UNIVERSITY OF LJUBLJANA | VF Veterinary Faculty

BIODIVERSITY OF PATHOGENIC FLAVOBACTERIA IN RAINBOW TROUT (*Oncorhynchus mykiss*) AND DYNAMICS OF INFECTIONS IN SELECTED SLOVENE FISH FARMS

Doctoral dissertation

Katarina Pavlin

Ljubljana, 2025

UNIVERSITY OF LJUBLJANA | VF Veterinary Faculty

The interdisciplinary doctoral programme in Biomedicine

veterinary medicine

UDK 582.23:597:616-022.3:620.266:639.3(494.4)(043.3)

Katarina Pavlin, DVM

BIODIVERSITY OF PATHOGENIC FLAVOBACTERIA IN RAINBOW TROUT (*Oncorhynchus mykiss*) AND DYNAMICS OF INFECTIONS IN SELECTED SLOVENE FISH FARMS

Doctoral dissertation

BIODIVERZITETA PATOGENIH FLAVOBAKTERIJ PRI ŠARENKAH (*Oncorhynchus mykiss*) IN DINAMIKA OKUŽB V NEKATERIH SLOVENSKIH RIBOGOJNICAH

Doktorska disertacija

Ljubljana, 2025

Katarina Pavlin

Biodiversity of pathogenic flavobacteria in rainbow trout (*Oncorhynchus mykiss*) and dynamics of infections in selected Slovene fish farms

The research was carried out at the Institute of Pathology, Wild Animals, Fish and Bees, and at the Institute of Microbiology and Parasitology of the Veterinary Faculty of the University of Ljubljana.

Date of public defence:

Mentor: Assoc. Prof. Dr. Tanja Švara, DVM
University of Ljubljana, Veterinary Faculty
Co-mentor: Sr. Res. Fell. Dr. Matjaž Ocepek, DVM
University of Ljubljana, Veterinary Faculty

The expert committee for evaluation and defence:
President: Prof. Dr. Vlasta Jenčič, DVM, University of Ljubljana, Veterinary Faculty
Member: Prof. Dr. Andrej Pengov, DVM, University of Ljubljana, Veterinary Faculty
Member: Prof. Dr. Heike Schmidt-Posthaus, DVM, University of Bern, Vetsuisse Faculty

I declare that the doctoral dissertation is the result of my own research work, that the results are correctly stated, and that I did not violate the copyrights and intellectual property of others. I declare that the electronic copy is identical to the printed copy.

ABSTRACT

Keywords: *Flavobacterium*; matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS); whole-genome sequencing (WGS); pathology; antimicrobial susceptibility; water quality.

This research aimed to investigate the biodiversity of *Flavobacterium* species and the aetiology of flavobacterial diseases in Slovene farmed rainbow trout (*Oncorhynchus mykiss*) throughout all seasons, assess the impact of infection on fish health by qualitative and semiquantitative evaluation of gross and histopathological lesions, determine antimicrobial susceptibility of flavobacteria using the standardised broth microdilution method and evaluate associations between water temperature, pH, dissolved oxygen, and flavobacterial infections. A total of 540 rainbow trout were sampled, namely longitudinally across all seasons in two farms and in two additional farms following mortality reports. We confirmed the presence of two known pathogens, *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum,* in 23.7% and 2.04% of fish, respectively, and described associated pathological lesions. Our results also show that *F. hercynium* and *F. hydatis* can cause pathological lesions in fish. Numerous other lesser-known flavobacteria and 41 putative new species were identified, although their pathogenic potential remains to be clarified. Antimicrobial susceptibility testing revealed that *Flavobacterium* species were predominantly multidrug-resistant. *F. psychrophilum* showed high non-wild-type frequencies for oxytetracycline and quinolones and high minimum inhibitory concentrations (MICs) for ceftazidime and trimethoprim/sulfamethoxazole. However, all isolates were wild type for florfenicol. Lesser-known species exhibited high MICs across at least three antimicrobial classes, and some for all 10 antimicrobials tested. Adjustment of the standardised method for *F. psychrophilum* enabled successful testing of *F. branchiophilum* and other fastidious flavobacteria. *F. psychrophilum* infections were associated significantly with elevated water pH at the inlet and winter season. In contrast, *F. branchiophilum* infections correlated with increased water temperatures, decreased dissolved oxygen at the outlet, and the summer season. This research

contributes to a better understanding of flavobacterial infections in Slovene aquaculture, enables informed strategies for disease treatment and control, and provides a foundation for future studies on flavobacterial biodiversity and their impact on fish health.

IZVLEČEK

Ključne besede: *Flavobacterium*; masna spektrometrija, ionizacija v matriksu z lasersko desorpcijo – čas preleta ionov (MALDI-TOF MS); sekvenciranje celotnega genoma (WGS); patologija; občutljivost za protimikrobna zdravila; kakovost vode.

Namen raziskave je bil preučiti biodiverziteto bakterij iz rodu *Flavobacterium* in etiologijo flavobakterijskih bolezni pri gojeni šarenki (*Oncorhynchus mykiss*) v Sloveniji v vseh letnih časih, raziskati vpliv okužbe na zdravje rib s kvalitativno in semikvantitativno oceno patoanatomskih in histopatoloških sprememb, določiti občutljivost flavobakterij za protimikrobna zdravila z uporabo standardizirane mikrodilucijske metode ter analizirati korelacije med temperaturo vode, pH in vsebnostjo raztopljenega kisika ter flavobakterijskimi okužbami. Skupno smo vzorčili 540 šarenk, in sicer v dveh ribogojnicah v okviru longitudinalnega vzorčenja v vseh letnih časih ter v dveh drugih ribogojnicah po prijavi povišanega pogina. V raziskavi smo ugotovili prisotnost znanih patogenov *Flavobacterium psychrophilum* in *Flavobacterium branchiophilum* pri 23,7 % oziroma 2,04 % rib ter opisali z okužbo povezane patološke spremembe. Poleg tega rezultati nakazujejo, da lahko vrsti *F. hercynium* in *F. hydatis* povzročata patološke spremembe pri ribah. Ugotovili smo tudi številne druge manj znane flavobakterije in identificirali 41 potencialno novih vrst, vendar njihova patogenost še ni pojasnjena. Testiranje protimikrobne občutljivosti (AST) je pokazalo, da so bakterije rodu *Flavobacterium* večinoma multirezistentne. Visok delež izolatov *F. psychrophilum* je bil zunaj območja divjega tipa za oksitetraciklin in kinolone; poleg tega so izolati imeli visoke minimalne inhibitorne koncentracije (MIC) za ceftazidim in trimetoprim/sulfametoksazol. Vsi izolati so bili divjega tipa za florfenikol. Manj znane vrste flavobakterij so imele visoke MIC za vsaj tri skupine protimikrobnih zdravil, nekatere pa za vseh deset testiranih učinkovin. Prilagoditev standardizirane metode za *F. psychrophilum* je omogočila uspešno AST *F. branchiophilum* in drugih za gojenje zahtevnih flavobakterij. Okužbe s *F. psychrophilum* so bile statistično značilno povezane s povišanim pH vode na dotoku bazena ter zimskim obdobjem. Nasprotno pa so bile okužbe s

F. branchiophilum povezane s povišanimi temperaturami vode, znižanjem raztopljenega kisika na iztoku bazena ter poletnim obdobjem. Raziskava prispeva k boljšemu razumevanju flavobakterijskih okužb v slovenskem ribogojstvu, omogoča oblikovanje premišljenih strategij za zdravljenje in obvladovanje bolezni ter predstavlja osnovo za nadaljnje raziskave biodiverzitete flavobakterij in njihovega vpliva na zdravje rib.

