by all organisms at different temperatures. This conclusion can be explained by the fact that 25 °C is the optimal growth temperature for most organisms used in this study. Although strains that were unable

to secrete siderophores at 25 °C were marginally able to do so at 37 °C, the ability of *C. gleum* to produce siderophores was adversely impacted (Fig. 4.3).

All six *Kaistella* test strains shared the ability to secrete siderophores at the three temperatures, although their siderophore production was not as pronounced as that of *C. gleum*. *Chryseobacterium gleum* will indeed outcompete its host for iron uptake in low-iron settings.

The production of siderophores by the four positive controls used in this study, *B. subtilis, B. megaterium, P. aeruginosa,* and *P. fluorescens*, was consistent at all three temperatures, as previously indicated. This is because, according to LaBauve and Wargo (2012), the optimal growth temperature range for *Bacillus* species is between 25 and 37 °C, while *Pseudomonas* species have a comprehensive growth temperature range from 4 to 42 °C but also grow well between 25 and 37 °C (Sidorova et al. 2020). The *Bacillus* species also produced siderophores in a stable and considerable amount, which was following Ferreira and colleagues (2019). The production of siderophores on CAS agar by *Pseudomonas* species following previous findings (Sayyed and Patel 2011, Peek et al. 2012, Kotasthane et al. 2017).

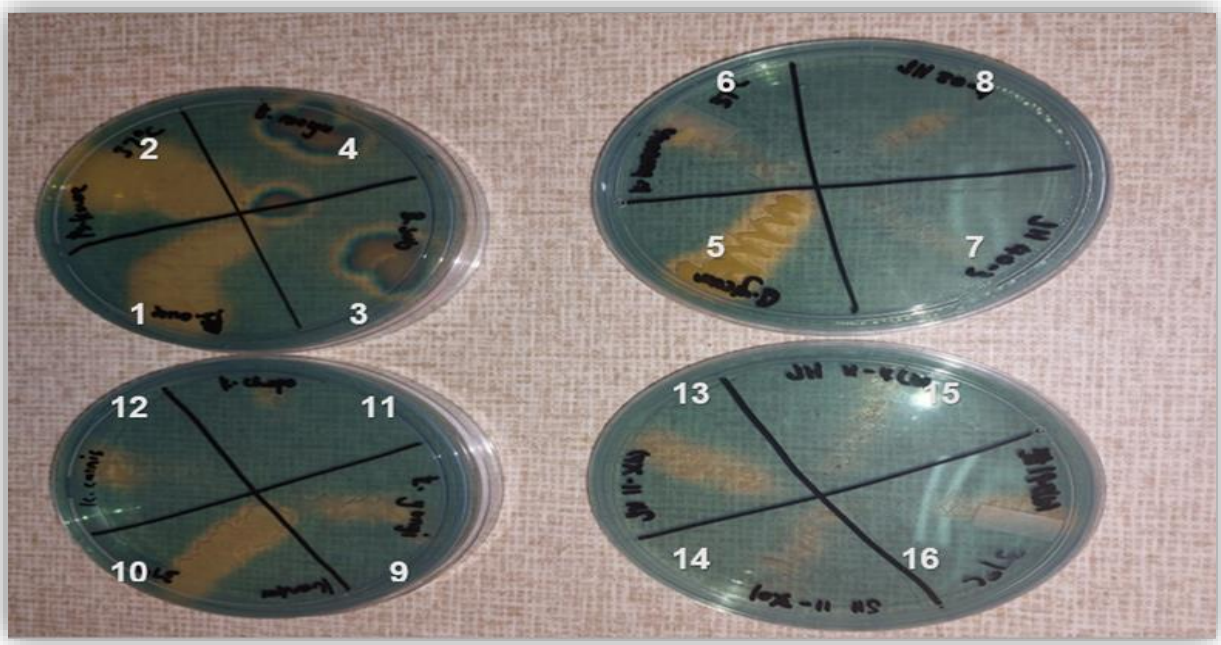

Figure 4.3: Siderophore production by the six *Kaistella* fish isolates [SH 11-3(a) (14), SH 11-3(b) (16), '*K. merluccii'* SH 11-4(b) (15), '*K. piscis*' SH 19-2(b) (16), '*K. frigidipiscis*' SH 20-4 (8) and SH 40-3 (7)] and reference strains [*C. gleum* (5)*, K. koreensis* (6)*, K. carnis* (12), *K. chaponensis* (11), *K. antarctica* (10)*, K. yonginensis* (9)*, B. subtilis* (3)*, B. megaterium* (4)*, P. aeruginosa* (1), and *P. fluorescens* (2)] at 37 °C.

Iron (Fe) chelation and biofilm formation are related because Fe is necessary for biofilm development and does so by regulating surface mobility and stabilizing the polysaccharide matrix (Weinberg 2004; Chhibber et al. 2013). According to Simões et al. (2007), microbial surface hydrophobicity diminishes under iron-deficient growth circumstances, changing the surface protein composition and limiting biofilm formation.

4.3.2. Enzyme activity

The qualitative (absence/presence) production of lecithinase, chondroitinase, hyaluronidase, and fibrinolysin enzymes, is presented in Table 4.4. Lecithinase was produced by 42% (5/12) strains ('*K. merluccii*' SH 11-4(b), '*K. piscis*' SH 19-2(b), '*K. frigidipiscis*' SH 40-3, *C. gleum* and *K. antarctica*) evaluated in this study. Lecithinases punch holes through or break down cell membranes, promoting the destruction of the targeted cell and/or increasing cellular invasion (Madigan et al. 2000, Zachary 2017).

Only 16% (2/12) (*C. gleum* and *K. antarctica)* of the strains produced the enzyme chondroitinase. None of the six *Kaistella* fish isolates shared this trait with *C. gleum* and *K. antarctica*. Hyaluronidase activity was observed in 42% (5/12) of the organisms, including *C. gleum, K. koreensis, K. antarctica,* '*K. merluccii*' SH 11-4 (b) and SH 20-4 (Table 4.4). Microbial hyaluronidase and chondroitinase have been studied because of their possible association with microbial mechanisms of pathogenicity (Smith and Willett 1968). Chondroitinase is a proposed virulence factor that may enhance pathogen invasion by degrading host tissues' structural components (i.e., cartilage) (Kunttu et al. 2011). Hyaluronidase is an enzyme that depolymerizes hyaluronic acid, which is a component of the ground substance of the mucoprotein and thus increases membrane permeability, reduces viscosity, and makes tissues more readily permeable (Weber et al. 2019). Therefore, organisms with this trait can overcome the measures put in place by their potential host to restrict entry by inducing membrane impermeability. Some bacteria have also been shown to use hyaluronan as a carbon source for growth and replication (Zachary 2017).

The enzyme activity assay was performed on 12 strains, but none of them could produce fibrinolysin. The coagulation system works with the inflammatory system to eliminate the microorganisms that cause bacterial infections (Loof et al. 2014). However, pathogenic bacteria have developed strategies to take advantage of elements of the haemostatic system for their gain, including fibrinolysin secretion (Loof et al. 2014). The proteolytic enzyme fibrinolysin, commonly known as plasmin, catalyses fibrin breakdown, a protein in blood clotting (Chapin and Hajjar 2015). Since clot formation also serves as a barrier against pathogen invasion, the analysis of this protein allows access and spread of the pathogen.

Thus, it was concluded that the strains in this study would have to use other mechanisms to invade their host since they could not produce this enzyme.

It was possible to calculate the production of gelatinase, lipase, proteinase, and DNase quantitively by determining the Z-scores (Table 4.4). A Z-score is a statistical measurement that describes a value's relationship to the mean of a group of values. The lower the value of the Z-score, the higher the production of the relevant enzyme. A Z-score of < 0.5 indicated high enzyme production (Mwanza et al. 2022). A Z-score of 1.00 was an indication that almost no enzyme was produced. The 12 strains evaluated in this study were able to produce gelatinase, lipase, proteinase, and DNase. The SH 40-3 *Kaistella* strain (0.35 ± 0.03) and *C. gleum* (0.33 ± 0.05) produced significantly ($p = 0.005$) more gelatinase than the rest of the 12 strains in this study, and *K. yonginensis* had the significantly ($p = 0.005$) lowest amount (0.55 ± 0.06). Gelatinases hydrolyse collagen, casein, haemoglobin, and other peptides (Coque et al. 1995). Gelatinase expression has been linked to enhanced biofilm formation (Wang et al. 2011, Guneser and Eldeniz 2016). Therefore, gelatinase secretion by these organisms means that they can disrupt and hydrolyse essential proteins.

Table 4.4: Qualitative (absence/presence) and quantitative (mean Z-score values ± standard deviation, n = 6) virulence factors (siderophore production, enzyme production, and haemolysis activity) of the six *Kaistella* fish isolates and six *Kaistella* reference strains in this study. (++), extremely positive; (+), positive; (-) negative; (w), weakly positive; N/A, not analysed. The means with different superscripts in the same column differed significantly.

Treatment	Siderophore	Lecithinase	Chondroitinase	Hyaluronidase	Fibrinolysin	Gelatinase	Lipase	Proteinase	DNase	β-Haemolysis
SH 11-3(a)	+	-	-	-	-	$0.38^{ab} \pm 0.06$	$0.60^{a} \pm 0.01$	$0.57^{ab} \pm 0.09$	$1.00^{d} \pm 0.01$	$0.46^{ab} \pm 0.01$
SH 11-3(b)	+	-	-	-	-	$0.39^{ab} \pm 0.10$	$0.56^{a} \pm 0.01$	$0.53^{ab} \pm 0.02$	$0.93^{d} \pm 0.12$	$0.52^{ab} \pm 0.04$
SH 11-4(b)	+	+	-	+	-	$0.38^{ab} \pm 0.06$	$0.55^{a} \pm 0.01$	$0.49^{ab} \pm 0.03$	$0.60^{bc} \pm 0.05$	$0.94^{c} \pm 0.10$
SH 19-2(b)	+	+	-	-	-	$0.42^{ab} \pm 0.10$	$1.00^{b} \pm 0.01$	$0.54^{ab} \pm 0.04$	$0.54^{abc} \pm 0.04$	$0.96^{c} \pm 0.08$
SH 20-4	+	-	-	+	-	$0.40^{ab} \pm 0.02$	$1.00^{b} \pm 0.01$	$0.58^{ab} \pm 0.09$	$1.00^{d} \pm 0.01$	$0.59^{b} \pm 0.08$
SH 40-3	+	+	-	-	-	$0.35^{a} \pm 0.03$	$0.59^{a} \pm 0.01$	$0.55^{ab} \pm 0.05$	$0.43^{a} \pm 0.02$	$0.52^{ab} \pm 0.04$
C. gleum	++	+	+	+	-	$0.33^{a} \pm 0.05$	$0.45^{a} \pm 0.04$	$0.44^{a} \pm 0.02$	$0.42^{a} \pm 0.04$	$0.41^{a} \pm 0.04$
K. koreensis	+	-	-	+	-	$0.47^{ab} \pm 0.07$	$0.63^{a} \pm 0.03$	$0.63^{b} \pm 0.03$	$0.94^{d} \pm 0.10$	$0.52^{ab} \pm 0.02$
K. carnis	w	-	-	-	-	$0.45^{ab} \pm 0.02$	$0.61^{a} \pm 0.04$	$0.56^{ab} \pm 0.03$	$1.00^{d} \pm 0.01$	$1.00^{c} \pm 0.01$
K. chaponensis	w	-	-	-	-	$0.49^{ab} \pm 0.05$	$0.89^{b} \pm 0.19$	$0.60^{b} \pm 0.09$	$0.68^{c} \pm 0.06$	$0.94^{c} \pm 0.10$
K. antarctica	+	+	+	+	-	$0.38^{ab} \pm 0.04$	$0.47^{a} \pm 0.02$	$0.56^{ab} \pm 0.02$	$0.45^{ab} \pm 0.03$	$0.43^{ab} \pm 0.02$
K. yonginensis	w	-	-	-	-	$0.55^{b} \pm 0.06$	$1.00^{b} \pm 0.01$	$0.62^{b} \pm 0.03$	$1.00^{d} \pm 0.01$	$1.00^{c} \pm 0.01$
Significance	N/A	N/A	N/A	N/A	N/A	$p = 0.005$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$

With lipase and proteinase production, only *C. gleum* (0.45 ± 0.04 and 0.44 ± 0.04, respectively) and *K. antarctica* (0.47 ± 0.02 for lipase production) produced these enzymes significantly ($p < 0.001$ for both) more than the rest of the strains in this study (Table 4.4). '*Kaistella piscis*' SH 19-2(b), SH 20-4, *K. chaponensis* and *K. yonginensis* produced the significantly ($p < 0.001$) least lipase, while *K. koreensis, K. chaponensis* and *K. yonginensis* produced the significantly ($p < 0.01$) least proteinase. The breakdown of biological components, particularly lipids, by lipases and other enzymes aids in the survival of the bacterium in the host by freeing up components necessary for the survival of the bacterium (Madigan et al. 2000). Proteinases, which are enzymes that hydrolyze peptide bonds and thus have the potential to degrade proteins and peptides that participate in a wide range of biological functions, including infection, are also essential candidates for virulence factors (Silva-Almeida et al. 2012). Proteinases are found throughout biological systems and perform various functions, ranging from protein digestion for nutritional purposes to exquisite control of protein function via hydrolysis of peptide bonds in a protein substrate (Barret 1994).

'*Kaistella frigidipiscis*' SH 40-3 (0.43 ± 0.02) was the only *Kaistella* test isolate that showed significant ($p < 0.001$) DNase activity and shared this characteristic with *C. gleum* (0.42 ± 0.04) and *K. antartica* (0.45 ± 0.03). Many pathogenic bacteria produce extracellular DNase. DNase secretion has been proposed to promote growth by increasing the pool of accessible nucleotides by DNA hydrolysis (Fox and Holtman 1968). Additionally, it has been suggested that extracellular DNase activity facilitates the dissemination and transmission of infectious microorganisms by liquifying pus (Sherry and Goeller 1987, Sumby et al. 2005).

In the category of enzyme production by all organisms used in this study, gelatinase was the most significantly produced (11/12), followed by lecithinase and hyaluronidase, which were secreted by 42% (5/12) of the tested organisms. Bacterial virulence is determined in part by the type and number of factors the bacterium expresses to complete its life cycle successfully in a host (Zachary 2017). In general, virulence factors are encoded by more than one bacterial gene. As previously indicated, *C. gleum* can be regarded as a hyper-virulent organism because it was able to synthesize seven of the eight enzymes that were investigated due to their capacity to act as virulence factors and shared this ability with *K. antarctica*. '*Kaistella merluccii*' SH 11-4(b), which produced more than 4/8 virulence enzymes, may be considered another potential pathogen. Other organisms that could synthesize more than two enzymes required for pathogen categorization (Zachary 2017) included *K. koreensis* (4/8), '*K. frigidipiscis*' SH 40-3 (3/8), SH 20-4 (3/8), '*K. piscis*' SH 19-2(b) (3/8), and SH 11-3(a) (3/8). The inability of *K. carnis* and *K. yonginensis* to significantly secrete more than two virulence-related enzymes indicated that these two strains should not be considered pathogens.

4.3.3. Blood Haemolysis

All organisms used in this study exhibited β-haemolysis (Table 4.4). Some of the tested bacterial strains ('*K. merluccii*' SH 11-4(b) and *C. gleum*) were able to display both the α- and β-haemolysis (Fig. 4.4). Alpha-haemolysis is the reduction of red blood cell haemoglobin to methaemoglobin in the medium surrounding the colony (Buxton 2005). It causes a green or brown discolouration in the medium, while β-haemolysis (β) results in the complete or accurate lysis of red blood cells. A clear zone, approaching the colour and transparency of the base medium, surrounds the colony (Buxton 2005).

Chryseobacterium gleum (0.41 ± 0.04) produced significantly ($p < 0.001$) the most β-haemolysis, while '*K. merluccii*' SH 11-4(b) (0.94 ± 0.10), '*K. piscis*' SH 19-2(b) (0.96 ± 0.06), *K. carnis* (1.00 ± 0.01), *K. chaponensis* (0.94 ± 0.10) and *K. yonginensis* (1.00 ± 0.01) produced significantly ($p < 0.001$) the least β-haemolysis (Table 4.4).