TABLE OF CONTENTS

ABSTRACT 5

IZVLEČEK 7

TABLE OF CONTENTS 9

LIST OF TABLES 13

LIST OF FIGURES 16

LIST OF APPENDICES 22

LIST OF ABBREVIATIONS AND SYMBOLS 23

PUBLISHED ARTICLE 25

1 INTRODUCTION 26

1.1 RESEARCH OBJECTIVES AND HYPOTHESES 27

2 LITERATURE REVIEW 28

2.1 THE GENUS *FLAVOBACTERIUM* 28

2.2 FLAVOBACTERIAL DISEASES, GROSS AND HISTOPATHOLOGICAL LESIONS 30

2.2.1 Well-known fish-pathogenic flavobacteria 33

2.2.1.1 *Flavobacterium psychrophilum* 33

2.2.1.2 *Flavobacterium columnare* 36

2.2.1.3 *Flavobacterium branchiophilum* 38

2.2.2 Other fish-pathogenic flavobacteria 39

2.3 PATHOGENESIS OF FLAVOBACTERIAL INFECTIONS 41

2.3.1 *Flavobacterium psychrophilum* 44

2.3.2 *Flavobacterium columnare* 46

2.3.3 *Flavobacterium branchiophilum* 47

2.4 DIAGNOSTIC METHODS 49

2.4.1 Culture 50

2.4.2 Identification of isolates 52
2.5 TREATMENT AND ANTIMICROBIAL RESISTANCE 54
2.5.1 Phenotypic characterization of antimicrobial resistance profiles 56
2.5.2 Genotypic characterization of antimicrobial resistance profiles 58
2.6 PREVENTION OF FLAVOBACTERIAL DISEASES AND INFLUENCE OF WATER QUALITY ON THEIR OCCURRENCE 60
3 MATERIALS AND METHODS 63
3.1 FISH SAMPLING AND WATER QUALITY MEASUREMENTS 63
3.2 NECROPSY AND SAMPLING 66
3.3 HISTOPATHOLOGICAL INVESTIGATION 68
3.4 BACTERIOLOGICAL INVESTIGATION 77
3.5 IDENTIFICATION OF ISOLATES 78
3.5.1 Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry 78
3.5.2 Whole-genome sequencing and bioinformatics analysis 79
3.6 ANTIMICROBIAL SUSCEPTIBILITY TESTING 80
3.6.1 Phenotypic AST of *Flavobacterium psychrophilum* 81
3.6.2 Phenotypic AST of *Flavobacterium branchiophilum* 82
3.6.3 Phenotypic AST of lesser-known *Flavobacterium* species 83
3.6.4 Determination of wild type (WT) and non-wild type (NWT) phenotypes 83
3.7 STATISTICAL ANALYSIS 84
4 RESULTS 89
4.1 SAMPLED FISH 89
4.2 DIVERSITY OF FLAVOBACTERIA IN SLOVENE FARMED RAINBOW TROUT 91
4.2.1 Well-known flavobacteria 96
4.2.2 Lesser-known flavobacteria 103

4.3 BIOMETRIC DATA, GROSS AND HISTOPATHOLOGICAL LESIONS IN FLAVOBACTERIAL DISEASES 116
4.3.1 Biometric data and gross lesions 116
4.3.1.1 *Flavobacterium psychrophilum* 116
4.3.1.2 *Flavobacterium branchiophilum* 122
4.3.1.3 Lesser-known *Flavobacterium* species 123
4.3.2 Histopathological lesions 124
4.3.2.1 *Flavobacterium psychrophilum* 124
4.3.2.2 *Flavobacterium branchiophilum* 146
4.3.2.3 Lesser-known *Flavobacterium* species 147
4.3.3 Histopathological and cytological investigation of filamentous bacteria 148
4.4 ANTIMICROBIAL SUSCEPTIBILITY 148
4.4.1 Quality control 148
4.4.2 Phenotypic AMR profiles of *Flavobacterium psychrophilum* 149
4.4.3 Phenotypic AMR profiles of *Flavobacterium branchiophilum* 154
4.4.4 Phenotypic AMR profiles of lesser-known *Flavobacterium* species 156
4.4.5 Genotypic AMR profiles of *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* isolates 163
4.5 WATER QUALITY PARAMETERS 164
4.6 OTHER FISH PATHOGENS 168
5 DISCUSSION 175
5.1 FLAVOBACTERIA IN SLOVENE FARMED RAINBOW TROUT AND THEIR ASSOCIATION WITH PATHOLOGICAL LESIONS 175
5.2 ANTIMICROBIAL SUSCEPTIBILITY OF SLOVENE *FLAVOBACTERIUM* SPECIES ISOLATES 185
5.3 INFLUENCE OF WATER QUALITY PARAMETERS ON THE ONSET OF FLAVOBACTERIOSIS 190

5.4 CONCURRENT INFECTIONS 193

6 CONCLUSIONS 195

7 SUMMARY 197

8 POVZETEK 204

9 ACKNOWLEDGMENTS 215

10 LITERATURE 217

APPENDICES 258

LIST OF TABLES

Table 1: General information on the fish farms (F) 65
Table 2: Description of organ variables and allocated score values for the assessment of gross lesions according to the adapted method developed by Adams et al. (1993) and Heath et al. (2004) 67
Table 3: Evaluated histopathological lesions and allocated importance factors (IF) according to the adapted method developed by Bernet et al. (1999), Saraiva et al. (2015), and Steinbach et al. (2016) 69
Table 4: Health-based categorisation of fish and associated inclusion criteria 88
Table 5: Information on the mean and range of body weight (W; g) and total length (L; cm), as well as the mean Fulton's condition factor (K) of diseased (D) and apparently healthy (H) fish from each fish tank (T) sampled during longitudinal sampling (LONG) in fish farms F1 and F2 89
Table 6: Information on the mean and range of body weight (W; g) and total length (L; cm), as well as the mean Fulton's condition factor (K) of diseased (D) and apparently healthy (H) fish from individual sampling (IND) in fish farms F3 and F4 91
Table 7: Number of diseased (D) and apparently healthy (H) fish and organs positive for *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* per sampling (LONG, longitudinal sampling; IND, individual sampling), fish farm (F), and fish tank 101
Table 8: Whole-genome sequencing data on seven *Flavobacterium psychrophilum* and two *Flavobacterium branchiophilum* isolates, including sequence read archive (SRA) run accession number, total genome size (GS), number of contigs (No.), N_{50}, GC content (GC), sequence type based on multilocus sequence typing (MLST ST), MLST allele profile, and molecular serotype 102
Table 9: Information on the isolation of the lesser-known flavobacteria identified with high confidence using whole-genome sequencing (WGS) and/or matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), including the fish farm (F), fish tank (T), number of isolates from diseased (D)

and apparently healthy (H) fish, organ of isolation and total number of positive fish (No.); putative new species are included if isolated from at least two fish 104
Table 10: Average nucleotide identity (ANI)-based species delineation of *Flavobacterium* isolates 108
Table 11: Necropsy variables associated with *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* infection, analysed per fish tank using the Mann–Whitney U test and Fisher's combined probability test, including mean, median, and standard deviation (sd) of each variable for infected (INF) fish and negative healthy (NH) fish; significant *p*-values ($p < 0.05$) are shown in red 118
Table 12: Histopathological (HP) variables associated with *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* infections analysed per fish tank using the Mann–Whitney U test and Fisher's combined probability test, including mean, median, and standard deviation (sd) of each variable, as well as percentage (%) of infected (INF) fish with a variable value above the median of negative healthy (NH) fish; significant *p*-values ($p < 0.05$) are shown in red 129
Table 13: Seven *Flavobacterium psychrophilum* isolates selected for whole-genome sequencing, representing six different resistotypes based on phenotypic antimicrobial susceptibility testing for ampicillin (AMP), enrofloxacin (ENR), erythromycin (ERY), florfenicol (FLO), oxolinic acid (OXO), and oxytetracycline (OXY); GyrA substitution associated with resistance to quinolones is also shown 164
Table 14: Measurements of water temperature (T), pH, and dissolved oxygen (O) at the inlet and outlet of the sampled fish tanks for each longitudinal (LONG) and individual (IND) sampling in fish farms F1 to F4 165
Table 15: Pearson and Spearman correlation analyses to investigate the correlation between *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* infections and water quality parameters, including water temperature (T), pH, and dissolved oxygen (O) at the inlet and outlet of sampled fish tanks, as well as sampled seasons; significant *p*-values ($p < 0.05$) are shown in red 168
Table 16: Information on the other pathogens identified with bacteriological and histopathological investigations of fish organs, including number (No.) of diseased (D) and apparently healthy (H) fish per fish farm (F) and fish tank (T), organ of

detection and connection with the isolation of *Flavobacterium psychrophilum* and/or *Flavobacterium branchiophilum* 169

LIST OF FIGURES

Figure 1: Study overview – a list of the methods used, along with a description of the purpose of each method 63
Figure 2: Sampling strategies including longitudinal (LONG) and individual (IND) samplings 64
Figure 3: The layout of the Sensititre custom AS1AQ microtitre plate (Trek Diagnostic Systems) 82
Figure 4: Considered statistical protocol 86
Figure 5: Necropsy fish index of diseased and apparently healthy fish and the determined threshold 87
Figure 6: Primary isolation of *Flavobacterium psychrophilum* on Anacker and Ordal's (AO) agar from gills (A), skin (B), and internal organs (C and D); note the different colony morphologies 93
Figure 7: Primary isolation of *Flavobacterium branchiophilum* (arrows) on Anacker and Ordal's (AO) agar from gills; multiple similar larger yellow colonies in figure A) represent putative new species FX10 94
Figure 8: Colony morphology of *Flavobacterium* spp. identified by whole-genome sequencing and subjected to antimicrobial susceptibility testing, namely a) *F. branchiarum* (F324), b) *F. bernardetii* (F609), c) *F. collinsii* (F1673), d) *F. celericrescens* (F1959), e) *F. difficile* (F1633), f) *F. erciyesense* (F562), g) *F. hercynium* (F2050), h) *F. oncorhynchi* (F1942), i) *F. piscis* (F1228), j) *F. tiangeerense* (F1485), k) *F. tructae* (F1900), l) FX2 (F334), m) FX3 (F1048), n) FX5 (F1140), o) FX9 (F945), p) FX10 (F1511), q) FX15 (F2167), r) FX18 (F626), s) FX23 (F1259), t) FX27 (F1527), u) FX32 (F1715) and v) FX36 (F2035) 96
Figure 9: Number of diseased (red) and apparently healthy fish (blue) testing positive for *Flavobacterium psychrophilum* per sampling (LONG, longitudinal sampling; IND, individual sampling) 97
Figure 10: Percentage of fish positive and infected with *Flavobacterium psychrophilum* per sampling (LONG, longitudinal sampling; IND, individual sampling); black full lines indicate standard deviations; grey dashed line and red X indicate overall rate across samplings and an average rate across other

samplings, respectively; colour of bars reflects significance based on Pearson chi-squared test, namely red for significantly higher, blue for significantly lower, and grey for no significant difference 99
Figure 11: Percentage of diseased and apparently healthy fish testing positive for *F. psychrophilum* per organ. 100
Figure 12: Dendrogram obtained by cluster analysis of MALDI-TOF MS spectra of selected *Flavobacterium* spp. identified using whole-genome sequencing 113
Figure 13: Primary isolation of A) FX3 (F1782) from the skin and B) *Flavobacterium difficile* (F1633), C) FX9 (F945), D) FX5 (F1140), E) FX10 (F1511) and F) FX15 (F2043) from the gills 113
Figure 14: Hierarchical clustering of fish (n = 482; at least one *Flavobacterium* species isolated) based on flavobacterial species isolated in more than 1% of fish, totalling 20 unique species and *Flavobacterium* spp. group; the top dendrogram shows fish that cluster based on similar species profiles, and the left dendrogram shows species that cluster based on co-occurrence in the same fish 116
Figure 15: Principal-component analysis of individual fish based on binary presence profiles of the top 1% most prevalent *Flavobacterium* species. Fish are coloured by sampling, showing that heterogeneous flavobacterial species were isolated at each sampling 116
Figure 16: Boxplots showing weight, length, and Fulton's condition factor in fish infected (INF) with *Flavobacterium psychrophilum* and negative healthy (NH) fish 117
Figure 17: Violin boxplots showing necropsy fish indices in fish testing positive (POS) for *Flavobacterium psychrophilum* and negative apparently healthy (NAH) fish; infected (INF) fish are POS fish at or above the threshold, and negative healthy (NH) fish are NAH fish below the threshold 118
Figure 18: Gross lesions in fish testing positive for *Flavobacterium psychrophilum* and negative for other identified systemic fish pathogens: A) severe ulceration of caudal fin and shallow skin ulceration, as well as skin hyperpigmentation; B) deep skin ulceration with reddish margins; C) perforative skin ulceration with yellowish margins; D) skin ulceration with yellowish margins; E) complete loss of caudal fin and deep ulceration of caudal peduncle with exposure of vertebral column; moderate shortening of the operculum is also evident; F) S-shaped tail 120

Figure 19: Gross lesions in fish testing positive for *Flavobacterium psychrophilum*: A) saddleback-like lesion due to skin ulceration behind the dorsal fin and shallow ulceration with scale loss extending laterally; anaemia of the gills and liver can also be observed; B) ulceration of the dorsal fin and skin hyperpigmentation; C) perisplenitis with loss of definition of the splenic capsule and haemorrhage in surrounding adipose tissue, as well as in the liver; exophthalmos is also evident; D) haemorrhage around the pyloric caeca and in the skeletal muscle tissue; moderate hepatic atrophy and mild splenic enlargement are also apparent; E) severe cachexia with severe hepatic atrophy and distended gall bladder; F) moderately enlarged kidney and spleen 122
Figure 20: Gross lesions in fish testing positive for *Flavobacterium branchiophilum*: A) severe gill hyperplasia with clubbing of the filaments; B) yellowish necrosis of the gill filament tips 123
Figure 21: Gross lesions in some of the fish that tested positive for lesser-known *Flavobacterium* species and negative for other identified systemic fish pathogens: A) shallow skin ulceration on the dorsal part of the caudal peduncle and caudal fin from which numerous *Flavobacterium bernardetii* colonies were isolated; B) mild erosion of the dorsal and caudal fins, multifocal scale loss, mild gill anaemia, light to dark grey-brown mottled liver, mild ascites and severe cachexia in a fish with numerous isolated FX3 colonies from the skin and individual colonies from the liver; C) large concave and hyperpigmented chronic skin lesion from which numerous FX10 colonies were isolated; D) deep skin ulceration from which numerous FX26 colonies were isolated 124
Figure 22: Violin boxplots showing the values of the organ indices for skin, gills, heart, spleen, kidney, liver, pancreas, stomach with pyloric caeca, and intestine in fish infected with *Flavobacterium psychrophilum* (INF) and negative healthy (NH) fish 125
Figure 23: Boxplots showing the values of the reaction indices for skin, gills, heart, spleen, kidney, liver, pancreas, stomach with pyloric caeca, and intestine in fish infected with *Flavobacterium psychrophilum* (INF) and negative healthy (NH) fish 129
Figure 24: Histopathological lesions in skin and gills of fish infected with *Flavobacterium psychrophilum*: A) shallow skin ulceration (thick arrow shows the