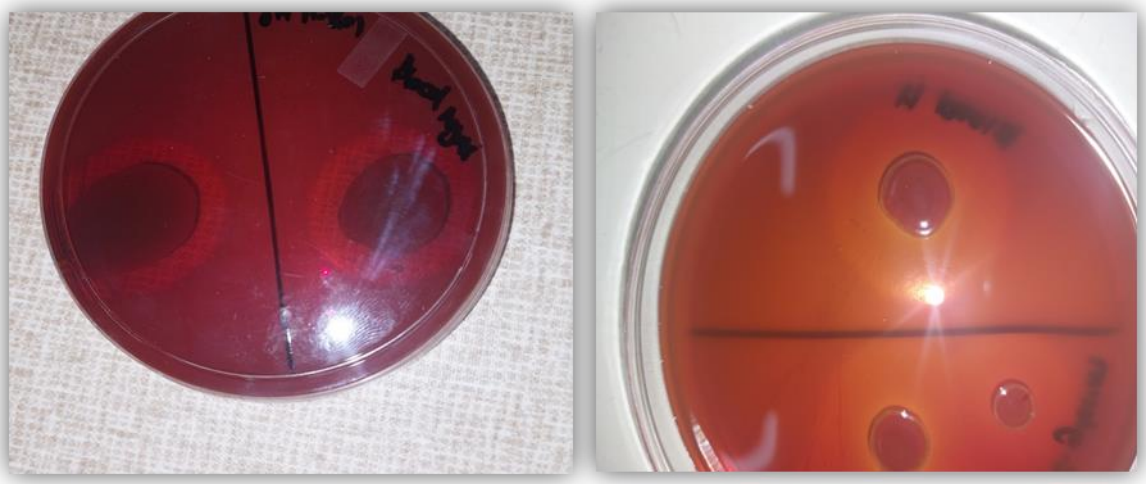

Figure 4.4: *'Kaistella merluccii'* SH 11-4(b) β-haemolysis in the picture on the left and both α- and β-haemolysis of *Chryseobacterium gleum* on the blood agar plate on the right.

4.3.4. Antimicrobial resistance

Chryseobacterium and *Kaistella* are two genera linked with antibiotic resistance (Vandamme et al. 1994, Kämpfer et al. 2009, Peng et al. 2021). Antimicrobial drugs were discovered and widely used to prevent the spread of diseases for a long time; however, this was before antimicrobial resistance emerged globally (Beceiro et al. 2013). The primary concern in veterinary medicine and human health is resistance to antimicrobial agents. The Kirby-Bauer disc diffusion susceptibility method determined if the *Kaistella* and *Chryseobacterium* species in this study were susceptible to or resistant to various antimicrobial substances. The results are shown in Table 4.5.

The antimicrobial disk inhibition zones were measured, and the test organism's susceptibility or resistance to the particular antibiotic was reported. The criteria to be considered resistant, intermediate, or susceptible are listed in Table 4.3. The resistance/susceptibility profiles of the organisms were also expressed as Z-scores and indicated in Table 4.6.

All organisms used in this investigation were resistant to ampicillin (10 µg), oxacillin, cephalothin, and streptomycin (10 µg) (Table 4.5). There were no significant differences in their mean Z-scores (Table 4.6). Organisms with a higher Z-score displayed higher antibiotic resistance, while those with a lower Z-score displayed antibiotic susceptibility (Table 4.6).

All organisms evaluated in this study were susceptible to piperacillin, cefepime, cefotaxime, and levofloxacin (Table 4.5). However, there were significant differences in the susceptibility of the organisms to these antimicrobials (Table 4.6). For piperacillin, *K. koreensis* was significantly ($p < 0.006$) more susceptible (Z-score of 0.15) than *C. gleum* (Z-score of 0.19). For cefepime, '*K. frigidipiscis*' SH 40-3 was significantly ($p < 0.001$) more susceptible (Z-score of 0.14) than '*K. piscis*' SH 19-2(b), *C. gleum, K. koreensis, K. carnis* and *K. chaponensis* (Z-scores between 0.18 and 0.19). For levofloxacin, strains SH 11-3(b) and '*K. merluccii*' SH 11-4(b) were significantly ($p < 0.001$) more susceptible (Z-scores of 0.16 for both) than *K. chaponensis* and *K. antarctica* (Z-scores of 0.21 for both) (Table 4.6). For cefotaxime, however, there were no significant differences between the organisms. Both cefepime and cefotaxime belong to the cephem antimicrobial group, which hinders the ability of bacterial strains to synthesize cell walls by creating substances that bind to cell membranes (Reygaert 2018).

Most organisms in this investigation were susceptible to ampicillin (25 µg) even though no CSLI standards are available (Table 4.5). *Chryseobacterium gleum* was significantly ($p < 0.001$) more sensitive to 25 µg ampicillin (Z-score of 0.19) than strains SH 11-3(a) and SH 11-3(b) (Z-scores of 0.29 and 0.26, respectively) (Table 4.6).

Table 4.5: Resistance and susceptibility patterns of the six *Kaistella* fish isolates and six reference strains to various antimicrobials according to the CLSI standards presented in Table 4.3. R, resistant, I, intermediate, S, susceptible, -, not reported.

Antimicrobial compound	**Concentration (µg)**	**SH 11-3(a)**	**SH 11-3(b)**	**'*K. merluccii*' SH 11-4(b)**	**'*K. piscis*' SH 19-2(b)**	**SH 20-4**	**'*K. frigidipiscis*' SH 40-3**	***C. gleum***	***K. koreensis***	***K. carnis***	***K. chaponensis***	***K. antarctica***	***K. yonginensis***
Ampicillin	10	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin	25	-	-	-	-	-	-	-	-	-	-	-	-
Oxacillin	1	R	R	R	R	R	R	R	R	R	R	R	R
Piperacillin	75	S	S	S	S	S	S	S	S	S	S	S	S
Cephalothin	30	R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline	30	-	-	-	-	-	-	-	-	-	-	-	-
Tetracycline	30	S	S	S	S	S	S	I	I	S	S	I	I
Gentamycin	10	R	R	I	I	R	R	R	R	S	S	R	I
Kanamycin	30	R	R	R	R	R	R	R	I	I	I	I	R
Streptomycin	10	R	R	R	R	R	R	R	R	R	R	R	R
Streptomycin	25	-	-	-	-	-	-	-	-	-	-	-	-
Cefepime	30	S	S	S	S	S	S	S	S	S	S	S	S
Cefotaxime	30	I	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	1	-	-	-	-	-	-	-	-	-	-	-	-
Levofloxacin	5	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	10	I	I	I	R	I	R	I	S	I	I	I	I
Vancomycin	30	S	S	I	I	S	S	R	S	S	I	R	R

Although no CSLI standards are available for oxytetracycline (Table 4.5), *'K. frigidipiscis'* SH 40-3, *C. gleum, K. carnis, K. chaponensis, K. antartica* and *K. yonginensis* were significantly ($p < 0.001$) more resistant to 30 µg oxytetracycline (Z-scores of 0.29 to 0.32) than strains SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b), '*K. piscis*' SH 19-2(b), SH 20-4 and *K. koreensis* (Z-scores of 0.16 to 0.20)(Table 4.6). For tetracycline, most of the organisms evaluated in this study were susceptible to this antibiotic (Table 4.5). However, *C. gleum* and *K. koreensis* were significantly ($p < 0.001$) more resistant (Z-scores of 0.29 and 0.31, respectively) than strains SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b), and '*K. frigidipiscis*' SH 40-3 (Z-scores between 0.13 and 0.18) (Table 4.6).

Table 4.6: Resistance/susceptibility patterns of the six isolates of *Kaistella* fish and the six reference strains used in this study. Values are the Z-scores of each antibiotic. The means with different superscripts in the same column differed significantly.

Sample name	Ampicillin (10 µg)	Ampicillin (25 µg)	Oxacillin (1 µg)	Piperacillin (75 µg)	Cephalothin (30 µg)	Oxytetracycline (30 µg)	Tetracycline (30 µg)	Gentamycin (10 µg)	Kanamycin (30 µg)	Streptomycin (10 µg)	Streptomycin (25 µg)	Cefepime (30 µg)	Cefotaxime (30 µg)	Ciproflaxin (1 µg)	Levofloxacilin (5 µg)	Erythromycin (10 µg)	Vancomycin (30 µg)
SH 11-3(a)	1.00^{a}	0.29^{c}	1.00^{a}	0.18^{abc}	1.00^{a}	0.19^{a}	0.18^{ab}	0.32^{abcd}	0.41^{cd}	1.00^{a}	0.29^{c}	0.16^{abc}	0.19^{a}	0.32^{d}	0.17^{ab}	0.25^{ab}	0.25^{bcd}
SH 11-3(b)	1.00^{a}	0.26^{bc}	1.00^{a}	0.17^{abc}	1.00^{a}	0.17^{a}	0.13^{a}	0.34^{abcde}	0.39^{abcd}	1.00^{a}	0.25^{abc}	0.17^{abcd}	0.13^{a}	0.28^{cd}	0.16^{a}	0.25^{abc}	0.23^{bcd}
SH 11-4(b)	1.00^{a}	0.24^{abc}	1.00^{a}	0.17^{abc}	1.00^{a}	0.17^{a}	0.14^{a}	0.29^{ab}	0.42^{d}	1.00^{a}	0.21^{ab}	0.17^{abcd}	0.17^{a}	0.18^{a}	0.16^{a}	0.23^{ab}	0.26^{abcd}
SH 19-2(b)	1.00^{a}	0.22^{ab}	1.00^{a}	0.17^{abc}	1.00^{a}	0.18^{a}	0.22^{abcd}	0.30^{abc}	0.34^{abcd}	1.00^{a}	0.28^{bc}	0.18^{cd}	0.17^{a}	0.29^{cd}	0.18^{abc}	0.31^{bc}	0.27^{cde}
SH 20-4	1.00^{a}	0.22^{ab}	1.00^{a}	0.17^{abc}	1.00^{a}	0.20^{a}	0.21^{abc}	0.42^{e}	0.34^{abcd}	1.00^{a}	0.27^{abc}	0.15^{ab}	0.17^{a}	0.28^{bcd}	0.17^{abc}	0.28^{abc}	0.22^{de}
SH 40-3	1.00^{a}	0.22^{ab}	1.00^{a}	0.15^{ab}	1.00^{a}	0.30^{b}	0.19^{ab}	0.40^{de}	0.31^{abc}	1.00^{a}	0.32^{c}	0.14^{a}	0.16^{a}	0.27^{bc}	0.19^{abc}	0.34^{c}	0.20^{abcd}
C. gleum	1.00^{a}	0.19^{a}	1.00^{a}	0.19^{c}	1.00^{a}	0.32^{b}	0.29^{cd}	0.38^{bcde}	0.41^{bcd}	1.00^{a}	0.30^{c}	0.18^{cd}	0.18^{a}	0.31^{cd}	0.18^{abc}	0.30^{bc}	0.31^{e}
K. koreensis	1.00^{a}	0.21^{ab}	1.00^{a}	0.15^{a}	1.00^{a}	0.16^{a}	0.31^{d}	0.38^{bcde}	0.29^{a}	1.00^{a}	0.25^{abc}	0.18^{cd}	0.15^{a}	0.27^{bc}	0.20^{bc}	0.24^{ab}	0.20^{a}
K. carnis	1.00^{a}	0.21^{ab}	1.00^{a}	0.19^{bc}	1.00^{a}	0.30^{b}	0.27^{bcd}	0.27^{a}	0.30^{a}	1.00^{a}	0.27^{abc}	0.19^{d}	0.18^{a}	0.23^{ab}	0.20^{bc}	0.20^{a}	0.21^{abc}
K. chaponensis	1.00^{a}	0.23^{abc}	1.00^{a}	0.17^{abc}	1.00^{a}	0.32^{b}	0.21^{abcd}	0.26^{a}	0.30^{a}	1.00^{a}	0.20^{a}	0.17^{bcd}	0.17^{a}	0.32^{d}	0.21^{c}	0.25^{abc}	0.27^{de}
K. antarctica	1.00^{a}	0.23^{abc}	1.00^{a}	0.18^{abc}	1.00^{a}	0.32^{b}	0.28^{bcd}	0.33^{abcd}	0.30^{ab}	1.00^{a}	0.29^{c}	0.17^{abcd}	0.18^{a}	0.29^{cd}	0.21^{c}	0.30^{bc}	0.31^{e}
K. yonginensis	1.00^{a}	0.21^{ab}	1.00^{a}	0.19^{bc}	1.00^{a}	0.29^{b}	0.28^{bcd}	0.30^{abc}	0.33^{abcd}	1.00^{a}	0.28^{bc}	0.16^{abc}	0.18^{a}	0.27^{bc}	0.18^{abc}	0.25^{abc}	0.32^{e}
Significance	p=0.474	p<0.001	p=0.474	p=0.006	p=0.474	p<0.001	p<0.001	p<0.001	p<0.001	p=0.474	p<0.001	p<0.001	p=0.520	p<0.001	p<0.001	p<0.001	p<0.001

Oxytetracycline and tetracycline belong to the tetracycline antimicrobial group (Table 4.2), which exerts its bacteriostatic activity by preventing protein synthesis in bacteria (Chopra and Roberts 2001).

The aminoglycoside group of antibiotics evaluated in this study consisted of gentamycin, kanamycin, and streptomycin. Most of the organisms used in this study were resistant to antibiotics in this group, and all organisms were resistant to 10 µg streptomycin (Table 4.5). The exceptions were *K. carnis* and *K. chaponensis*, which were significantly ($p < 0.001$) susceptible to gentamycin with Z-scores of 0.27 and 0.26, respectively; *K. koreensis, K. carnis, K. chaponensis* and *K. antarctica*, which were significantly ($p < 0.001$) intermediate susceptible to kanamycin (Z-scores from 0.29 to 0.30); and *K. chaponensis* and '*K. merluccii*' SH 11-4(b), which were significantly ($p < 0.001$) susceptible to 25 µg streptomycin (Z-scores of 0.21 and 0.20, respectively)(Table 4.6). Aminoglycosides are solid bactericidal antibiotics that block bacterial genes, bind to the 30S or 50S ribosomal subunits, and prevent translocation of the peptidyl-tRNA from the A-site to the P-site, and causing mRNA misreading (Kotra et al. 2000, Dowling et al. 2017, Ullah et al. 2017). Although members of the aminoglycoside class have different specificities for different regions of the A-site, their attachment results in its structural conformation (Krause et al. 2016).

There were no CLSI standards available for ciprofloxacin (Table 4.5); however, strains SH 11-3(a), SH 11-3(b), '*K. piscis*' SH 19-2(b), *C. gleum, K. chaponensis* and *K. antarctica* were significantly ($p < 0.001$) more resistant than the rest of the strains evaluated in this study with Z-scores between 0.28 and 0.32 (Table 4.6). '*Kaistella merluccii*' SH 11-4(b) was significantly ($p < 0.001$) the most susceptible to ciprofloxacin, with a Z-score of 0.18 (Table 4.6). As already mentioned, all strains in this study were sensitive to levofloxacin. Ciprofloxacin and levofloxacin belong to the fluoroquinolone antimicrobial group (Table 4.2) and block bacterial replication using efflux pumps as a defence mechanism (Dowling et al. 2017, Ullah et al. 2017).

Most (9/12) of the organisms in this study were intermediately susceptible to erythromycin (Table 4.5). The strains '*K. piscis*' SH 19-2(b) and '*K. frigidipiscis*' SH 40-3 were the only two strains that were significantly ($p < 0.001$) resistant to erythromycin (Z-scores of 0.31 and 0.34, respectively) (Table 4.6). *Kaistella carnis* was significantly ($p < 0.001$) inhibited by erythromycin since it had the significantly lowest Z-score (0. 20) of all organisms in this study (Table 4.6). Erythromycin belongs to the macrolide antimicrobial group (Table 4.2). The macrolides class of antibiotics inhibit bacterial protein synthesis by blocking DNA gyrase binding (Mazzei et al. 1993, Dowling et al. 2017, Ullah et al. 2017).