ulceration and thin arrow its margin) next to the dorsal fin with Zenker's degeneration of the underlying skeletal muscle tissue (star) as well as mild to moderate macrophage and neutrophil infiltration in the affected area and surrounding tissues (X) (100× magnification); B) numerous filamentous *F. psychrophilum* bacteria (star) adhering to and surrounding the caudal vertebra (X), which is exposed due to severe peduncle ulceration and necrosis (400× magnification); C) severe necrosis of the gill filament tip with exposed cartilage surrounded by filamentous *F. psychrophilum* bacteria (arrow), along with severe hyperplasia and lamellar fusion in the proximal part of the affected filament (100× magnification); D) severe gill epithelial hyperplasia with lamellar fusion and multifocal small areas of necrosis (long thin arrow), multifocal haemorrhage (short thin arrow) and mild lymphocyte infiltration (thick arrow) (200× magnification); E) thrombus (thick arrow) adherent to the endothelium of the central venous sinus of the gill filament with a mild macrophage infiltrate (long thin arrow) in the surrounding connective tissue as well as mild hypertrophy, hyperplasia (short thin arrows) and vacuolar degeneration of the epithelium (400× magnification); F) lymphocytic and neutrophilic perivasculitis of the gill arch efferent branchial arteriole with multifocal small areas of necrosis (arrows) (200× magnification); haematoxylin and eosin staining 140
Figure 25: Histopathological lesions in internal organs of fish infected with *Flavobacterium psychrophilum*: A) thrombus adherent to the endocardium overlying the spongy myocardium with mild hyaline (long arrows) and hydropic (short arrows) degeneration (400× magnification); B) necrotising endocarditis of the atrioventricular valves (X) with vegetations (star) infiltrated by neutrophils and lymphocytes (200× magnification); C) necrosis (star) at the compact/spongy interface of the ventricular myocardium and moderate infiltration of epicardium, myocardium and endocardium with lymphocytes and neutrophils (arrows) (HE, 200× magnification); D) necrosis and haemorrhage (star) in the splenic parenchyma with severe lymphocytic and neutrophilic perisplenitis (100× magnification); E) infiltration of kidney haematopoietic tissue with neutrophils (X), haemorrhage (star) and necrosis of tubules (short thin arrow) in the affected area, hyaline droplet degeneration of the surrounding kidney tubules (long thin arrow) and increased number and size of melanomacrophage centres (thick arrow) (200×

magnification); F) small liver necrosis with mild neutrophil (thick arrow) infiltration and erythocytes (thin arrow) indicating haemorrhage; note the lack of hepatocellular vacuolation (400× magnification); haematoxylin and eosin staining 145
Figure 26: Histopathological lesions in gills of fish infected with *Flavobacterium branchiophilum*: A) a group of *F. branchiophilum* bacteria and sloughed epithelial cells (thick arrow) in a pseudocyst, lamellar aneurysms (star) and hydropic or vacuolar degeneration (thin arrows) of gill epithelium (400× magnification); B) filamentous *F. branchiophilum* bacteria (thick arrow) along the gill epithelium with hyperplasia and fusion of gill lamellae; the epithelium shows mild lymphocytic infiltration (short thin arrows) and multifocal single-cell necrosis (long thin arrows) (400× magnification); haematoxylin and eosin staining 146
Figure 27: Microtitre plates for two selected *Flavobacterium psychrophilum* isolates. A) Isolate F1824 with smooth margins; B) Isolate F341 with rhizoid margins. For each microtitre plate, two images were merged between columns 6 and 7 so that all 96 wells are clearly visible 150
Figure 28: The distribution of minimum inhibitory concentrations (MICs) of 54 *Flavobacterium psychrophilum* isolates for 10 antimicrobials (A-J) determined using the broth microdilution method. The tested concentration range (µg/ml) is presented on the x-axis and the number of isolates on the y-axis. The solid black and dashed blue vertical lines represent the epidemiological cut-off values (ECVs) and the provisional epidemiological cut-off values for wild type (CO_{WT}) for *F. psychrophilum*, respectively. Where possible, the green bars represent the wild type (WT) isolates, and the red bars represent the non-wild type (NWT) isolates 153
Figure 29: Microtitre plates for two selected *Flavobacterium branchiophilum* isolates. A) Isolate F1517 on Anacker and Ordal (AO) broth; B) Isolate F1628 on diluted cation-adjusted Mueller-Hinton broth supplemented with additional cations (DCAMHB+C). For each microtitre plate, two images were merged between columns 5 and 6 so that all 96 wells are clearly visible 155
Figure 30: The distribution of minimum inhibitory concentrations (MICs) of eight *Flavobacterium branchiophilum* isolates determined using the broth microdilution method for AMP, ampicillin; CTZ, ceftazidime; ENR, enrofloxacin; ERY,

erythromycin; FLO, florfenicol; GEN, gentamicin; MER, meropenem; OXO, oxolinic acid; OXY, oxytetracycline; TRS, trimethoprim/sulfamethoxazole. The range of dilutions tested (µg/ml) for each antimicrobial is shaded gray. MICs higher than the highest concentration tested are given as the first concentration above the tested range, and MICs equal to or lower than the lowest concentration tested are underlined. The red and green numbers represent the MICs determined using diluted cation-adjusted Mueller-Hinton broth with additional cations (DCAMHB+C) and Anacker and Ordal (AO) broth, respectively 156
Figure 31: Mean water quality parameters per sampling; heatmap with normalised scale indicates low (0) or high (1) values, allowing comparison across water parameters and sampling points (LONG, longitudinal sampling; IND, individual sampling)..... 166
Figure 32: Hierarchical clustering of fish (n = 351; at least one other fish pathogen) based on semiquantitatively assessed infection with other fish pathogens. The heatmap shows infection severity from 0 (none) to 3 (severe). In the top dendrogram, fish are clustered based on similar infection profiles, and in the left dendrogram, fish pathogens are clustered based on their co-occurrence in the same fish 174

LIST OF APPENDICES

Appendix 1: Owner's informed consent form 258
Appendix 2: Questionnaire for fish farmers 260
Appendix 3: Information on the *Flavobacterium* isolates subjected to whole genome sequencing and/or antimicrobial susceptibility testing, including sampling time, fish farm, fish tank, health status of the fish, source of isolation, and other positive organs 272
Appendix 4: Pairwise comparison of blast-based average nucleotide identity (ANIb) values between *Flavobacterium* species isolates and their closest phylogenetic relatives 276
Appendix 5: Raw data on phenotypic antimicrobial susceptibility testing (minimum inhibitory concentrations in µg/ml) for the *Flavobacterium psychrophilum* ATCC 49418^T reference strain (Ref), the 54 Slovene *F. psychrophilum* isolates and other *Flavobacterium* spp., determined using the standardised method recommended for *F. psychrophilum* (CLSI, 2020a) for AMP, ampicillin; CTZ, ceftazidime; ENR, enrofloxacin; ERY, erythromycin; FLO, florfenicol; GEN, gentamicin; MER, meropenem; OXO, oxolinic acid; OXY, oxytetracycline; TRS, trimethoprim/sulfamethoxazole 285
Appendix 6: Raw data on phenotypic antimicrobial susceptibility testing (minimum inhibitory concentrations in µg/ml) for the eight Slovene *Flavobacterium branchiophilum* isolates and other *Flavobacterium* spp., determined using the adjusted method recommended for *F. psychrophilum* (CLSI, 2020a) for AMP, ampicillin; CTZ, ceftazidime; ENR, enrofloxacin; ERY, erythromycin; FLO, florfenicol; GEN, gentamicin; MER, meropenem; OXO, oxolinic acid; OXY, oxytetracycline; TRS, trimethoprim/sulfamethoxazole 288

LIST OF ABBREVIATIONS AND SYMBOLS

AMP	ampicillin
AMR	antimicrobial resistance
ANI analysis	average nucleotide identity analysis
AO medium	Anacker and Ordal's medium
AST	antimicrobial susceptibility testing
BCWD	bacterial cold-water disease
BGD	bacterial gill disease
CD	columnaris disease
CLSI	Clinical and Laboratory Standards Institute
CO_{WT}	epidemiological cut-off value for wild type
CTZ	ceftazidime
DCAMHB	diluted cation-adjusted Mueller-Hinton broth
ECV	epidemiological cut-off value
EGCs	eosinophilic granular cells
FLO	florfenicol
ENR	enrofloxacin
ERY	erythromycin
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GEE	generalised estimating equation
GEN	gentamicin
HE	haematoxylin and eosin
IND sampling	individual sampling
INF	infected fish
IPDRČ	Institute of Pathology, Wild Animals, Fish and Bees
K	Fulton's condition factor
LONG sampling	longitudinal sampling
MALDI-TOF MS	matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry
MER	meropenem
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing

MMCs	melanomacrophage centres
MS medium	modified Shieh's medium
MSP	mass spectrum profile
NAH fish	negative apparently healthy fish
NH fish	negative healthy fish
NRI method	normalised resistance interpretation method
NWT	non-wild type
OXO	oxolinic acid
OXY	oxytetracycline
POS fish	positive fish
RES	reticuloendothelial system
RTFS	rainbow trout fry syndrome
TRS	trimethoprim/sulfamethoxazole
WGS	whole-genome sequencing
WT	wild type

PUBLISHED ARTICLE

Pavlin K, Papić B, Zdovc I, Knific T, Gruntar I, Sitar R, Žele Vengušt D, Seničar M, Ocepek M, Švara T. Phenotypic and Genotypic Antimicrobial Resistance Profiles of *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* Isolated From Rainbow Trout (*Oncorhynchus mykiss*) in Slovenia. J Fish Dis 2025; e14119.
doi: 10.1111/jfd.14119

1 INTRODUCTION

Flavobacteria are one of the main causes of bacterial diseases in salmonid aquaculture worldwide. Flavobacterial diseases are traditionally attributed to three bacterial species, namely *Flavobacterium psychrophilum* (Davis, 1946), *Flavobacterium columnare* (Davis, 1922), and *Flavobacterium branchiophilum* (Wakabayashi et al., 1989). Additionally, several other *Flavobacterium* spp. have been described to cause disease in fish, and their numbers are rapidly increasing (Loch and Faisal, 2015; Heckman et al., 2023). Nevertheless, studies investigating the histopathological lesions in fish infected with these lesser-known flavobacteria are scarce (Faisal et al., 2011; Loch, 2012). Additionally, previous research highlights the complexity of the aetiologies associated with flavobacteriosis (Good et al., 2015; Loch and Faisal, 2015; Li et al., 2021). Therefore, complete identification of the causative pathogen is always recommended. Flavobacteria can cause disease in a variety of fish species, albeit salmonids, particularly rainbow trout (*Oncorhynchus mykiss*), are most susceptible (Bernardet and Bowman, 2006; Loch and Faisal, 2015). In Slovene aquaculture, rainbow trout is the most commonly farmed fish species and is therefore of great economic importance (Statistical Office of the Republic of Slovenia, 2025). Because of the worldwide distribution of flavobacteria as well as the clinical signs and gross lesions observed in farmed fish, flavobacterial diseases are probably also common in Slovene fish farms. However, this has not yet been investigated, and there are currently no data on *Flavobacterium* spp. in Slovene fish.

Over the past decade, the Clinical and Laboratory Standards Institute (CLSI) has published standardised guidelines for antimicrobial susceptibility testing (AST) of *F. psychrophilum* (CLSI, 2014, 2020a) and *F. columnare* (CLSI, 2020a), allowing comparability of results between studies. However, AST and interpretation of results for other *Flavobacterium* spp. remain challenging, with limited research available. The results of some studies suggest that most flavobacteria are multidrug resistant (Mwega et al., 2020; Heckman et al., 2023). Research on the antimicrobial resistance (AMR) in flavobacteria is needed to enable the selection of appropriate treatment against these important fish pathogens.

Environmental factors are particularly important in aquatic ecosystems where both the host and the pathogen are strongly influenced by changes in water conditions. Although water quality is considered an important factor contributing to the occurrence of flavobacterial diseases (Barnes and Brown, 2011; Strepparava et al., 2014), a limited number of studies have investigated this relationship. Therefore, the role of water parameters in the initiation and pathogenesis of flavobacterial diseases is still poorly understood (Straus et al., 2015; Elmore, 2016).

1.1 RESEARCH OBJECTIVES AND HYPOTHESES

To better understand the importance of flavobacteria in Slovene rainbow trout aquaculture, we aimed to investigate the biodiversity of *Flavobacterium* spp. and the aetiology of flavobacterial diseases throughout the year, to assess the impact of infection with different *Flavobacterium* spp. on rainbow trout health by qualitative and semiquantitative evaluation of gross and histopathological lesions, to determine the antimicrobial susceptibility of *Flavobacterium* spp. to the antimicrobials commonly used in aquaculture using the latest standardised broth microdilution method (CLSI, 2020) and to evaluate the association between selected environmental factors (water temperature, pH, and dissolved oxygen) and the occurrence of flavobacterial infections in selected Slovene fish farms.

The research aimed to test the following hypotheses:

1. Flavobacteria are widespread in rainbow trout from Slovene fish farms and affect fish health.
2. Environmental factors, such as water temperature, pH, and dissolved oxygen in the water, influence the dynamics of flavobacterial infections.
3. Flavobacteria isolated from rainbow trout in Slovene fish farms are multidrug-resistant.