Chryseobacterium gleum, K. antarctica and *K. yonginensis* were significantly ($p < 0.001$) resistant to vancomycin with Z-scores of 0.31, 0.31, and 0.32, respectively (Tables 4.5 and 4.6). *Kaistella koreensis* and *K. carnis* were significantly ($p < 0.001$) the most susceptible to vancomycin due to their significantly lowest Z-scores of 0.20 and 0.21, respectively (Table 4.6). Vancomycin belongs to the glycopeptide antimicrobial group (Table 4.2). Glycopeptides inhibit cell wall synthesis in Gram-positive bacteria. Still, they are relatively ineffective against Gram-negative bacteria because they cannot penetrate the outer membrane barrier due to their large size and high molecular weight (Antonoplis et al. 2019, Selim 2022). However, significant progress has been made in improving antibiotic efficacy against Gram-negative bacteria by modifying antibiotics and conjunction. Antonoplis and colleagues (2019), for instance, discovered that the covalent attachment of a single arginine to vancomycin demonstrated efficacy against actively growing Gram-negative bacteria.

Together with *C. gleum* and *K. antarctica*, strains SH 11-3(a) and '*K. frigidipiscis*' SH 40-3 demonstrated resistance to 9 of the 17 antimicrobials used in this study, omitting those to which they also demonstrated partial or intermediate resistance. In a study conducted by Peng et al. (2021), *Kaistella antartica* also showed resistance to most antibiotics. Therefore, since only antimicrobials from these classes were 100% effective against all organisms utilized in this investigation, it followed that relatively few antimicrobials from the cephem and fluoroquinolone classes would be able to suppress these organisms during infection. *Kaistella yonginensis* was the most sensitive organism because it was susceptible to four antimicrobials despite showing some resistance to nine antimicrobials.

4.4. Conclusions

Although members of the genus *Kaistella* have been isolated from clinical, environmental, industrial, and food sources, little is known about their virulence traits. The goal of this investigation was to determine the virulence characteristics of six *Kaistella* strains which were isolated from fresh marine fish. The virulence factors of the organisms were assessed through tests on the resistance to antibiotics, haemolysis, enzyme activity, and ability to produce siderophores.

All the examined bacterial strains could produce siderophores; however, the amount produced varied depending on the specific strain's temperature and optimum growth temperature. Siderophore production was more pronounced at 25 °C and less at 37 °C. Compared to all the other organisms used in this investigation, *C. gleum* produced more significant siderophores, and its ability to produce many iron chelators makes it hypervirulent.

In most cases, more than one bacterial gene codes for virulence factors. The kind and quantity of components that a bacterium produces to complete its life cycle in an animal effectively determine, in part, its pathogenicity. Since *C. gleum*, as previously noted, could produce 7/8 enzymes and shared this property with *K. antarctica*, '*K. merluccii*' SH 11-4(b) could be added to the list of potential pathogens, as it produced more than four enzymes.

Antimicrobials that significantly inhibited all 12 bacterial strains included cefotaxime (30 μg), cefepime (30 μg), levofloxacin (5 μg), and piperacillin (75 μg), while cephalothin (30 μg), ampicillin (10 μg), streptomycin (10 μg) and oxacillin (1 μg), all belonging to the penicillin class, could not inhibit any of the organisms used in this study. The strains SH 11-3(a) and '*K. frigidipiscis*' SH 40-3, together with *C. gleum* and *K. antarctica,* showed resistance to most of the antimicrobials used in this study. Antimicrobials belonging to the cephem, and fluoroquinolone classes must be considered when treating infections caused by these organisms since they showed 100% bactericidal activity against all the microorganisms used in this study.

CHAPTER 5

BIOFILM FORMATION ABILITY OF *Kaistella* FISH ISOLATES AND THE EFFICACY OF ANTI-BIOFILM COMPOUNDS

Abstract

Complex sessile microbial communities, or biofilms, can be firmly embedded in an extracellular matrix as aggregates or adhere to a surface. Biofilm formation promotes pathogenicity, making bacteria more resilient to antibiotics and antimicrobial substances. The first purpose of this study was to examine the potential for biofilm formation in six *Kaistella* fish isolates used in Chapter 4. The tube method (TM), the tissue culture plate (TCP) and Congo red agar (CRA) methods were used to identify biofilm development. The TCP method was regarded as the best method for determining biofilm formation. Although all the organisms evaluated in this study could form biofilms, according to the TCP method, *Kaistella* strain SH 20-4, *K. carnis* and *K. antarctica* were regarded as strong biofilm producers.

The second purpose was to evaluate the effectiveness of five anti-biofilm compounds (fucoidan, proteinase K, EDTA, D-leucine, and D-glucose) in preventing biofilm formation at minimum inhibitory concentrations (MIC) and sub-MICs by using the crystal violet staining assay. Glucose was the compound most efficient at inhibiting the *Kaistella* test and reference strains used in this investigation. Some organisms were also inhibited at sub-MIC levels, and significant inhibition was observed at the MIC 50 mM and 100 mM. The lowest inhibition index was found in *K. antarctica* (26.5%), *P. aeruginosa* (34.1%) and SH 20-4 (34.7%), while the highest inhibition index was found in *C. gleum (*81.7%), *K. yonginensis* (71.1%), *K. koreensis* (65.8%) and *K. carnis* (61.0%). The least effective substance was proteinase K, with a percentage inhibition range of 21.2% to 34.7%. Strains SH 20-4, '*K. frigidipiscis*' SH 40-3, *K. antarctica* and *P. aeruginosa* were among the organisms whose biofilms showed the most outstanding resilience to inhibitory chemicals, while the vulnerability was mainly seen in SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b), and '*K. piscis*' SH 19-2(b) shared this trait with *C. gleum, K. koreensis,* and *K. chaponensis*.

5.1. Introduction

The term biofilm refers to the complex communities of microbes that may be found attached to a surface or form aggregates without adhering to a surface (Bjarnsholt et al. 2009, Hassan et al. 2011, Haaber et al. 2012). The biofilm lifestyle allows bacteria to withstand hostile environmental conditions, such as starvation and desiccation (Roy et al. 2018).

In addition to the protection offered by the matrix, biofilms provide resistance to many antimicrobials and protection from protozoan grazing. Bacteria in biofilms can use various survival strategies to overcome the host defence mechanisms and are also responsible for the spoilage of food products in food processing industries (Matz and Kjelleberg 2005, López et al. 2010, Vestby et al. 2020). Susceptibility tests with *in vitro* biofilm models have shown the survival of bacterial biofilms after treatment with antibiotics at hundreds or even a thousand times the minimum inhibitory concentration of the bacteria measured in suspension culture (Cery et al. 1999). *In vivo*, antibiotics might suppress symptoms of infection by killing free-floating bacteria shed from the attached population but fail to eradicate those bacterial cells still embedded in the biofilm (Stewart and Costerton 2001). Therefore, high antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antimicrobial resistance can increase 1,000-fold (Stewart and Costerton 2001).

Temperature plays an essential role in an organism's ability to induce biofilm formation (Olson et al. 2002). Environmental factors have an effect not only on development and sustainability, but also on virulence. An unfavourable temperature may affect the transcriptional levels of some of the essential general stress genes (*rpo*S, *rpo*H), thus affecting an organism's stress response during biofilm formation (Bezek et al. 2019, Roy et al. 2021).

Biofilms can contain spoilage and pathogenic organisms that increase post-processing contamination and risk to public health and are more resistant to cleaning and decontamination procedures in the food industry (Shi and Zhu 2009, Carrascosa et al. 2021). Therefore, there is a greater need for developing anti-biofilm strategies. Extracts derived from natural plants or related synthetic molecules used at subminimum inhibitory concentrations (subMIC) have been shown to effectively inhibit and/or disperse bacterial biofilms through non-microbicidal mechanisms, for example, by disrupting the biofilm matrix (Das et al. 2017). Such studies have shown that plant extracts could induce resistance to compounds in bacteria less frequently compared to antibiotics.

Several tests to detect biofilm formation are available and include quantitative methods, such as tissue culture plate (TCP), which is considered the gold standard method for biofilm detection (Christensen et al. 1985), and qualitative methods, such as the tube method (TM) (Christensen et al. 1982), and Congo red agar (CRA) (Freeman et al. 1989). Crystal violet (CV) staining of biofilms formed within microtiter plate wells, or on other abiotic surfaces, is the most commonly used quantitative method to analyse biofilm formation, inhibition and eradication (Haney et al. 2018).

Biofilm formation was found to play a role in the pathogenesis of *E. meningoseptica*, *C. indologenes* and *C. gleum* (Lin et al. 2010a, Kodama et al. 2013, Lo and Chang 2014), which

are close relatives of the *Kaistella* genus. Therefore, detecting the biofilm-forming potential of the six *Kaistella* fish isolates will be meaningful. The study was conducted to detect biofilm formation by the six *Kaistella* fish isolates and their reference strains and to evaluate the effect of fucoidan, proteinase K, EDTA, D-leucine, and D-glucose as inhibition compounds on these biofilms using three different methods (TCP, TM, CRA).

5.2. Materials and methods

All Oxoid and Difco products were sourced from Thermo Fisher Scientific, Johannesburg, South Africa. In contrast, all Sigma and Merck products were sourced from Merck Life Science (Pty) Ltd, Johannesburg, South Africa.

5.2.1. Cultures used and their maintenance

Six *Kaistella* bacterial fish isolates, namely, SH 11-3 (a), SH 11-3 (b), '*K. merluccii*' SH 11-4(b), '*K. piscis*' SH 19-2 (b), SH 20-4, and '*K. frigidipiscis*' SH 40-3, all obtained from a previous study (Engelbrecht 1992), were used in this study section. *Chryseobacterium gleum* NCTC 11432^T, *Kaistella koreensis* KCTC 12107^T, *K. carnis* CCUG 60559^T, *K. chaponensis* CCUG 58959^T, *K. antarctica* LMG 24720^T, *K. yonginensis* KCTC 22744^T, *Pseudomonas aeruginosa* ATCC 27853^T, and *P. fluorescens* DSM 4358^T were the reference strains used in this study. They were maintained and cultivated as previously described (Chapters 3 and 4). *Pseudomonas aeruginosa* and *P. fluorescens* were used as positive controls of biofilm production (Mann et al. 2012, Rasamiravaka et al. 2015, Milivojevic et al. 2018).

5.2.2. Biofilm formation

In this study, three methods were evaluated to detect biofilm formation by the strains. The Congo red agar (CRA) and tube (TM) methods were qualitative methods with positive and negative reactions. In contrast, the tissue culture plate (TCP) method was quantitatively measured by OD.

5.2.2.1. Congo red agar (CRA) method

Congo red agar (CRA) medium was prepared as previously described by Freeman et al. (1989). CRA medium was prepared with 37 g/l brain heart infusion broth (Oxoid CM1135), 50 g/l sucrose (Sigma S0389), 10 g/l agar (Oxoid LP0011) and 8 g/l Congo red indicator (Merck 0142825). First, Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121 °C for 15 min). The rest of the medium components were mixed and autoclaved. The sterile Congo red stain was added to the sterile brain heart infusion agar with sucrose at 55 °C (Reid 1999). The CRA plates were inoculated by streak plating the test organisms and incubated at 25, 30 and 37 °C for 48 h. Black colonies, with a dry crystalline

consistency, indicated biofilm production (Reid 1999). The experiment was carried out in triplicate and repeated thrice to produce nine data points per organism.

5.2.2.2. Tube method (TM)

Biofilm formation of the 14 isolates was tested using the tube method previously described by Christensen et al. (1982) with slight modifications. A loopful from a 48 h nutrient broth culture was inoculated in 10 ml of trypticase soy broth (TSB, Oxoid CM0129) with 1% (w/v) glucose (Sigma G8270) in test tubes. The tubes were incubated at 25, 30 and 37 °C for 48 h. After incubation, the tubes were decanted, washed with phosphate buffer saline (pH 7.3), and inverted to dry for 30 min. The tubes were stained with 0.1 % crystal violet (Sigma C0775) and allowed to stand for 30 min. Subsequently, the excess stain was washed off with deionized water, and the tubes were dried again in an inverted position for 2 h. The scoring for the TM was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as weak (w), negative/none (-) and positive (+). The experiment was carried out in triplicate and repeated thrice to produce nine data points per organism.

5.2.2.3. Tissue culture plate (TCP) method

The biofilm formation ability of the *Kaistella* fish isolates, and reference strains was tested according to a method described by Christensen et al. (1982) and Alvarez et al. (2006) with some modifications. Organisms isolated from 48 h old TSA culture agar plates were inoculated in 10 ml of TSB with 1% (w/v) glucose and incubated for 48 h at 25 °C for the *Chryseobacterium* and *Kaistella* strains and 32 °C for the two *Pseudomonas* positive controls. Following incubation, cultures were washed three times with 10 ml of phosphate-buffered saline and vortexed. The TSB cell solution was standardized with a spectrophotometer (OD_{600}) to 0.05, then 1:100 serially diluted with fresh medium. Individual wells of sterile 96-well flat-bottom polystyrene tissue culture plates (ThermoScientific 174366) were filled with 200 µl of the diluted cultures. The control organisms were also incubated, diluted, and added to tissue culture plates. Negative control wells contained uninoculated sterile broth. The plates were then incubated at the respective optimum temperatures of the isolates for 48 h. The contents of each well were removed by gently tapping after incubation. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) three times to remove free-floating bacteria. The biofilms formed by bacteria adherent to the wells were fixed for 15 min with 2% sodium acetate (w/v) (Saarchem 5821010) and stained with 0.1% crystal violet (w/v) for 30 min. The excess stain was removed by washing the plates three times with 0.2 ml of deionized water and inverting the plates. The plates were then left to dry at room temperature for 2 h. Crystal violet was solubilized by adding 200 µl of 96% ethanol (w/v) (Merck 1029177). The adhesion ability

of the bacterial cells was quantified by measuring the absorbance value of the inoculated wells at 595 nm using a microplate reader (Victor 1420, Perkin Elmer, USA). The absorbance values were finally corrected by subtracting the average absorbance value of the negative controls.

5.2.3. Biofilm inhibition

5.2.3.1. Minimum inhibitory compound (MIC) determination

The MIC of each biofilm inhibition compound against all isolates was determined using a broth microdilution method in 96-well round bottom microtiter plates (Nunc; Thermo Scientific), as previously described by Rangdale et al. (1997) but with minor modifications. Adhesion inhibitor compounds with their final concentrations used in this study are given in Table 5.1. Compounds were dissolved in phosphate-buffered saline (PBS) (pH 7.2) and then filter-sterilized (0.22 µm, Millipore SLGS025OS).

Twenty-five microliters of a 1:100 serially diluted bacterial cell suspension (10^8 CFU/ml) were added to 175 µl of a serial two-fold dilution of the compound in TSB at final concentrations ranging from 100 to 25 mM for D-glucose; from 2.5 to 0.63 mg/ml for fucoidan; from 1.0 to 0.25 mg/ml for proteinase K and from 6.0 to 1.5 mM for D-leucine and EDTA. The plates were sealed with foil to maintain humidity and incubated for 48 h at 25 and 32 °C under gentle agitation (135 cycles/min). Plates were read with the naked eye over a dark background, and visible growth in the form of opaque spots on the bottom, or turbidity of a well, was considered a positive reaction. The MIC value of each compound was defined as the lowest concentration that inhibited bacterial growth.

Table 5.1: Compounds and concentrations used for the biofilm inhibition assay of the six *Kaistella* isolates and reference strains.

Compound	Concentration
D(+)-glucose	100 mM
D-leucine	6 mM
Ethylenediaminetetraacetic acid (EDTA)	6 mM
Fucoidan	2.5 mg/ml
Proteinase K	1 mg/ml

5.2.3.2. Inhibition assay

With some modifications, biofilm adhesion inhibition of the test strains and reference strains was assessed according to Högfors-Rönnholm et al. (2014). Bacterial cells were suspended in 10 ml TSB with 1% (w/v) glucose and incubated at their optimum temperatures for 48 h.