2 LITERATURE REVIEW

2.1 THE GENUS *FLAVOBACTERIUM*

The genus *Flavobacterium*, first proposed in 1923 by Bergey et al. (1923), belongs to the family Flavobacteriaceae in the phylum Bacteroidota. The description of the genus has been emended several times (Bernardet et al., 1996; Dong et al., 2013; Kang et al., 2013; Kuo et al., 2013). Since the emendation of the genus *Flavobacterium* based on 16S rRNA sequence comparisons (Bernardet et al., 1996), the taxonomy of several validly described species within the genus remains unresolved. Even well-studied species may still be misclassified (Bernardet and Bowman, 2006; Gélinas et al., 2024). Notably, *F. columnare*, one of the three known flavobacterial fish pathogens, was only recently, in 2022, reclassified into four distinct species: *F. columnare*, *Flavobacterium covae* sp. nov., *Flavobacterium davisii* sp. nov., and *Flavobacterium oreochromis* sp. nov. (LaFrentz et al., 2022). In addition, the genus *Chryseobacterium*, once the most common non-*Flavobacterium* genus within the family Flavobacteriaceae recovered from freshwater fish, with several species causing pathological lesions similar to pathogenic *Flavobacterium* spp. (Loch, 2012; Loch and Faisal, 2017) was reclassified to a new family Weeksellaceae fam. nov. in 2019 (García-López et al., 2019). These ongoing changes require continuous vigilance to remain aligned with current taxonomic classifications.

The minimal standards for describing new taxa of the family Flavobacteriaceae were established by Bernardet et al. (2002). The characteristics that distinguish *Flavobacterium* species from members of other genera in the family Flavobacteriaceae were listed by Bernardet and Bowman in 2006 (Bernardet and Bowman, 2006). The genus *Flavobacterium* is the type genus of the family Flavobacteriaceae, and the type species of the genus is *Flavobacterium aquatile* (Bernardet and Bowman, 2006, 2015). The genus has expanded rapidly in the last two decades, from 26 species in 2006 (Bernardet and Bowman, 2006) to 329

validly published species in June 2025 (GBC, 2025), and new species are still being discovered at a rapid pace.

Members of the genus *Flavobacterium* are gram-negative rods that are typically 0.3 to 0.5 µm wide and 2–5 µm long. Cells may also be shorter (1 µm long), filamentous (10–40 µm long), or pleomorphic. A capsular layer was observed in several species. They exhibit pale to bright yellow or orange pigmentation due to the production of flexirubin and/or carotenoid pigments. Most species are strictly aerobic and display a slow gliding motility, and few are nonmotile (*F. branchiophilum*). They are ubiquitous, chemoorganotrophic organisms widely distributed in soil, freshwater, and seawater in warm, temperate, and polar regions. Optimal growth temperature varies widely, although most temperate species prefer temperatures between 20 and 30 °C and most psychrophilic species between 15 and 20 °C. No member of the genus requires sodium chloride (NaCl) or seawater for growth, and NaCl even inhibits the growth of several species. All tested species are catalase-positive, indole-negative, and predominantly cytochrome oxidase-positive. Menaquinone-6 is the only or predominant respiratory quinone, and phosphatidylethanolamine is the major polar lipid. Whole-cell fatty acid profiles include predominantly $C_{15:0}$, $C_{15:0}$ iso, $C_{15:0}$ iso 3-OH, $C_{15:0}$ anteiso, $C_{15:1}$ ω6c, $C_{15:1}$ iso G, $C_{16:0}$ iso 3-OH, and $C_{17:0}$ iso 3-OH, additionally any combination of $C_{15:0}$ iso 2-OH, $C_{16:1}$ ω7c, and/or $C_{16:1}$ ω7t. However, it is difficult to assess whether all *Flavobacterium* species display similar fatty acid profiles, mostly due to considerable heterogeneity in the conditions used to cultivate flavobacteria. The DNA's G + C content ranges between 30 and 41 mol% (Bernardet and Bowman, 2006, 2015). *Flavobacterium* spp. exhibit high genetic diversity, which is supported by the presence of an open pangenome and a predominance of accessory genes. Recombination events and, to a lesser extent, horizontal gene transfer play an important role in shaping this diversity and allow adaptation to a wide range of ecological niches (Kim et al., 2023a).

Most species also have strong proteolytic activity, and about half of the species can reduce nitrate to nitrite and oxidise carbohydrates. Numerous species can degrade gelatine and casein, hydrolyse starch and esculin, and some also other

polysaccharides, whereas only a few species can degrade DNA and urea. Some flavobacteria can utilise a wide range of carbohydrates, whereas others utilise few or no carbohydrates and prefer proteins. For example, *F. psychrophilum* is unable to use carbohydrates but produces proteases and lipases and can uptake and process the degradation products of host proteins and lipids. The ability of many *Flavobacterium* species to degrade a variety of biomacromolecules by producing polysaccharide-degrading enzymes and extracellular proteases, and lipases contributes to the severe necrotic lesions commonly observed in fish infected with pathogenic species (Bernardet and Bowman, 2006, 2015).

2.2 FLAVOBACTERIAL DISEASES, GROSS AND HISTOPATHOLOGICAL LESIONS

The diseases of fish caused by various bacterial species of the genus *Flavobacterium* are known as flavobacterioses (Bernardet and Bowman, 2006; Loch and Faisal, 2015). Flavobacteria are ubiquitous in nature and are part of the normal bacterial flora on the surface of fish and fish eggs, albeit several species are opportunistic pathogens that may exhibit various degrees of virulence (Bernardet and Bowman, 2006, 2015). Nonetheless, high-intensity rearing systems create favourable conditions for opportunistic infections, and flavobacterial diseases are notoriously challenging to treat and control (Kumru et al., 2020; Heckman et al., 2023). Flavobacteriosis was first reported by Davis in 1922 (Davis, 1922), and flavobacteria have since been recognized as one of the main causes of bacterial diseases in freshwater fish worldwide and are responsible for considerable economic losses, particularly in salmonid aquaculture. Despite extensive scientific efforts over the last century to prevent and control outbreaks of flavobacterioses, progress has been hindered by the rapid expansion and constant restructuring of genera, as well as species and strains associated with the disease (Heckman et al., 2023).

Flavobacterial diseases are traditionally attributed to three bacterial species, namely *F. psychrophilum* (Davis, 1946), *F. columnare* (Davis, 1922), and *F. branchiophilum* (Wakabayashi et al., 1989). However, several other