After incubation, the cell suspension was standardized with a spectrophotometer to an OD_{595} of 0.05, and 15 µl of bacterial cell suspension and 25 µl of inhibitor compound were mixed in 210 µl TSB and incubated at the respective optimal temperatures (25 and 32 °C) of the isolates for 48 h. Subsequently, the adhesion assay was performed using the tissue culture plate method described in 5.2.2.3. The biofilm mass was calculated from the mean OD_{595} of four wells. Negative control wells were filled with 200 µl of the selected inhibitor compound and TSB separately and treated the same way as wells containing bacteria.

5.2.3.3. Quantification of biofilm inhibition

After incubation for the MIC determination and the inhibition assay, the total biomass of the biofilms was quantified using crystal violet staining as described by Álvarez et al. (2006). The biofilms in the wells were washed three times with PBS, and then 150 µl of a 0.1% (w/v) crystal violet solution was added to each well. After incubation at room temperature for 45 min, the crystal violet solution was discarded. The biofilms were washed thrice with 200 µl of deionised water to remove the dye and dried for 2 h. The stained biofilms were dissolved with 200 µl of 96% ethanol (v/v), and the mass of the biofilm was quantified by measuring the OD of the de-staining solution at 595 nm using an EZ Read 800 microplate reader (Biochrom, UK). The biofilms produced were calculated as the five wells' mean absorbance values ± standard deviation (SD). The percentage of biofilm inhibition by each of the compounds was calculated according to the formula developed by (Das et al. 2017):

$$\text{Biofilm inhibition index \%} = [(A_1 - A_2) / A_1] \times 100$$

where A_1 is the mean of the OD_{595} values of the positive control wells, and A_2 is the mean OD_{595} value of the biofilms exposed to each compound.

5.2.4. Statistical analysis

All experiments were carried out in biological triplicates, each with at least four technical replicates, and the averages and standard deviations were calculated. Microsoft Excel student's t-test was used to assess the significance of the differences, and p-values of at least 0.05 were considered significant.

5.3. Results and Discussion

5.3.1. Biofilm formation

A total of 14 bacterial isolates were screened for biofilm formation using the TCP method (Christensen et al. 1985), the TM (Christensen et al. 1982), and the CRA method (Freeman et al. 1989). The results are discussed below.

5.3.1.1. Congo red agar method

When screening for the ability of organisms to form biofilms using the CRA method, the two positive control organisms, *P. aeruginosa* and *P. fluorescens*, were able to produce biofilm at 25 and 30°C but not at 37°C. '*Kaistella merluccii*' SH 11-4(b), '*K. piscis*' SH 19-2(b), SH 20-4, *K. koreensis* and *K. antarctica* were able to form biofilms at 25 °C, and SH 20-4, *K. koreensis* and *K. antarctica*, at 30 °C and 37 °C (Table 5.2). Using the CRA method, strains SH 11-3(a), SH 11-3(b), *C. gleum*, *K. carnis*, *K. chaponensis,* and *K. yonginensis* were unable to biofilms at any of the three temperatures in this study, while strains SH 20-4 and K. koreensis could produce biofilms at the three temperatures. The results in Table 5.2 show that temperature affected the ability of all strains used in this study to form biofilms.

Table 5.2: Biofilm formation by the six *Kaistella* fish isolates and reference strains using the Congo red agar method at different temperatures. +, positive; -, negative; w, weakly positive.

Sample Name	25 °C	30 °C	37 °C
SH 11-3(a)	-	-	-
SH 11-3(b)	-	-	-
'*K. merluccii*' SH 11-4(b)	+	w	-
'*K. piscis*' SH 19-2(b)	+	w	w
SH 20-4	+	+	+
'*K. frigidipiscis*' SH 40-3	w	w	-
Chryseobacterium gleum	-	-	-
Kaistella koreensis	+	+	+
Kaistella carnis	-	-	-
Kaistella chaponensis	-	-	-
Kaistella antarctica	+	+	+
Kaistella yonginensis	-	-	-
Pseudomonas aeruginosa	+	+	w
Pseudomonas fluorescens	+	+	w

5.3.1.2. Tube method

With the TM, the two positive control organisms, *P. aeruginosa* and *P. fluorescens*, formed biofilms at all three temperatures (Table 5.3). Even though some of the isolates could produce biofilms only weakly, all of the organisms in this study could form biofilms at 25 °C and 30 °C, unlike with the CRA method. Only strains '*K. merluccii'* SH 11-4(b), *K. carnis*, *K. chaponensis* and *K. yonginensis* could not produce biofilm at 37 °C.

Table 5.3: Biofilm formation by the six *Kaistella* fish isolates and reference strains using the tube method at different temperatures. +, positive; -, negative; w, weakly positive.

Sample Name	25 °C	30 °C	37 °C
SH 11-3(a)	w	w	w
SH 11-3(b)	w	w	w
'*K. merluccii*' SH 11-4(b)	+	w	-
'*K. piscis*' SH 19-2(b)	+	+	w
SH 20-4	+	+	+
'*K. frigidipiscis*' SH 40-3	w	w	w
Chryseobacterium gleum	+	+	+
Kaistella koreensis	+	+	+
Kaistella carnis	w	w	-
Kaistella chaponensis	w	w	-
Kaistella antarctica	+	+	+
Kaistella yonginensis	w	w	-
Pseudomonas aeruginosa	+	+	+
Pseudomonas fluorescens	+	+	+

5.3.1.3. Tissue culture plate method

Figure 5.1 overviews biofilm formation by *Kaistella* fish isolates and reference strains detected by the TCP method. All strains and reference strains evaluated in this study significantly ($p < 0.050$) produced biofilms at their optimum growth temperatures (25 °C for the *Kaistella* strains and 32 °C for the *Pseudomonas* species) when evaluated with the TCP method. Strains SH 20-4 ($OD_{595} = 3.542$), *K. carnis* ($OD_{595} = 3.287$), and *K. antarctica* ($OD_{595} = 3.543$) produced significantly ($p < 0.050$) the most biofilm biomass of all the organisms used in this study, including the two *Pseudomonas* positive controls; while biofilm formation by strains SH 11-3(a) ($OD_{595} = 0.916$) and '*K. merluccii*' SH 11-4(b) ($OD_{595} = 0.972$) was significantly ($p < 0.050$)

less than that of the other *Kaistella* test and reference strains. Positive controls, *P. aeruginosa* (OD_{595} = 1.234) and *P. fluorescens* (OD_{595} = 1.442), moderately produced biofilm biomass.

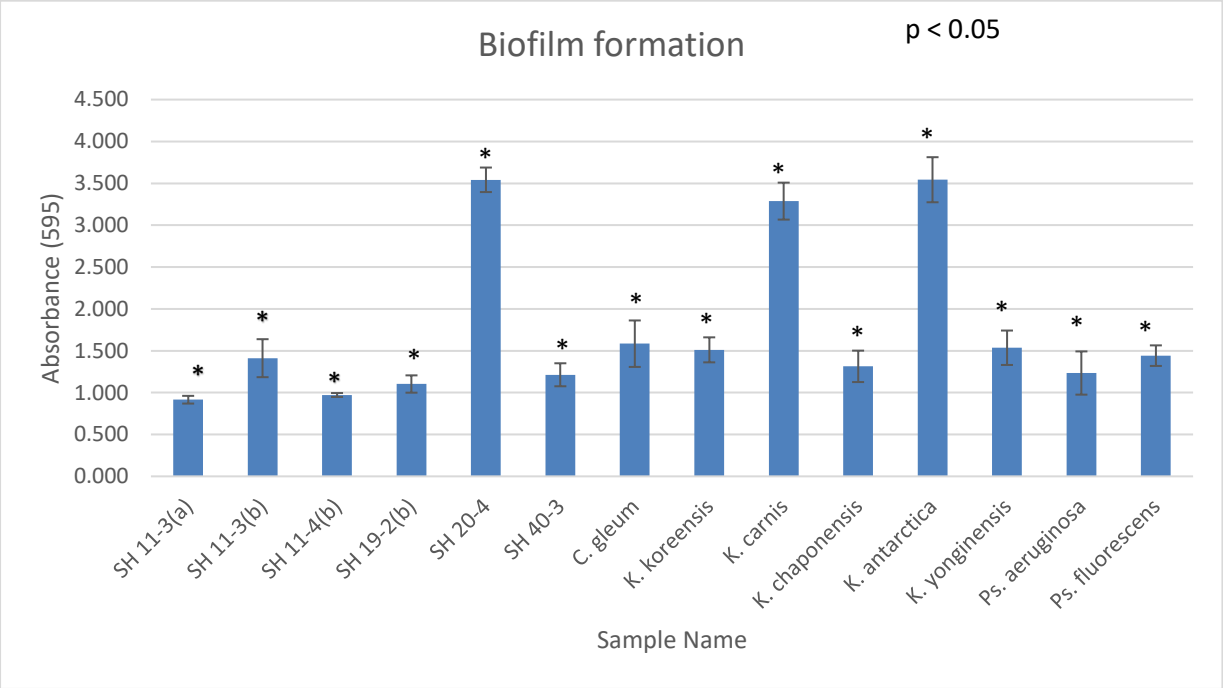

Figure 5.1: Adhesion ability of *Kaistella* fish isolates and reference strains in the tissue culture plate method, measured as absorbance at 595 nm of crystal violet stained cells. Values are averages of three independent experiments with the standard deviations indicated by the error bars. *, $p < 0.05$.

The development of biofilms by strain SH 20-4, *K. koreensis*, and *K. antarctica* was observed using all three methods in this investigation, indicating that these strains were definite biofilm formers. It was also possible that these organisms were not necessarily biofilm formers; however, their adhesion ability could have been due to the modification of the tissue culture surface of the plates, which makes the polystyrene surface more hydrophilic, thus facilitating maximum adhesion.

Given that their average OD_{595} was below 1, strains SH 11-3(a) and '*K. merluccii*' SH 11-4(b) were the least successful in forming biofilms. It appears likely that virulence in terms of antimicrobial resistance will be minimised because these organisms cannot form stable biofilms and, therefore, will have to use other mechanisms to overcome host defences or antimicrobial treatments.

5.3.2. Biofilm inhibition

Bacterial biofilms harbour persisting dormant cells protected from the host defense mechanism and highly resistant to antibiotics or antimicrobials (Lewis 2001). Small molecule agents that can prevent the development of biofilms, or disperse current formations, are thus required.

The initial concentrations of the biofilm inhibition compounds used in this study were those described in a study conducted in 2015 by Papadopoulou and colleagues to assess the impact of D(+)-glucose, D-leucine, ethylenediaminetetraacetic acid (EDTA), fucoidan and proteinase K on biofilm development. All compounds used in this study were reported to be able to fully inhibit biofilm formation in *Flavobacterium psychrophilum* at the respective MIC concentrations (Papadopoulou et al. 2015). However, it was observed that none of the inhibitory substances at the initial concentrations could prevent biofilm formation by all organisms used in this study. Following MIC analysis, a visual examination of plates revealed inhibition at double concentrations, as indicated in Table 5.1.

According to Simes et al. (2010), most biofilm inhibitory chemicals are not bactericidal; they do not try to eradicate bacteria that have already formed biofilms but rather prevent biofilm formation through different processes. Recently, numerous antibacterial substances have been discovered to avoid the development of biofilms by inhibiting quorum sensing (Bjarnsholt and Givskov 2007). In the research carried out in 2012 by Robertson and colleagues and in 2015 by Papadopoulou and colleagues, EDTA was used as a biofilm suppression agent. Similarly, Eladawy and colleagues (2020) assessed the impact of proteinase K on biofilm development.

Glucose could only significantly suppress biofilm formation at 100 mM and not at the sub-MICs. Jahid and colleagues also observed significant inhibitory effects in a 2012 investigation using glucose at its highest inclusion level. *Kaistella koreensis* (90.5%), *K. antarctica* (58.1%), *C. gleum* (53.0%), *K. chaponensis* (50.7%), and SH 20-4 (50.1%) had the highest percentages of glucose inhibition index (Table 5.4).

Only at the highest concentration of D-leucine (6 mM) pronounced inhibitory effects were observed. The lowest inhibition index was found in *K. antarctica* (26.5%), *P. aeruginosa* (34.1%) and SH 20-4 (34.7%). In comparison, the highest inhibition index was found in *C. gleum* (81.7%), *K. yonginensis* (71.1%), *K. koreensis* (65.8%) and *K. carnis* (61.0%) (Table 5.4). All the strains used in this study showed minimal to no inhibitory effects at sub-MICs. In their 2011 study, Hochbaum and colleagues also found that D-amino acids, including D-leucine, had little or no inhibitory effects. They also concluded that D-amino acids prevent

biofilm development at a stage after initial attachment to the surfaces and continue to inhibit biofilm growth for at least 48 h.

Table 5.4: Percentage inhibitory index of different compounds on biofilm formation of *Kaistella* fish isolates and reference strains at three different concentrations of D(+)-glucose, D-leucine, EDTA, fucoidan, proteinase K.

Sample name+concentration	D(+)-Glucose (%)	D-Leucine (%)	EDTA (%)	Fucoidan (%)	Proteinase K (%)
SH 11-3(a)					
MIC/8	1.66	16.9	12.66	10.79	-10.25
MIC/2	7.05	34.57	27.27	18.87	11.69
100 mM	40.75	41.13	44.5	35.81	23.7
SH 11-3(b)					
MIC/8	-6.08	24.63	18.36	13.86	3.38
MIC/2	14.47	17.86	28.8	22.78	8.48
100 mM	47.58	40.78	43.78	28.97	9.05
'*K. merluccii*' **SH 11-4(b)**					
MIC/8	17.34	22.8	21.03	8.74	-3.49
MIC/2	33.08	35.47	37.04	19.83	1.43
100 mM	46.83	45.67	43.4	40.95	16.72
'*K. piscis*' **SH 19-2(b)**					
MIC/8	12.2	20.19	14.03	6.57	1.66
MIC/2	25.7	30.68	17.47	12.91	12.12
100 mM	50.1	43.02	38.53	31.69	4.44
SH 20-4					
MIC/8	-11.67	20.45	4.73	33.24	-28.33
MIC/2	13.78	16.48	20.93	34.52	-17.97
100 mM	42.36	34.72	35.12	31.06	-21.19
'*K. frigidipiscis'* **SH 40-3**					
MIC/8	3.47	15.82	15.13	9.29	3.00
MIC/2	20.8	28.83	26.05	20.47	11.83
100 mM	36.4	37.94	55.38	19.9	11.88
C. gleum					
MIC/8	-2.34	13.81	1.71	4.03	-4.82
MIC/2	6.56	17.01	30.92	14.47	8.06

100 mM	52.97	81.68	44.02	27.86	7.64
K. koreensis					
MIC/8	18.335	17.4	34.17	11.78	6.98
MIC/2	54.175	24.71	23.39	20.47	27.66
100 mM	90.485	65.78	50.43	29.76	33.63
K. carnis					
MIC/8	34.36	-6.02	13.13	-5.54	-8.93
MIC/2	50.9	10.51	21.35	22.76	17.60
100 mM	50.72	61.04	43.00	27.85	9.44
K. chaponensis					
MIC/8	9.89	-5.83	10.649	10.09	10.38
MIC/2	21.75	11.76	22.109	15.16	14.09
100 mM	38.73	51.15	33.659	28.64	30.71
K. antarctica					
MIC/8	-0.44	4.42	10.68	-16.02	-9.19
MIC/2	0.32	2.38	14.88	-3.85	5.63
100 mM	58.05	26.52	23.20	-6.20	4.71
K. yonginensis					
MIC/8	-3.79	15.74	18.01	15.09	5.16
MIC/2	29.08	30.6	19.88	22.39	22.84
100 mM	41.72	71.13	24.76	38.73	33.93
P. aeruginosa					
MIC/8	2.09	2.68	10.3	9.91	4.39
MIC/2	20.5	22.53	13.43	19.56	15.61
100 mM	35.03	34.12	35.78	34.04	34.68
P. fluorescens					
MIC/8	12.52	-7.32	12.39	-4.09	2.80
MIC/2	41.63	13.24	17.92	32.57	12.3
100 mM	49.96	43.37	46.23	40.18	23.68

Cell-to-cell adhesion is necessary for biofilm formation, and cations, notably calcium, are believed to play a significant role in the bonding of polymer molecules in biofilms, promoting the polymer layer's cohesiveness. In 2012, Chaudhary and Payasi found that EDTA at 10 mM completely disrupted biofilms. This explains why EDTA had insufficient inhibitory effects on all organisms used in this study, given that the concentration was lower (6 mM). '*Kaistella frigidipiscis*' SH 40-3 (55.4%), *K. koreensis* (51.4%) and *P. fluorescens* (46.2%) showed the

highest levels of inhibition (Table 5.4). Inhibition of cell-to-surface and cell-to-cell contacts by EDTA was demonstrated by Chang and colleagues (2012), and this at least partially contributed to the repressed early transcription. They stated that the chelating properties of EDTA did not cause biofilm inhibition but rather that EDTA functions in the early stage of the biofilm process by affecting the initial adherence. They also reported that adding enough cations to saturate EDTA did not restore biofilm formation.

Fucoidan is a polysaccharide from brown seaweed that contains significant amounts of l-fucose and sulfate ester groups (Chizhov et al. 1999). Due to its wide range of fascinating biological actions, fucoidan has been the subject of extensive research for the past ten years (Li et al. 2008). In this work, the biofilm-forming bacteria *Chryseobacterium, Pseudomonas*, and representatives of the genus *Kaistella* were treated with fucoidan as an inhibiting compound. As shown in Table 5.4, a slight inhibition was observed at 2.5 mg/ml in all organisms used in this study. A negative inhibition index percentage was obtained for *K. antarctica* (-6.2%). The ability of this organism to generate pellicles was not affected by any of the concentrations. The 2014 investigation by Chmit and colleagues also showed relatively mild inhibitory effects of fucoidan. In this study, Fucoidan's ability to remove biofilms ranged from 3.7% to 33.5%.

In contrast, fucoidan was one of the most effective inhibitors of cell adhesion at even lower concentrations (1 mg/ml), according to a 2015 study by Papadopoulou and colleagues. In a 2019 study, Khan and colleagues reported significant antibiofilm effects. Their findings showed a more substantial dispersion of mature biofilm developed at higher concentrations (from 128 to 256 g/ml) than lower concentrations (16 to 64 g/ml).

Proteinase K is a proteolytic enzyme and may facilitate biofilm dissemination by breaking surface proteins (Shukla and Rao 2013). Proteinase K is a highly reactive serine protease, which is stable in a broad range of conditions, such as pH, buffer salts, detergents (SDS) and temperature (Kristjánsson et al. 1999). This makes proteinase K an ideal choice for disassembly among the various proteases. Shukla and Rao (2013) reported 36% biofilm dispersal with 2 µg/ml proteinase K treatment, while the highest dose of 250 µg/ml showed ~76% biofilm dispersal. Therefore, they concluded that 100% biofilm dispersal could not be achieved since increasing the concentration of proteinase K did not result in a proportional improvement of dispersion. In contrast, proteinase K did not significantly affect biofilm mass reduction at any of the concentrations tested, according to Eladawi and Colleagues (2020).

Proteinase K antibiofilm inhibitory effects were evaluated in this study, and most organisms showed resistance to proteinase K since inhibitory percentages ranged between -21.2% and

34.7% (Table 5.4). Inhibition was not observed in all tested concentrations of strain SH 20-4 (-21.2%).

The highest inhibitory effects were observed in *P. aeruginosa* (34.7%), *K. yonginensis* (34.0%), *K. koreensis* (33.6%), and *K. chaponensis* (30.71%). It could be concluded that these organisms can develop with proteinase K since, regardless of the proteinase K concentrations examined, all proteinase K-treated biofilms had microbial growth that was at least as great as that of the untreated control organisms. Proteinase K appeared to promote their gain rather than prevent the development of biofilms.

It is clear from Table 5.4 that D(+)-glucose was the most efficient inhibition compound against the *Kaistella* test strains and reference strains utilized in this investigation. Some organisms were also inhibited at sub-MIC levels, and significant inhibition was observed at the MIC (100 mM) and sub-MIC (50 mM). The lowest inhibition index was found in *K. antarctica* (26.5%), *P. aeruginosa* (34.1%) and SH 20-4 (34.7%), while the highest inhibition index was found in *C. gleum* (81.7%), *K. yonginensis* (71.1%), *K. koreensis* (65.8%) and *K. carnis* (61.0%).

Proteinase K was the least effective compound, and its percentage inhibition ranged between -21.2%-34.7%. Organisms whose biofilms showed the highest resistance to inhibition compounds include SH 20-4, '*K. frigidipiscis*' SH 40-3, *K. antarctica* and *P. aeruginosa*, while susceptibility was primarily observed in SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b), '*K. piscis*' SH 19-2 (b) shared this trait with *C. gleum, K. koreensis* and *K. chaponensis*.

5.4 CONCLUSIONS

Persistent (biofilm-forming) microorganisms have become a global challenge due to their ability to withstand antibiotics, host defence systems, and other external stresses. The ability of certain microbes to form biofilms has been associated with virulence since organisms that have this ability show resistance to a wide range of antimicrobials. This study determined the ability of the six *Kaistella* strains isolated from fish using three methods. The potential of five biofilm inhibition compounds (D(+)-glucose, D-leucine, EDTA, fucoidan, proteinase K) to inhibit biofilm formation by these organisms was also determined.

The TCP method detected 100% biofilm production (14/14) in the six test isolates and eight reference species at their optimum growth temperatures (25 °C for the *Kaistella* strains and 32 °C for the two *Pseudomonas* species). However, strain SH 20-4, *K. carnis* and *K. antarctica* produced significantly ($p < 0.05$) the most biofilm. In the TM, significant and consistent biofilm formation was observed in strains SH 20-4, *K. koreensis, K. antarctica, P. aeruginosa* and *P. fluorescens* at 25, 30 and 37 °C.

The CRA method detected biofilm production only in eight isolates, including the positive control organisms, *P. aeruginosa* and *P. fluorescens*, with strain SH 40-3 that only formed biofilms weakly at 25 and 32 °C. Six out of 14 isolates (42.9%) did not form biofilms. Therefore, based on the results, it can be concluded that the tissue culture plate is a more quantitative and reliable method for detecting biofilm formation compared to the TM and CRA methods.

The antibiofilm activity of the five compounds used in this study, revealed that D(+)-glucose was the most effective inhibitor compound against the *Kaistella* test strains and the reference strains used. Significant inhibition was observed at the MIC (100 mM), and some organisms were also inhibited at the sub-MIC (50 mM). The highest inhibition index was observed in *C. gleum* (81.7%), *K. yonginensis* (71.1%), *K. koreensis* (65.8%), *K. carnis* (61.0%), while the lowest was observed in *K. antarctica* (26.5%), *P. aeruginosa* (34.1%), and SH 20-4 (34.7%). Proteinase K was the least effective compound; its percentage inhibition ranged between 21.2% and 34.7%. Organisms whose biofilms showed the highest resistance to inhibition compounds included SH 20-4, '*K. frigidipiscis'* SH 40-3, *K. antarctica* and *P. aeruginosa*, while susceptibility mainly was observed in strains SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b) and '*K. piscis*' SH 19-2 (b) shared this trait with *C. gleum, K. koreensis* and *K. chaponensis*.

CHAPTER 6

THE ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES INFECTING *Kaistella* FISH ISOLATES

Abstract

In both human and veterinary medicine, concerns have been raised about the global rise in multidrug-resistant diseases. This has rekindled interest in developing antibiotic-alternative techniques, such as using bacteriophages (phages) for treating bacterial infections, eradicating pathogenic bacteria in livestock production, and acting as biocontrol agents to manage foodborne pathogens, and lowering contamination on surfaces in contact with food. Phage therapy uses naturally occurring phages to infect and kill microorganisms at the site of the occurrence. Chapters 4 and 5 show *Kaistella* fish isolates' capability to produce virulence factors, and biofilms were proven. This chapter isolated phages infecting various strains of *Kaistella* fish isolates from sewage water samples and fishpond water. Thirty-four phages were isolated, and most of the *Kaistella* fish isolates, and reference strains used in this investigation were susceptible to their lytic activity. Twenty phages were isolated from sewage samples, while 14 were isolated from the fishpond samples. The most comprehensive host strain range was exhibited by phage isolate 11-3(b)-S2, which infected five of the 12 *Kaistella* strains used in this study. Strains '*K. piscis*' SH 19-2(a), *K. koreensis, K. carnis,* and *K. yonginensis* displayed the most resistance toward phage infection. This implied that these strains might be phage resistant. This study was the first to examine bacteriophages that infect the *Kaistella* species.

6.1. Introduction

Before antibiotics were developed, bacterial infections seriously threatened human health (D'Accolti et al. 2021). Unfortunately, the widespread and occasionally inappropriate use of antibiotics has sparked the emergence, evolution, and dissemination of drug-resistance mechanisms by which bacteria can withstand and survive antimicrobial attacks. As a result, antimicrobial resistance and drug residues in food have continued to grow, one of the main reasons that have sparked research interest in risk-free solutions (D'Accolti et al. 2021).

Microbial control strategies are needed in the food industry to prevent foodborne diseases and outbreaks and prolong the product's shelf life (Techathuvanan et al. 2013). Phage therapy, which employs lytic bacteriophages, can potentially replace antibiotics to inactivate bacteria (O'Flynn et al. 2004, Soliman et al. 2019). Bacteriophages, which were first identified by William Twort and Felix d'Herelle in 1915 and 1917, are tiny viruses that can only infect

prokaryotic bacterial cells, making them safe for humans and, more broadly, for all eukaryotic cells (Hendrix 2002, Jamal et al. 2019).

Due mainly to their intrinsic low toxicity, using phages to inactivate harmful bacteria in, for example, farmed fish, has gained steam in recent years (Pereira et al. 2021). This expanding pattern indicates a rise in curiosity about the commercial uses of phages in aquaculture (Pereira et al. 2021). Bacteriophages, first identified by William Twort and Felix d'Herelle in 1915 and 1917, are tiny viruses that can only infect prokaryotic bacterial cells, making them safe for humans and, more broadly, for all eukaryotic cells (Hendrix 2002, Jamal et al. 2019).

Phages are the most prevalent and diverse living organisms in the biosphere, and they are present in virtually every setting where bacteria can grow and reproduce. As a result, they help keep ecosystems balanced and prevent the overgrowth of bacteria (Hendrix, 2002, El-Shibiny and El-Sahhar 2017, Jamal et al. 2019). They have also been isolated from human and animal samples, like faeces, urine, saliva, and serum, in addition to being frequently found in water, soil, and sewage (Gantzer et al. 2002, Bachrach et al. 2003, Breitbart et al. 2004). Phages are widely employed in various agricultural settings and human and veterinary health (Sillankorva et al. 2012, Mahn et al. 2021). As obligatory parasites, phages can either cause cell lysis, release newly produced virus particles (lytic pathway) or lead to the integration of genetic information into the bacterial chromosome without causing cell death (lysogenic way) (Sillankorva et al. 2012, Stone et al. 2019, Cristobal-Cueto et al. 2021). Strictly lytic phages are likely one of the most innocuous antibacterial techniques accessible regarding food safety (Sillankorva et al. 2012).

Phage therapy, as a bacterial infection control mechanism, has increased dramatically in recent years, mainly owing to the emergence of bacterial resistance to a wide range of antimicrobial drugs. After years of neglect in the Western world, phage therapy has resurfaced as a viable treatment option. Although phages are already being used to treat humans and animals, there is little literature on studies, including industrial environments and the use of phages as sanitation agents. No research has yet been conducted using phages capable of infecting food-related isolates in the genus *Kaistella*.

This part of the study aimed to isolate phages from sewage samples and fishpond water, which could infect the six *Kaistella* fish isolates and their reference strains from Chapters 3, 4 and 5. The host strain range specificity, lytic profiles, and classification of these bacteriophages using electron microscopy were then evaluated.

6.2. Materials and Methods

6.2.1. Cultures used and their maintenance

The six *Kaistella* fish isolates, SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b), '*K. piscis*' SH 19-2(b), SH 20-4, and '*K. frigidipiscis'* SH 40-3, evaluated in Chapters 3, 4 and 5 and obtained from a previous study (Engelbrecht 1992), were also used in this study section. The reference strains used in this study were *Chryseobacterium gleum* NCTC 11432^T, *Kaistella koreensis* KCTC 12107^T, *K. carnis* CCUG 60559^T, *K. chaponensis* CCUG 58959^T, *K. antarctica* LMG 24720^T, and *K. yonginensis* KCTC 22744^T. All strains were maintained and cultivated as previously described (Chapters 3, 4 and 5).

6.2.2 Isolation of bacteriophages

6.2.2.1 Bacteriophage enrichment

The sewage water sample was collected at a residence with a sewage leak at Universitas, Bloemfontein. The other was collected at a residential fishpond at Langenhoven Park, Bloemfontein. The two water samples were enriched to isolate specific bacteriophages infecting the *Kaistella* fish isolates. Briefly, the *Kaistella* and the reference strains were cultivated to the exponential phase (overnight incubation in nutrient broth at 25 °C). A 100 ml sample of each sewage and fishpond water was centrifuged at 2 419 x *g* for 30 min at 4 °C. The supernatant was then collected, with care taken to prevent the movement or inclusion of pelleted debris. Following incubation, 1 ml of the bacterial culture, 0.02 M $CaCl_2$, and approximately 100 ml of centrifuged water sample were added to 250 ml of SM buffer. The SM buffer contained 0.01% (w/v) gelatin, 100 mM NaCl, 8 mM $MgSO.7H_2O$, and 50 mM Tris-HCl (pH 7.5) to obtain lysates of bacteriophages able to propagate in cells of the *Kaistella* hosts. This mixture was then incubated for 48 h at 25 °C. Ten millilitres of the enrichment broth was centrifuged (2 419 x *g,* 30 min, 4 °C) and filtration using a 0.45 µm membrane filter. The bacterial culture was infected by adding 1 ml of the filtered supernatant to 100 ml exponential growing culture, and this mixture was incubated for 48 h at 25 °C followed by centrifugation and filtering.

6.2.2.2. Plaque assay

The plaque assay was conducted according to Kropinski et al. (2009) with some modifications. Briefly, the enriched phage culture was diluted serially in SM buffer (10^{-1}–10^{-9}). One millilitre of the respective phage dilutions was added to the same exponential phase bacterial culture volume. The dilution-mixture samples were vortexed and incubated for 15 min at 25 °C. The

phage-bacteria culture (different diluted phages) was added to 5 ml 0.8% trypticase soy (TSA) molten soft agar (Oxoid CM0131, ThermoFisher) and mixed by swirling the tube.

The molten soft agar mixture was poured onto pre-poured TSA plates, and the agar plate was swirled to ensure coverage of the agar plate and to allow it to solidify. The plates were incubated at 25 °C and observed for three days for plaque formation.

Plaques were individually picked with an inoculation loop and added to 2 ml SM buffer when observed. This was followed by adding three drops of chloroform to remove any remaining bacteria. The mixture was shaken gently and centrifuged at 14 500 x *g* for 10 min. The supernatant was carefully removed and filtered through a 0.45 µm filter. A 100 µl of the lysate and 100 µl bacterial culture (exponential phase) was added to 5 ml molten 0.8% TSA at 45 °C, and the plaque assay was repeated. When the same plague formation was observed, the process was repeated. However, the samples were prepared for visualization with transmission electron microscopy instead of reinfecting the phages.

6.2.2.3. Bacteriophage isolation and purification

The phage lysate was centrifuged for 3 h at 4 °C at 16 000 x *g*. The supernatant was decanted, and the resulting pellet was suspended in 1 M ammonium acetate buffer and incubated overnight at 4 °C. The ammonium acetate purification step was repeated three times (three days), and purified concentrated samples were kept at 4 °C until transmission electron microscopy.

6.2.2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out at the Electron Microscope Unit based at the University of the Free State. A drop of the concentrated sample (6.2.2.3) was placed onto a formvar carbon-coated copper grid (Sigma-Aldrich) and absorbed in the desiccator overnight. The phages were visualized using the Phillips CM100 transmission electron microscope.