Flavobacterium species suspected of causing disease in freshwater fish have also been described and continue to be discovered at a rapid pace (Heckman et al., 2023; Lee et al., 2023). Most of these species were associated with disease due to their isolation from the external or internal tissues/organs of clinically diseased fish. However, studies investigating the gross and histopathological lesions associated with the proposed pathogenic *Flavobacterium* species are scarce. Only a few of these species, namely *Flavobacterium bernardetii* (Saticioglu et al., 2021a; Lee et al., 2023), *Flavobacterium collinsii* (Lee et al., 2023), *Flavobacterium inkyongense* (de Alexandre Sebastião et al., 2019), *Flavobacterium johnsoniae* (Carson et al., 1993; Soltani et al., 1994; Rintamäki-Kinnunen et al., 1997; Flemming et al., 2007), and *Flavobacterium tructae* (synonym *Flavobacterium spartansii*) (Zamora et al., 2014; Loch and Faisal, 2016; Kämpfer et al., 2020; Mallik et al., 2023), have had their pathogenic potential investigated through experimental infection, including re-isolation of the causative agent from external and internal tissues and evaluation of gross and histopathological lesions (Heckman et al., 2023). However, the pathogenicity of *F. bernardetii*, *F. collinsii* (Lee et al., 2023), and *F. inkyongense* (de Alexandre Sebastião et al., 2019) has not been conclusively proven. Several other *Flavobacterium* spp. have been isolated from diseased fish, including *Flavobacterium araucananum* (Kämpfer et al., 2012), *Flavobacterium bizetiae* (Mühle et al., 2021), *Flavobacterium chilense* (Kämpfer et al., 2012), *Flavobacterium frigidimaris* (Loch and Faisal, 2014), *Flavobacterium hydatis* (Mantareva et al., 2022), *Flavobacterium kayseriense* (Saticioglu et al., 2021b), *Flavobacterium oncorhynchi* (Zamora et al., 2012; Loch and Faisal, 2018), *Flavobacterium piscis* (Zamora et al., 2014; Loch and Faisal, 2018), *Flavobacterium plurextorum* (Zamora et al., 2013; Loch and Faisal, 2014), *Flavobacterium succinicans* (Good et al., 2015; Adamek et al., 2018; Loch and Faisal, 2018; Einarsdottir et al., 2020), and *Flavobacterium turcicum* (Saticioglu et al., 2021b). However, experimental infection or histopathological investigation was not performed.

The sole presence of flavobacteria in fish does not necessarily mean that a true infection is present. Therefore, the use of histopathology in disease aetiology

studies is important to differentiate between pathogenic and commensal flavobacteria (Burreson, 2008; Gupta et al., 2009). The complex taxonomy of flavobacteria and the absence of consistently effective experimental challenge models obscure our understanding of the potential risks these putative pathogens pose to fish aquaculture (Heckman et al., 2023).

It is becoming increasingly evident that one or more aetiological agents can contribute to the onset of flavobacterial diseases (Good et al., 2015; Loch and Faisal, 2015; Li et al., 2021). In addition, different species may cause similar pathology and present similar morphology, thus complete identification of the causative pathogen is always recommended (Bernardet and Bowman, 2006; Faisal et al., 2011; Loch and Faisal, 2015). However, many flavobacteria may not be easily identified using conventional diagnostic methods (Gélinas et al., 2024).

Flavobacteria can cause disease in all life stages of fish and a variety of fish species, with salmonids, especially rainbow trout, being the most susceptible (Bernardet and Bowman, 2006; Loch and Faisal, 2015). Depending on the bacterial species and other factors, particularly the species and life stage of the fish, flavobacteria can infect the skin or gills of the fish, cause systemic infection, or both (Smith, 2019). The course of flavobacteriosis can be from peracute to chronic and can lead to high mortality (up to 90% in infections with *F. psychrophilum*), especially in juvenile fish, or low but continuous mortality, which is more common in adult fish (Loch, 2012; Loch and Faisal, 2017). The optimal temperature range for different species varies widely. Psychrophilic species, such as *F. psychrophilum*, cause disease at cold temperatures (< 15 °C), while temperate species, such as *F. columnare*, prefer warm temperatures (> 12 °C). Therefore, flavobacterial diseases may occur in fish farms year-round (Bernardet and Bowman, 2006; Loch and Faisal, 2015).

Flavobacteria are considered extremely rare human pathogens, mostly in immunocompromised people (Zurbuchen et al., 2023). *F. oncorhynchi* has recently been identified as the causative agent of necrotising chorioamnionitis and intrauterine fetal death (Ferreira et al., 2024). Notably, some flavobacteria reported

to cause infections in humans have since been reclassified to other genera, including *Flavobacterium meningosepticum* (now known as *Elizabethkingia meningoseptica*) and *Flavobacterium indologenes* (now classified as *Chryseobacterium indologenes*) (Vandamme et al., 1994; Kim et al., 2005).

2.2.1 Well-known fish-pathogenic flavobacteria

2.2.1.1 *Flavobacterium psychrophilum*

F. psychrophilum was first described in North America in 1946 (Davis, 1946) and has since been reported as an important fish pathogen worldwide (Nematollahi et al., 2003a; Barnes and Brown, 2011; Li et al., 2021). Naturally present in freshwater environments, it can likely survive for several months or even years without a host (Madetoja et al., 2003). It is also a common commensal organism on the gills and skin of apparently healthy fish and fish eggs (Jarau et al., 2018; Calvez et al., 2021). *F. psychrophilum* is the aetiological agent of bacterial cold-water disease (BCWD) or rainbow trout fry syndrome (RTFS), causing skin, fin, and gill lesions, as well as systemic infections (Nematollahi et al., 2003a; Barnes and Brown, 2011). The peracute and acute disease can lead to high mortality, especially in juvenile fish (up to 90%) (Nilsen et al., 2011), with survivors suffering from poor growth and spinal deformities. In addition, subacute and chronic infections cause low but persistent mortality and reduced market value, resulting in ongoing economic losses (Loch, 2012; Loch and Faisal, 2017). Infections most commonly occur at water temperatures between 4 and 15 °C (Holt et al., 1993). Salmonids are most susceptible to infection with *F. psychrophilum*. However, the disease has also been reported in several other fish species, including ayu (*Plecoglossus altivelis*), common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), European eel (*Anguilla anguilla*), pale chub (*Zacco platypus*), perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), and tench (*Tinca tinca*) (Smith, 2019).

The terms RTFS and BCWD describe different aspects of the same disease caused by *F. psychrophilum*, the distinction being based mainly on the life stage of the affected fish, the course of the disease, and regional terminology. RTFS

applies primarily to young fish (fry and fingerlings), while BCWD is more commonly associated with older fish. RTFS is more commonly used to describe the acute, systemic form of the disease, whereas BCWD refers to the more chronic form with mainly skin involvement. Additionally, RTFS is the preferred term in the European Union, while BCWD is more commonly used in the United States of America (Barnes and Brown, 2011; Smith, 2019).

Infection with *F. psychrophilum* is characterized by the formation of erosions and deep ulcers in the skin, particularly at the caudal peduncle and the caudal fin, often involving the whole tail and revealing the underlying musculature and bones (Bruno et al., 2013). Additionally, large cystic lesions filled with serohaemorrhagic or pus-like fluid can develop in the skin and musculature (Nilsen et al., 2011). One of the first clinical signs is yellowish-white spots on the skin or fins, which progress to necrosis. Other clinical signs and/or gross lesions include lethargy, anorexia, increased skin mucus secretion, hyperpigmentation, especially on the caudal part of the body (black tails), corneal opacity, ocular haemorrhage and exophthalmos, ascites, anaemia of the gills, liver, and kidneys, gill necrosis, enlarged spleen, intestinal hyperaemia, haemorrhage and oedema of the vent, spinal deformities, and neurological signs leading to erratic or spiral swimming (Kent et al., 1989; Lorenzen et al., 1991; Bruno, 1992; Ekman and Norrgren, 2003; Bruno et al., 2013; Loch and Faisal, 2017). Spinal deformities, such as kyphosis, lordosis, and scoliosis due to compressed or fused vertebrae and collapsed intervertebral spaces, and neurological signs may persist even after recovery from the disease (Kent et al., 1989; Madsen et al., 2001; Loch and Faisal, 2017).