6.3. Results and Discussion

6.3.1 Isolation and host range characterization of promising lytic phages

6.3.1.1 Sewage water sample phages

This study intended to isolate, identify, and characterize efficient lytic broad host range bacteriophages active against various *Kaistella* fish isolates. By using a two-fold agar overlay in a plaque experiment, lytic bacteriophages were successfully extracted. This was demonstrated by the clear zones (plaques) on bacterial lawns that appeared when agar plates

were incubated for three days. There were variations in the plaques' sizes, clearness, and turbidity. Because this characteristic is exclusive to particular phages, variation in plaque shape and size suggested the existence of numerous phage strains (Gallet et al. 2011).

Different plaque formation was observed on plates treated with sewage water samples, which could indicate the presence of several phage types. This was anticipated as sewage collects waste from a large human population, considerably increasing the diversity of microorganisms growing in these systems. It has also been demonstrated that sewage and sewage-contaminated settings are the most credible sources for isolating phages (Hyman 2019, Aghaee et al. 2021).

Figures 6.1 and 6.2 show plaque formation against *Kaistella* strains SH 11-3(a) and '*K. merluccii*' SH 11-4(b), which is a positive indicator of the presence of phages against specific members of the genus *Kaistella*. Compared to the other test strains, SH 11-3(a) and SH 11-3(b) consistently showed the most significant lysis and shared this trait with *K. antarctica*. The plaques varied in size and morphology, which could indicate that the organisms were susceptible to lysis by different phages. As a result, it is reasonable to assume that the susceptibility of these strains to lysis was due to the presence of receptors that were easily recognized by phages. As previously stated, phage receptor binding proteins on the bacterial membrane is critical for initiating the lytic pathway (Hyman 2019, Stone et al. 2019).

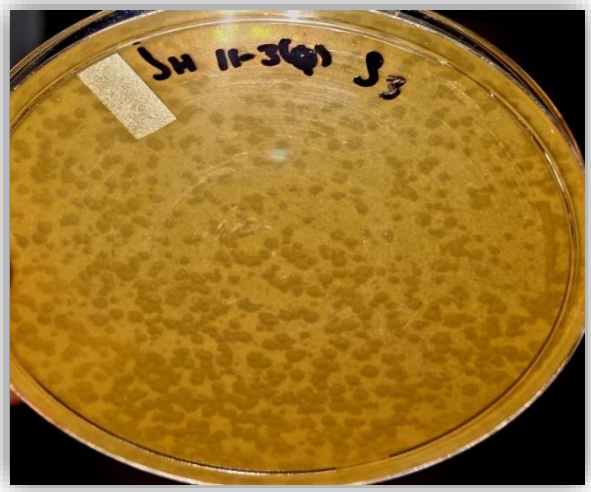

Figure 6.1: *Kaistella* SH 11-3(a) plaques from sewage water

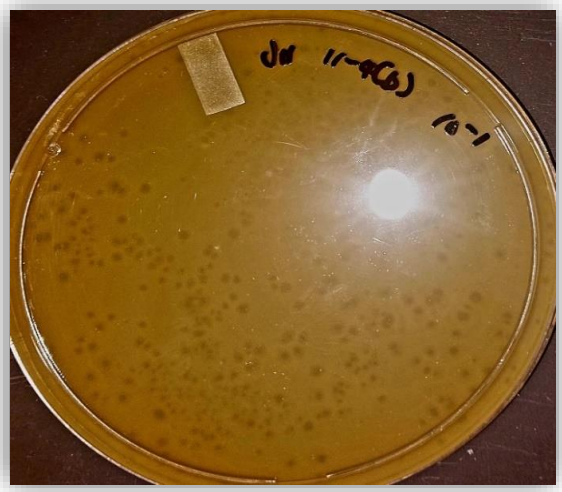

Figure 6.2: '*Kaistella merluccii'* SH 11-4(b) plaques from sewage water

Most phages recovered from the sewage sample infected a wide range of hosts (Table 6.1). Phage strains 11-3(a)-S1, 11-3(a)-S2, 11-3(a)-S3, 11-3(b)-S1, 11-3(b)-S2, 11-3(b)-S3, 19-2(b)-S1, 40-3-S1, K-ch-S1, K-ch-S2, K.a-S1 and K.a-S2 formed clear plaques against their

hosts while phage strains 11-4(b)-S1, 11-4(b)-S2, 11-4(b)-S3, 20-4-S1, C.g-S1, C.g-S2, K.k-S1 and K.c-S1 formed both clear and turbid plaques (Table 6.1).

Virulent phages produce clear plaques, and the plaques formed by phages with large genomes tend to be smaller than those created by phages with small genomes. Temperate phages give rise to turbid plaques due to the growth of surviving lysogenic bacteria within the lysis zone (Payne 2017, Hyman 2019). Plaques with a turbid appearance may also result from partially lysed cells or accumulation of plasma membranes rather than the growth of surviving bacteria (Payne 2017, Hyman 2019).

Table 6.1: Sensitivity of *Kaistella* fish isolates and reference strains to sewage water phages.

Phage Name	Original bacterial host	Degree of lysis
11-3(a)-S1	*Kaistella* SH 11-3(a)	Clear
11-3(a)-S2	*Kaistella* SH 11-3(a)	Clear
11-3(a)-S3	*Kaistella* SH 11-3(a)	Clear
11-3(b)-S1	*Kaistella* SH 11-3(b)	Clear
11-3(b)-S2	*Kaistella* SH 11-3(b)	Clear
11-3(b)-S3	*Kaistella* SH 11-3(b)	Clear
11-4(b)-S1	*'Kaistella merluccii'* SH 11-4(b)	Clear and turbid
11-4(b)-S2	*'Kaistella merluccii'* SH 11-4(b)	Clear and turbid
11-4(b)-S3	*'Kaistella merluccii'* SH 11-4(b)	Clear and turbid
19-2(b)-S1	*'Kaistella piscis'* SH 19-2(b)	Clear
20-4-S1	*Kaistella* SH 20-4	Clear and turbid
40-3-S1	*'Kaistella frigidipiscis'* SH 40-3	Clear
C.g-S1	*Chryseobacterium gleum*	Clear and turbid
C.g-S2	*Chryseobacterium gleum*	Clear and turbid
K.k-S1	*Kaistella koreensis*	Clear and turbid
K.c-S1	*Kaistella carnis*	Clear and turbid
K.ch-S1	*Kaistella chaponensis*	Clear
K.ch-S2	*Kaistella chaponensis*	Clear
K.a-S1	*Kaistella antarctica*	Clear
K.a-S2	*Kaistella antarctica*	Clear

The names of the phage isolates included part of the host strain designation and the source of the phages. The "S" indicated that the phage was recovered from sewage. The numbers at the end of the name of the phage isolate represented a different phage infecting the same organism.

Twenty phages were isolated from the sewage water sample. Eight isolated phages from the sewage water were used in further screening assays, using the phage spot test on bacterial lawns of all the strains, to characterize the host range of each phage and select a phage with the broadest host range towards the *Kaistella* fish isolates. Several phages could lyse most of the *Kaistella* fish isolates; however, some were more host-specific, such as strains 19-2(b)-S1, 20-4-S1, and 40-3-S1.

6.3.1.2. Fishpond water sample phages

According to Figures 6.3 and 6.4, a heterogeneous variety of plaques was visible on the several bacterial strains amenable to lysis. This suggested that various phage species were present in the fishpond water sample from which they were extracted. Along with variances in diameter, the plaques' clarity varied in some instances. While some of the plaques were murky, the majority were clear zones.

Although the phages were comparable in size, those recovered from the fishpond against strain SH 11-3(a) in Figure 6.4 varied slightly and differed from the plaque formation of all the other examined isolates. With a broader phage spectrum than the other test isolates and reference strains, strain SH 11-3(a) demonstrated susceptibility to phages collected from sewage and fishpond samples, indicating that this organism was the most vulnerable to phage attack.

Figure 6.3: SH 11-3(b) fishpond plagues

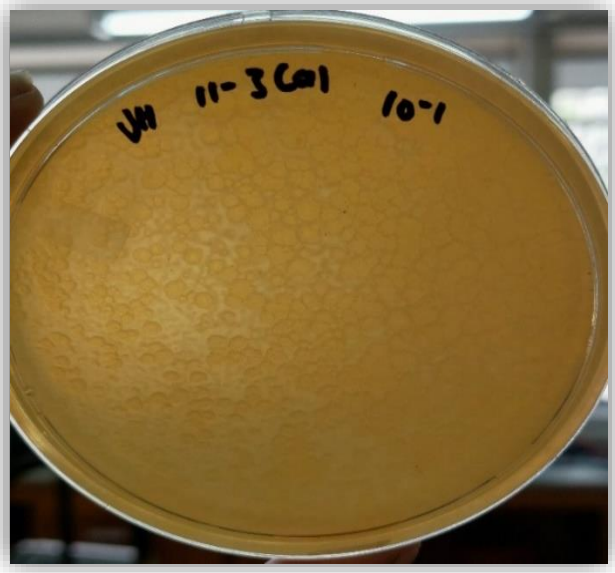

Figure 6.4: SH 11-3(a) fishpond plaques

The phage 11-3(a)-S2 (Table 6.2) had a broad host spectrum since it was also able to lyse strains SH 11-3(b), SH 20-4, '*K. merluccii*' SH 11-4(b), *C. gleum*, and *K. antarctica*. A phage will only lyse a bacterium if it is sufficiently virulent and productive to prevent the bacterium from simply outpacing the phage's ability to lyse them (Hyman 2019). Bacteria have developed many diverse mechanisms for resistance and tolerance, such as ones that stop phage adsorption and protect the host against viral infection (Labrie et al. 2010). Similarly, phages have developed several strategies to get around bacterial resistance mechanisms (Samson et al. 2013).

Eight of the 12 isolates utilized in this study (*C. gleum*, SH 11-3(a), SH 11-3(b), '*K. merluccii*' SH 11-4(b), SH 20-4, '*K. frigidipiscis*' SH 40-3, *K. chaponensis* and *K. antarctica*) showed susceptibility to lyses by various fishpond phages (Table 6.2). Phage strains 11-3(a)-FP1, 11-3(a)-FP2, 11-3(b)-SFP1, 11-3(b)-FP2, 11-4(b)-FP1, 11-4(b)-FP2, 40-3-FP1, 40-3-FP3, K.a-FP1 and K.a-FP2 formed clear plaques against their hosts, while strains 20-4-FP1, C.g-FP1, C.g-FP2 and K.ch-FP1 formed both clear and turbid plaques.

Table 6.2: Sensitivity of *Kaistella* fish isolates and reference strains to fishpond phages.

Phage Name	Original bacterial host	Degree of lysis
11-3(a)-FP1	*Kaistella* SH 11-3(a)	Clear
11-3(a)-FP2	*Kaistella* SH 11-3(a)	Clear
11-3(b)-FP1	*Kaistella* SH 11-3(b)	Clear
11-3(b)-FP2	*Kaistella* SH 11-3(b)	Clear
11-4(b)-FP1	*'Kaistella merluccii'* SH 11-4(b)	Clear
11-4(b)-FP2	*'Kaistella merluccii'* SH 11-4(b)	Clear
20-4-FP1	*Kaistella* SH 20-4	Clear and turbid
40-3-FP1	*'Kaistella frigidipiscis'* SH 40-3	Clear
40-3-FP3	*'Kaistella frigidipiscis'* SH 40-3	Clear
C.g-FP1	*Chryseobacterium gleum*	Clear and turbid
C.g-FP2	*Chryseobacterium gleum*	Clear and turbid
K.ch-FP1	*Kaistella chaponensis*	Clear and turbid
K.a-FP1	*Kaistella antartica*	Clear
K.a-FP2	*Kaistella antartica*	Clear

The names of the phage isolates included part of the host strain designation and the source of the phages. The "FP" indicates that the phage was recovered from a fishpond, and the numbers at the end of the name of the phage isolate represent a different phage infecting the same organism.

Plaque formation was absent in strain '*K. piscis*' SH 19-2(b), *K. koreensis, K. carnis*, and *K. yonginensis*. Plaque formation was also not observed for *K. yonginensis* when the sewage water sample was screened for lysis. This could indicate that these organisms lacked receptors that matched those of the phages, preventing the phages from multiplying. Another reason could be that the phages could not overcome the host's defensive mechanisms and were thus degraded by the organisms. It was, therefore, also possible that these strains had an integrated phage (prophage) since it has been proven that, when bacteria are infected with an integrated phage, it renders the bacterial host immune to antibiotics and infection by other phages (Bondy-Denomy et al. 2016, Colavecchio et al. 2017, Fillol-Salom et al. 2019). The prophages code for proteins that interact with the cytoplasmic membrane to inhibit further phage genome injection and phage binding by interacting with the phage receptor on the bacterial outer membrane (Bondy-Denomy et al. 2016).

Only eight of the 34 phage isolates (11-3(a)-S1, 11-3(a)-S2, 11-3(b)-S1, 11-3(b)-S2, 20-4-P1, 20-4-P2, and 40-3-P2) were chosen for the host strain identification assay. Phages 11-3(a)-S2 and 11-3(b)-S2, which were isolated from the sewage water sample, had a powerful antibacterial impact against many of the isolates and could produce distinct lysis zones on more than three bacterial isolates (Table 6.3). As was previously indicated, phage 11-3(b)-S2 exhibited a wider host spectrum than other phage isolates since it showed lytic activity against 5/12 isolates tested in this study. This phage isolates required additional characterization since it may be a promising option for sanitizing applications, given its capacity to lyse a variety of *Kaistella* hosts.

'*Kaistella piscis'* SH 19-2(a), *K. koreensis, K. carnis*, and *K. yonginensis* displayed the most significant resistance towards phage infection since they were unaffected by any of the isolated phages (Table 6.3). Phage resistance was also observed by Fang et al. (2022). Bacteria have developed an astounding array of strategies, including spontaneous mutations, restriction-modification systems, and adaptive immunity via the CRISPR-Cas system, to combat phage attacks at every stage of infection (Labrie et al. 2010). As phage adsorption to cell receptors is the first step in infection, some bacterial strains have evolved mechanisms to prevent it (Labrie et al. 2010). Strains SH 11-3(a) and SH 11-3(b) exhibited sensitivity to a variety of phage isolates, including 11-3(a)-S1, 11-3(a)-S2, 11-3(b)S1 and 11-3(b)-S2. It can, therefore, be deduced that these strains had a large variety of receptors that the phages could recognize, which is why a more significant number of plaques were observed.

In Chapter 4, the virulence of several *Kaistella* strains was evaluated. '*K. merluccii*' SH 11-4(b) was considered another potential pathogen with *C. gleum* and *K. antarctica*.

Strains SH 11-3(a), '*K. frigidipiscis*' SH 40-3, *C. gleum*, and *K. antarctica* demonstrated resistance to 9 of the 17 antimicrobials used in this study, indicating a need for alternative antimicrobial agents. The results of Chapter 6 indicated that strains SH 11-3(a), '*K. merluccii*' SH 11-4(b), and '*K. frigidipiscis'* SH 40-3 can be controlled by phage therapy because they showed susceptibility to phage strains obtained from the sewage and fishpond water samples.

Table 6.3: Lytic activity of sewage and fishpond water phages against all the *Kaistella* fish isolates and reference strains. C, clear phage plaque; T, turbid phage plaque; -, no phage plaque.

Sample name	**11-3(a)-S1**	**11-3(a)-S2**	**11-3(b)-S1**	**11-3(b)-S2**	**20-4-P1**	**20-4-P2**	**40-3-P1**	**40-3-P2**
SH 11-3(a)	C	C	C	C	-	-	-	-
SH 11-3(b)	C	C	C	C	-	-	-	-
'*K. merluccii*' SH 11-4(b)	C	C	C	C	-	-	-	-
'*K. piscis*' SH 19-2(b)	-	-	-	-	-	-	-	-
SH 20-4	T	-	T	C	C	C	-	-
'*K. frigidipiscis*' SH 40-3	-	-	-	-	T	T	C	C
C. gleum	-	-	-	C	-	-	-	-
K. koreensis	-	-	-	-	-	-	-	-
K. carnis	-	-	-	-	-	-	-	-
K. chaponensis	-	-	-	-	T	T	-	-
K. antarctica	-	-	-	C	-	-	-	-
K. yonginensis	-	-	-	-	-	-	-	-

It will be a challenge to treat strain '*K. piscis*' SH 19-2(b) with any of the phages isolated in this study since the organism, together with *K. koreensis, K. carnis* and *K. yonginensis,* were resistant to plaque formation. In Chapter 3, it was discovered that a phage protein was among the genes identified in '*K. piscis*' SH 19-2(b), which, therefore, explains the immunity of the

organism to be attacked by phage, as bacteria with an integrated phage, have been shown to demonstrate immunity to inhibition compounds and infection by other phages (Bondy-Denomy et al. 2016, Colavecchio et al. 2017, Fillol-Salom et al. 2019).

6.3.2. Morphology of the phage isolates

One of the crucial components in determining a microorganism's identity is its morphological characterization. The identification and description of viruses have long been accomplished using electron microscopy (EM) (Goldsmith and Miller 2009). However, because the virus particles are so tiny, transmission electron microscopy (TEM) has been crucially significant (Goldsmith and Miller 2009). The basis for the identification and establishment of bacteriophage families was provided by TEM, which is also one of the crucial criteria for categorizing new viruses into families (Ackermann 2012). It enables prompt diagnosis and is thus the fastest diagnostic technique in virology.

6.3.2.1 Phages isolated from sewage water samples

Phage 11-3(b)-S2's morphological characterization by TEM suggested it might be a member of the *Plasmaviridae* or *Cystoviridae* families (Figure 6.5). *Plasmaviridae* are pleomorphic, lack a head-tail structure, and have not yet been assigned to a higher rank, as seen in Chapter 2, Table 2.5. The icosahedral members of the family *Cystoviridae* are grouped in the order *Mindivirales*. Unlike plasma viruses, which leave their host by budding and do not lyse their hosts, these organisms are particularly virulent in that they cause the lysis of their bacterial host cells towards the end of the viral reproduction cycle (Mäntynen et al. 2018).

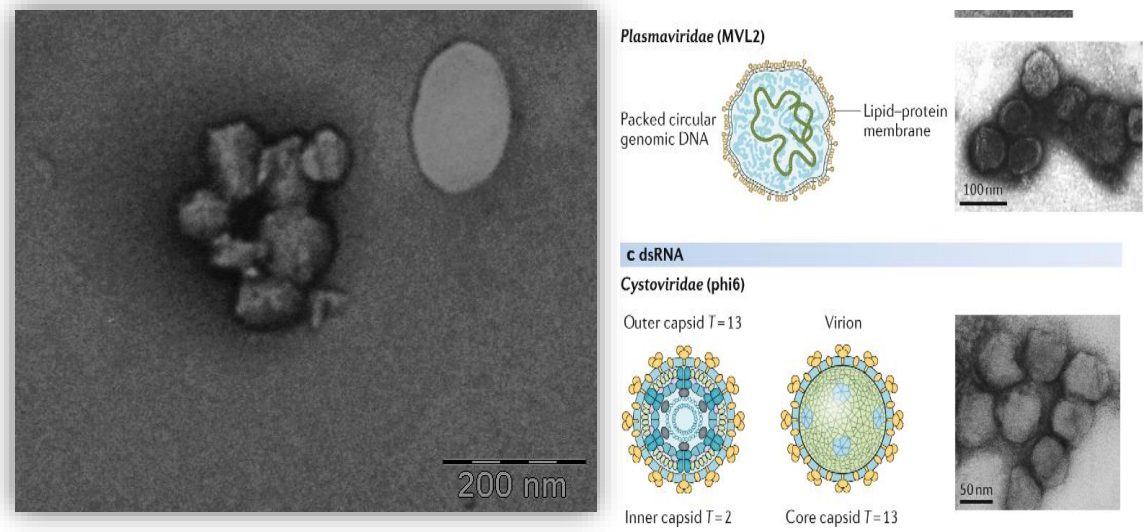

Figure 6.5: The transmission electron micrograph of phage 11-3(b)-S1 from this study, on the left, and comparison to electron micrographs from literature (Dion et al. 2020), on the right.

The morphology of phage 11-4(b)-S2 was similar to the one of the organisms belonging to the family *Siphoviridae* (Figure 6.6). These organisms have a head-tail morphology indicating that they form part of the 96% tailed viruses belonging to the order *Caudovirales*.

Bacteriophages in the order *Caudovirales* have a tail extension used to identify the host and provide special genome delivery (Veesler and Cambillau 2011, Bebeacua et al. 2013). *Caudovirales* phages are divided into three different families based on the shape of their tails: *Siphoviridae*, which has long non-contractile tails, and *Podoviridae*, which has short non-contractile tails. And *Myoviridae* has complex contractile tails (Hatfull et al. 2008, Veesler and Cambillau 2011, Bebeacua et al. 2013). The temperate *Siphoviridae* phages usually integrate at predetermined loci (Hatfull and Sarkis 1993, Hatfull et al. 2008, Badaway et al. 2020).

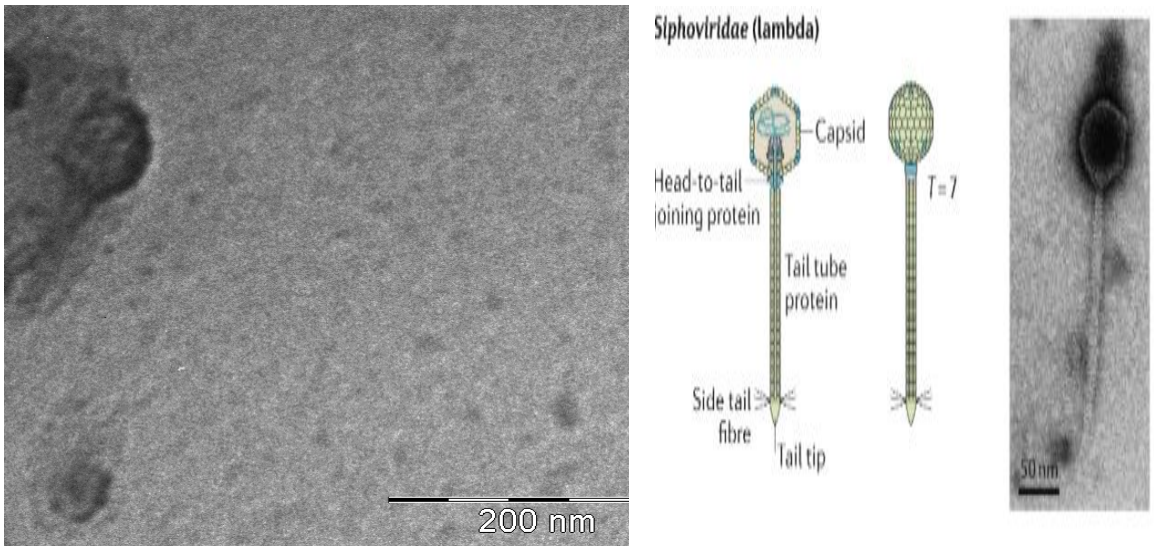

Figure 6.6: The transmission electron micrograph of phage 11-4(b)-S2 from this study, on the left, and comparison to the electron micrographs from literature (Dion et al. 2020), on the right.

6.3.2.2. Phages isolated from fishpond water samples

Figure 6.7 gives an overview of the morphology of phage 11-3(a)-FP1. The variation in the morphology observed suggested that there was more than one specific phage. Their morphology resembled those belonging to one of the two groups depicted in the picture on the right, which was taken from a research study carried out in 2020 by Dion and colleagues. The members of the *Tectiviridae* family are double-stranded DNA (dsDNA) phages without tails distinguished by an internal protein-rich lipid membrane encased in an icosahedral proteinaceous capsid not enveloped (Mäntynen et al. 2019). The *Tectiviridae* includes both virulent and temperate species.

There are two categories into which the *Tectiviridae* members can be placed: i) Gram-negative bacteria are hosts for a wide variety of bacteriophages, and ii) bacteriophages with a specific host range infecting Gram-positive *Bacillus* species (Bamford and Butcher 2008).

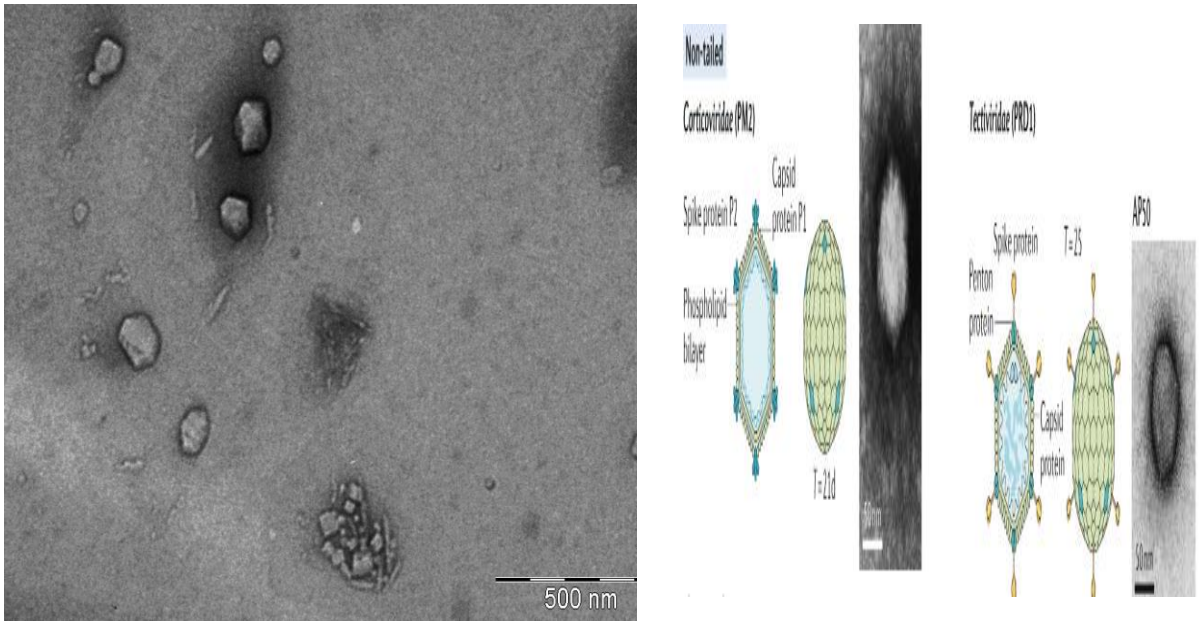

Figure 6.7: Transmission electron micrograph of phage 11-3(a)-FP1 from this study, on the left, and comparison to the electron micrographs from literature (Dion et al. 2020), on the right.

Contrarily, the *Corticoviridae* family of virulent, icosahedral viruses with internal membranes and double-stranded circular DNA genomes are extremely supercoiled (Chapter 2, Table 2.5). Compared to the *Tectiviridae* family, which was previously mentioned and may infect both Gram-negative and Gram-positive bacterial isolates, members of this family have a narrower host spectrum. The *Corticoviridae* family only has one identified species in the genus *Corticovirus*, the virulent *Pseudoalteromonas* virus PM2, which infects Gram-negative *Pseudoalteromonas* species (Oksanen and ICTV Report Consortium 2017).

The morphology of phage 11-3(b)-FP1 (Figure 6.8) showed that the virus is a member of the family *Microviridae. Microviridae* members are small icosahedral viruses with circular single-stranded DNA genomes. The *Microviridae* family consists of icosahedral lytic viruses with circular single-stranded DNA genomes. The family is divided into two groups based on genome characteristics and virion structure (Roux et al. 2012).

Viruses that infect *Enterobacteria* are classified as *Microviruses*, whereas those that infect obligate parasitic bacteria, such as *Chlamydia*, *Spiroplasma,* and *Bdellovibrio*, are classified as *Gokushovirinae* (Roux et al. 2012). Initially, *Enterobacteriaceae* was used to isolate the organisms that make up the genus *Microvirus*. However, recent research has shown that *Microviridae* appears widespread since they have been isolated from human faeces, the gut, and freshwater and marine environments (Roux et al. 2012). They have also been discovered as temperate phages integrated into the genomes of the *Bacteroidota* species (Krupovic and Forterre 2011).

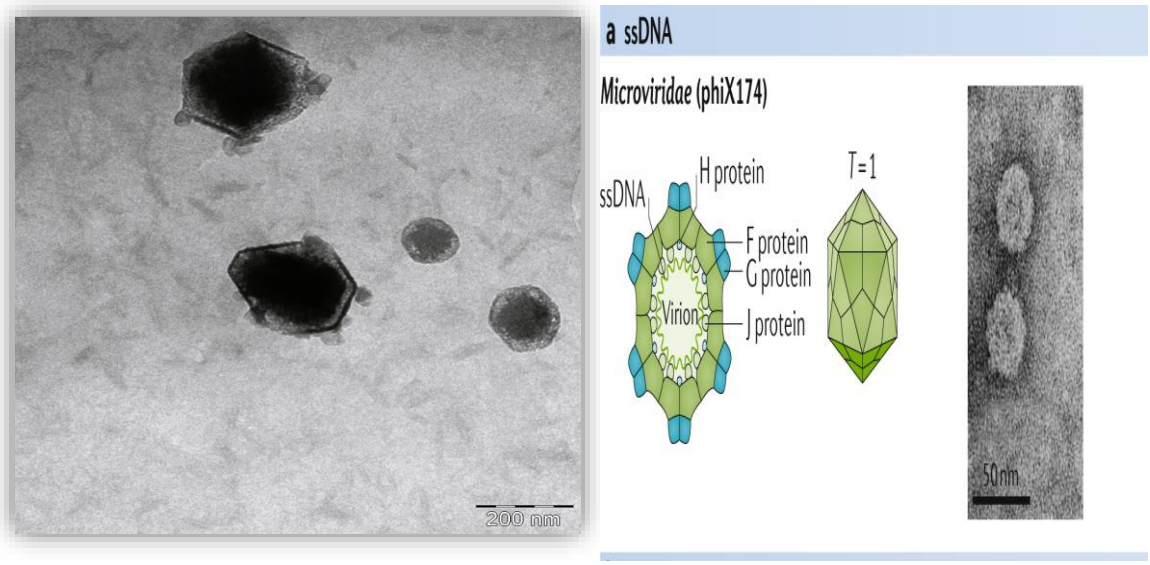

Figure 6.8: Transmission electron micrograph of phage 11-3(b)-FP1 from this study, on the left, and comparison to the electron micrographs from literature (Dion et al., 2020), on the right.

Despite difficulties visualizing every phage sample, which led to hazy images, several phage types were found, possibly belonging to *Corticoviridae, Plasmaviridae, Microviridae, Siphoviridae and Tectiviridae* and therefore, TEM was an effective method for identifying the morphology of the phages. Low viral concentrations and/or the purification stage using ammonium acetate may have accounted for the difficulties in visualizing the bacteriophages. The phages can also be sequenced to aid in the identification.

6.4. Conclusions

This part of the study aimed to extract phages from sewage and fishpond water samples, which were able to lyse the six *Kaistella* fish isolates and the reference strains and then evaluate their host strain range specificity through their lytic profiles and classify the phages based on electron microscopy. Thirty-four phage isolates were obtained from the sewage and fishpond water samples using a simple isolation procedure. For the lytic spectrum analysis, only eight phage isolates were used. The various lytic spectra showed that the isolated phages could lyse the *Kaistella* isolates and could, therefore, be used to control the growth of these *Kaistella* isolates. However, additional characterization studies are required before this ability can be thoroughly exploited.

Strains SH 11-3(a) and SH 11-3(b) showed sensitivity toward a few phage isolates, meaning these strains had many receptors the phages could recognize. In addition, phage 11-3(b)-S2 showed a broader host spectrum than the other phage isolates. It displayed lytic activity against 5 out of 12 *Kaistella* isolates used in this study. Therefore, due to its ability to form clear large plaques on a broad host range, this phage isolate may be an exciting candidate for sanitizing applications and needs further characterization. A great diversity of phages recovered from the sewage water sample were able to infect members in the genus *Kaistella* including the reference strains. '*Kaistella piscis*' SH 19-2(a), *K. koreensis, K. carnis*, and *K. yonginensis* displayed the most significant resistance towards phage infection since they were unaffected by any of the isolated phages.

It was possible to identify most isolated phages as members of the *Cystoviridae, Corticoviridae, Plasmaviridae, Siphoviridae*, and *Tectiviridae* families using transmission electron microscopy to examine morphological traits. Some viruses were only provisionally classified into certain families since drawing firm judgments about their identities was impossible. To the author's knowledge, this was the first study to report on bacteriophages that were active against *Kaistella* isolates.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

The genus *Kaistella* was previously regarded to belong to the family *Flavobacteriaceae*; however, the latest findings from a whole genome sequence (WGS) analysis of 1000 type strain genomes from the phylum *Bacteroidota* demonstrated that they belong to the family *Weeksellaceae* (García-López et al. 2019). *Kaistella koreensis* was identified as the type species of the genus when it was first described by Kim et al. (2004), but the taxonomic status of the genus has changed since that time. Eleven additional species of *Chryseobacterium* were recently transferred to the genus *Kaistella* (Nicholson et al. 2020). The genus *Kaistella* currently comprises 15 validly published species (Parte et al. 2020).

In Chapter 2 of this study, a comprehensive literature review was performed, which investigated the history and current taxonomy of the *Kaistella* genus and methods used to describe novel species. The significance of organisms being present in specific sources was explored regarding the production of virulence factors. Enzyme production, siderophore production, biofilm production, and antibiotic resistance were discussed. As a biocontrol mechanism, bacteriophage therapy was last investigated as a possible biocontrol method.

Members of the genus *Kaistella* are aerobic, Gram-staining-negative rods isolated from various clinical, environmental, industrial and food sources (Hantsis-Zacharov and Halpern 2007, Kämpfer et al. 2009, Kämpfer et al. 2011, Peng et al. 2021, Nicholson et al. 2020). Six species, namely *K. antarctica* (Yi et al. 2005, Kämpfer et al. 2009, Nicholson et al. 2020), *K. flava* (Peng et al. 2021), *K. gelatinilytica* (Ren et al. 2021), *K. montana* (Guo et al. 2016, Nicholson et al. 2020), *K. palustris* (Pires et al. 2010, Nicholson et al. 2020) and *K. solincola* (Benmalek et al. 2010, Nicholson et al. 2020) were isolated from various soil environments. *Kaistella* species have also been identified in aquatic environments, forming part of the bacterial community isolated from aquatic animals (Kämpfer et al. 2011). For instance, *Kaistella chaponensis* recovered from diseased Atlantic salmon farmed in Lake Chapo, was identified as a fish pathogen after being isolated from exterior lesions, gills, and fins of diseased fish in mixed cultures with the infamous salmonid pathogen, *Flavobacterium psychrophilum* (Kämpfer et al. 2011). *Kaistella haifensis*, formerly *Chryseobacterium haifense,* has been associated with proteolytic activity. The organism was reported to have a wide range of proteolytic and lipolytic abilities during an investigation of the psychrotolerant bacterial community in raw milk (Hantsis-Zacharov and Halpern 2007).

An organism must first have its taxonomy investigated to be identified. The theory and procedure of classifying organisms is taxonomy (Prakash et al. 2007). The techniques used to categorize and classify microorganisms include automated biochemical analysis, conventional techniques, and molecular-based analysis (Vandamme et al. 1996, Morgan et al. 2009, Donelli et al. 2013, Hugenholtz et al. 2021).

In Chapter 3, the first and second objectives of this study were to subject four *Kaistella* strains previously isolated from fish (Engelbrecht 1992, Gavu 2019) to the latest taxonomic techniques and to describe and name any new species by using a polyphasic approach that included the following methods: whole-genome and 16S rRNA gene sequencing, determination of the nearest neighbours, the use of conventional and commercial phenotypic methods to determine the phenotypic characteristics of strains SH 11-4(b), SH 19-2(b), SH 20-4 and SH 40-3.

Phylogenetic analysis based on 16S rRNA gene sequences was obtained by constructing phylogenetic trees, which were then used to determine the genus to which the strains belonged and their closest neighbours. It was confirmed that this study's four unidentified bacterial strains represented members of the genus *Kaistella.* The phylogenetic trees revealed that the four test isolates were most closely related to *K. carnis;* however, strain SH 20-4 clustered more closely with *K. carnis.* The results of the GenBank blast and EzBioCloud for 16S rRNA gene sequences for these organisms showed that strains SH 11-4(b) (*K. antarctica* 97.01%, *K. carnis* 97.41%), SH 19-2(b) (*K. antarctica* 97.01%, *K. carnis* 97.41%), and SH 40-3 (*K. antarctica* 97.01%, *K. carnis* 97.41%) might be novel species of the genus *Kaistella* since they shared similarity percentages lower than 98.7%, the cut-off value for species delineation (Kim et al. 2014). Strain SH 20-4 was regarded as another member of *Kaistella carnis* since it shared a 16S rRNA gene sequence similarity percentage of 99.30%. However, more data was required to confirm the 16S rRNA gene sequencing results.

Whole-genome sequence analysis of the test strains further supported the affiliation of the organisms with the genus *Kaistella*. The G+C content of strains SH 11-4(b) (35.10%), SH 19-2(b) (35.00%), SH 20-4 (36.29%), and SH 40-3 (35.00%) fell within the ranges of the members in the genus (31.3–41.6 mol%) (Kim et al. 2004, Nicholson et al. 2020). Overall genome similarity metrics (dDDH, ANI and AAI) revealed the most significant similarity of strain SH 11-4(b) to *Kaistella antarctica* (25.40%, 81.94%, 85.55%) and *Kaistella carnis* (27.70%, 82.32%, 85.34%); strain SH 19-2(b) to *Kaistella antarctica* (25.40%, 81.83%, 85.52%) and *Kaistella carnis* (26.00%, 82.17%, 75.24%); strain SH 20-4 to *Kaistella antarctica* (22.30%, 79.19%, 81.07%) and *Kaistella carnis* (79.70%, 97.84%, 96.98%), and lastly, that of strain SH 40-3 to *Kaistella antarctica* (25.40%, 81.93%, 85.30%) and *Kaistella carnis* (20.30%, 7.92%, 79.17%).

These results confirmed that strains SH 11-4(b), SH 19-2(b), and SH 40-3 were novel members of the genus *Kaistella* and cemented strain SH 20-4 as another species of *Kaistella carnis*. It was also clear that the four fish isolates had very close relationships since they shared the same nearest neighbours and clustered tightly on the phylogenetic trees.

According to the Venn diagrams generated with the coding sequences with bidirectional best hits, strains SH 11-4(b), SH 19-2(b), and SH 40-3 contained genes associated with hydrolytic activity such as amidases (nicotinamidases), *S41* peptidases, and phosphatases, metallopeptidases and multidrug resistance proteins (MRP) which further confirms their affiliation to the genus *Kaistella,* since these characteristics are common to the members of the genus *Kaistella* (Peng et al. 2021). Based on the above phenotypic, genotypic, and phylogenetic characterizations, strains SH 11-4(b), SH 19-2(b) and SH 40-3 represented novel species of the genus *Kaistella* for which the names *Kaistella merluccii*, *Kaistella piscis*, *Kaistella frigidipiscis,* respectively, were proposed.

The third objective of this study was to investigate the potential pathogenicity of the six *Kaistella* fish isolates. Although the pathogenicity of *K. chaponensis* has been documented, little is known about the pathogenicity of another genus *Kaistella*. There was, therefore, a need to investigate the ability of these species to act as pathogens and/or food spoilage organisms. Virulence factors and determination of spoilage potentials, such as blood haemolysis, enzyme production, siderophore production, and biofilm production, were used to determine these characteristics.

An overview of the ability of the test isolates and the reference strains to produce siderophores at 25, 32, and 37 °C indicated that all the bacterial strains could produce siderophores; however, the amount produced varied depending on the temperature and the optimal growth temperature of the specific strain. Siderophore production was more pronounced at 25 °C and less at 37 °C. Compared to all the other organisms used in this investigation, *C. gleum* produced more significant siderophores, and its ability to produce abundant iron chelators makes it hypervirulent.

Multiple bacterial genes typically encode virulence factors. The type and number of proteins that a bacterium expresses to complete its life cycle in a host play a role in determining the organism's pathogenicity (Zachary 2017). Gelatinase was produced by 11/12 organisms used in this study, followed by lecithinase and hyaluronidase, which were expressed by 42% (5/12) of the microorganisms. As previously mentioned, the ability of *C. gleum* to synthesize 7/8 virulence enzymes selected for this study, which it shared with *K. antarctica,* made it possible to classify it as a hypervirulent organism.

Another potential pathogen was '*K. merluccii*' SH 11-4(b), which was able to produce more than 4/8 virulence enzymes. *Kaistella koreensis* (4/8), '*K. frigidipiscis*' SH 40-3 (3/8), SH 20-4 (3/8), '*K. piscis*' SH 19-2(b) (3/8) and SH 11-3(a) (3/8) were among the other organisms that could synthesise more than two enzymes, which indicated that they could be categorized as pathogens (Zachary 2017). It was concluded that *K. carnis* and *K. yonginensis* should not be classified as pathogens due to their inability to express more than two enzymes related to virulence effectively.

All organisms used in this study exhibited β-haemolysis on horse blood agar. Two tested bacterial strains ('*K. merluccii*' SH 11-4(b) and *C. gleum*) showed both α- and β-haemolysis. An essential component of virulence is the ability of bacteria to synthesize haemolysins, compounds that disrupt membranes, cause cell lysis, and destroy nearby cells and tissues (Mogrovejo et al. 2020).

Another step in determining the possible pathogenicity of the test isolates was determining whether they were resistant to antibiotics since antibiotic resistance has become one of the significant public health issues of the twenty-first century and threatens to prevent and cure a wide range of bacterial infections effectively (Prestinaci et al. 2015). The strains SH 11-3(a), '*K. frigidipiscis*' SH 40-3, *C. gleum*, and *K. antarctica* were the most resistant, while *K. yonginensis* showed the highest susceptibility to the antimicrobials evaluated in this study. Based on the findings of the antimicrobial tests, fluoroquinolone and cephem antimicrobials will be the most effective for treating *Kaistella* infections.

In Chapter 5, the last step used in this study to determine the potential pathogenicity of the *Kaistella* species was to evaluate their ability to form biofilms and the efficacy of five inhibitory compounds against biofilm formation by these organisms. Biofilms may contain spoilage and pathogenic microorganisms that increase post-processing contamination and risk to public health and are, in many cases, most resistant to cleaning and decontamination procedures in the food industry (Shi and Zhu 2009, Carrascosa et al. 2021). The Congo red agar (CRA), tube (TM) and tissue culture plate (TCP) methods were used to screen for the ability of the organisms to form biofilms. The TCP method was regarded as the most effective, reliable, and gold standard for biofilm detection. Strains SH 20-4, *K. koreensis*, and *K. antarctic*a were considered significant biofilm formers since they gave positive results for biofilm formation with all three methods used in this investigation, while strains SH 11-3(a) and ''*K. merluccii*'' SH 11-4(b) were the least successful in forming biofilms.

Antibiofilm activity by the five compounds used in this study (D (+)-glucose, D-leucine, EDTA, fucoidan, proteinase K) revealed that glucose was the most effective inhibition compound against the *Kaistella* test strains and reference strains used in this study.

Significant inhibition was observed at the minimum inhibitory concentration (MIC, 100 mM), and some organisms were also inhibited at sub-minimum inhibitory concentrations (50 mM). The highest inhibition index was observed in *C. gleum* (81.7%), *K. yonginensis* (71.1%), *K. koreensis* (65.8%), *K. carnis* (61.0%), while the lowest was observed in *K. antarctica* (26.5%), *P. aeruginosa* (34.1%) and SH 20-4 (34.7%). Proteinase K was the least effective compound; its percentage inhibition ranged between -21.2% and 34.7%. Organisms whose biofilms showed the most significant resistance to inhibition compounds included SH 20-4, '*K. frigidipiscis*' SH 40-3, *K. antarctica* and *P. aeruginosa*, while susceptibility was mostly observed in SH 11-3(a), SH 11-3(b),'*K. merluccii*' SH 11-4(b),'*K. piscis*' SH 19-2(b), and they shared this trait with *C. gleum, K. koreensis,* and *K. chaponensis*.

In Chapter 6, the last objective of this study was to use phage therapy as a control measure against any novel *Kaistella* species that may be pathogenic. Although the use of bacterial viruses to treat bacterial infections has been known for about a century, it has only recently gained prominence due to the advent of multidrug-resistant pathogens (Lin et al. 2017). They have been proposed as bio preservatives in the food industry, as alternatives to antibiotics in animal health, and as tools for identifying harmful bacteria throughout the food chain (Garca et al. 2008).

Bacteriophages infecting various strains of *Kaistella* fish isolates were isolated from sewage water samples and fishpond water. Strains SH 11-3(a) and SH 11-3(b) showed sensitivity to a few phage isolates, meaning these strains had many receptors that the phages could recognize. The various lytic spectra showed that the isolated phages could lyse the *Kaistella* isolates and, therefore, could be used to control the growth of these *Kaistella* isolates. However, additional characterization studies will be required before this ability can be fully exploited. Phage 11-3(b)-S2 showed a broader host spectrum than the other phage isolates in that it had lytic activity against 5 out of 12 *Kaistella* isolates used in this study. Therefore, due to its ability to form large clear plaques in a broad host range, this phage isolate may be an exciting candidate for sanitizing applications. It was possible to identify most isolated phages as members of the *Corticoviridae, Plasmaviridae, Microviridae, Siphoviridae,* and *Tectiviridae* families using transmission electron microscopy.

It is evident that '*K. merluccii*' SH 11-4(b) and '*K. frigidipiscis*' SH 40-3 had exceptional characteristics, as they both formed part of strains that produced visible siderophores, '*K. merluccii*' SH 11-4(b) produced 4/8 virulence enzymes, while '*K. frigidipiscis*' SH 40-3 demonstrated resistance to 9/17 antimicrobials.

'*Kaistella frigidipiscis*' SH 40-3 was among the organisms whose biofilms were resistant to the five antibiofilm compounds. Results from Chapter 6 showed that strains SH 11-3(a), '*K. merluccii*' SH 11-4(b), and '*K. frigidipiscis*' SH 40-3 can be controlled by phage therapy because they showed susceptibility to phage strains obtained from the sewage and fishpond water samples. However, it will be a challenge to treat '*K. piscis*' SH 19-2(b) with any of the phages isolated in this study, since the organism, together with *K. koreensis, K. carnis* and *K. yonginensis,* were resistant to all the phages isolated in this study. In Chapter 3, it was discovered that a phage protein was among the genes identified in '*K. piscis*' SH 19-2(b), which, therefore, explains the immunity of the organism to be attacked by phage, as bacteria with an integrated phage, have been shown to demonstrate immunity to inhibition compounds and infection by other phages (Bondy-Denomy et al. 2016, Colavecchio et al. 2017, Fillol-Salom et al. 2019).

In conclusion, using a polyphasic approach, it was possible to accurately classify, describe, and name three new members of the genus *Kaistella*. It was possible to determine the significance of six *Kaistella* strains in virulence. Lastly, this study demonstrated that some isolated phages could potentially prevent, eliminate, or reduce *Kaistella* infections in fish or on food industry surfaces.

Future research

i. Estimating the spoilage potential of the three novel *Kaistella* isolates by determining the effect of other parameters such as pH, water activity, oxygen concentration, and nutrient content of the food on the growth of *Kaistella* species.

ii. The genome of the novel strains can be further investigated for the presence of spoilage, phage, and virulence-associated genes and their functions.

iii. Determination of the complete genome sequence of newly isolated phage strains that demonstrated lysis of *Kaistella* species.

iv. Characterisation of phage isolates and performance of efficacy tests in *vivo.*

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