Histopathological investigation reveals necrosis and haemorrhage in most internal organs (Lorenzen et al., 1991; Bruno, 1992; Ekman and Norrgren, 2003; Park et al., 2023). Notably, in the early stages of infection, an inflammatory reaction is usually absent (Ferguson, 2006), although various numbers of neutrophils, macrophages, and lymphocytes may be observed (Ekman and Norrgren, 2003; Miwa and Nakayasu, 2005; Soares et al., 2019). Filamentous bacteria may be visible in the affected tissues and the phagocytes of the spleen and kidney

(Lorenzen et al., 1991; Decostere et al., 2001; Wiklund and Dalsgaard, 2003; Marancik et al., 2014).

The skin is often eroded and ulcerated, while the surrounding preserved epidermis may become hyperplastic, with vacuolar or ballooning degeneration and necrosis of epithelial cells. In the underlying or surrounding dermis, there are haemorrhage, oedema, and necrosis, as well as Zenker's degeneration of the underlying musculature (Ekman and Norrgren, 2003; Nilsen et al., 2011; Aly et al., 2013; Marancik et al., 2014). In addition, *F. psychrophilum* has an affinity for cartilage, causing lysis and inflammation, particularly in cranial cartilage and the intervertebral discs of the spinal column in juveniles (Ferguson, 2006). In the gills, epithelial and goblet cell hyperplasia can occur, which can eventually lead to fusion of gill lamellae and filaments (Soares et al., 2019). Haemorrhage, degenerative lesions, and necrosis can be found in the gills and internal organs, especially the spleen, kidney, and liver (Lorenzen et al., 1991; Bruno, 1992; Nilsen et al., 2011; Aly et al., 2013; Soares et al., 2019).

The spleen is usually the most severely affected internal organ with congestion, haemorrhage, haemosiderosis, oedema, necrosis, and a loss of definition of the splenic border due to inflammation and fibrin deposition (Bruno, 1992; Ekman, 2008; Bruno et al., 2013). Perisplenitis is a very common and possibly pathognomonic lesion in *F. psychrophilum* infection (Nilsen et al., 2011). Inflammatory infiltrates composed of neutrophils, macrophages, and lymphocytes can also be observed within vascular channels and splenic parenchyma. Activation of the splenic reticuloendothelial system (RES) is described as one of the earliest signs of systemic cellular damage (Marancik et al., 2014). Lesions described in the kidney include hyaline droplet degeneration and necrosis of tubular epithelium, necrosis of haematopoietic tissue, and inflammatory infiltrates composed of neutrophils, macrophages, and lymphocytes within the vascular channels and haematopoietic tissue (Lorenzen et al., 1991; Bruno, 1992; Ekman and Norrgren, 2003; Marancik et al., 2014). Degeneration, coagulative necrosis, and circulatory disturbances were reported in the liver (Aly et al., 2013). Inflammation of the heart, from mild lymphocytic epicarditis to severe neutrophilic

and necrotising myocarditis, can be observed (Nilsen et al., 2011). Additionally, the gastric glands may show vacuolation (Park et al., 2023), which is thought to be a result of anorexia, one of the clinical signs of *F. psychrophilum* infection (Nematollahi et al., 2003a).

Histopathological investigation of disease-resistant and disease-susceptible rainbow trout lines infected with *F. psychrophilum* revealed the same pathological lesions. However, the lesions in the disease-resistant line were less severe, suggesting a difference in disease severity rather than an altered disease course between genetic lines (Marancik et al., 2014).

2.2.1.2 *Flavobacterium columnare*

F. columnare was first described in North America in 1922 (Davis, 1922) and has since been reported as an important fish pathogen worldwide (Declercq et al., 2013a; Ma et al., 2019). The bacterium can also be a part of the normal microbiota of freshwater fish and fish eggs (Barker et al., 1990), and it can survive in the water and remain infectious for weeks or even months (Ross and Smith, 1974; Kunttu et al., 2012). *F. columnare* is the aetiological agent of columnaris disease (CD), and it has a high affinity for gill, skin, and fin tissues. The disease can develop at both low (12 °C) and high (25 °C) temperatures (Starliper, 2011; Declercq et al., 2013a). In rainbow trout, the disease occurs most frequently in the summer when water temperatures rise above 15 °C to around 20 °C (Suomalainen et al., 2005; Declercq et al., 2013a). CD affects a variety of freshwater fish species; it is believed that all freshwater fish are susceptible under conditions that favour the bacterium and stress the host (Wakabayashi, 1993; Smith, 2019).

In juvenile fish, the disease occurs more frequently in peracute or acute form, with a mortality rate of up to 100% within 12–24 hours, while in adult fish it can manifest in acute, subacute, or chronic form. The peracute and acute forms mainly affect the gills, with the development of large, yellowish necrotic areas, while the chronic form is characterised by extensive skin erosions, ulcerations, and gill necrosis (Rucker et al., 1954; Declercq et al., 2013a). Skin erosions typically first

appear at the base of the dorsal fin as pale, poorly pigmented patches of skin, usually surrounded by a thin reddish band. Fin deterioration then occurs, resulting in deep ulceration that spreads laterally from the dorsal fin and forms a saddleback-like lesion. In rainbow trout, the adipose fin is often affected, and the lesions may extend to the peduncle. As in infections with *F. psychrophilum*, the skin lesions can extend into deeper layers, including the skeletal muscles (Pacha and Ordal, 1967; Decostere, 2002; Bernardet and Bowman, 2006). Ulceration of the oral mucosa with inflammation and resorption of the maxilla and mandible have also been observed (Ferguson, 2006). At necropsy, ascites may be present (Bruno et al., 2013). However, no other gross lesions are reported in the internal organs.

Histopathological lesions that may initially occur in the gills include hyperplasia of epithelial and goblet cells with fusion of gill lamellae, vacuolar degeneration, congestion, and oedema resulting in epithelial lifting, and infiltration of lamellae with inflammatory cells, predominantly neutrophils. In later stages, clubbing and fusion of gill filaments may be observed, as well as extensive haemorrhage and necrosis. Large groups of filamentous bacteria can be observed on the surface of the epithelial cells, which are characteristically arranged in columnar structures (Pacha and Ordal, 1967; Foscarini, 1989; Bruno et al., 2013; Singh et al., 2021). Acute ulcerative dermatitis can develop, leading to oedema, haemorrhage, and necrosis of the dermis as well as Zenker's degeneration of the skeletal muscle. Numerous filamentous bacteria, staining blue-purple with haematoxylin and eosin (HE), can be observed in the affected tissues (Pacha and Ordal, 1967; Ferguson, 2006).

F. columnare has occasionally been reported to cause systemic infections, particularly in severe cases, across various fish species, including rainbow trout. In these cases, the bacteria are most commonly isolated from the spleen, kidney, and liver (Pacha and Ordal, 1967; Hawke and Thune, 1992; Decostere et al., 1999a; Bader et al., 2003; Darwish et al., 2008; Kunttu, 2010; Bruno et al., 2013; Evenhuis et al., 2014). However, studies describing histopathological lesions in internal organs are scarce. In rainbow trout, Evenhuis et al. (2014) observed

splenic necrosis and granulomatous splenitis and nephritis, while Singh et al. (2021) described hypertrophy, vacuolation, and necrosis of hepatocytes and hepatic congestion, dilatation of Bowman's space and tubular degeneration and necrosis in the kidney, as well as increased numbers of melanomacrophage centres (MMCs) and congestion in the spleen. Pacha and Ordal (1967) also observed an enlargement of Bowman's space with eosinophilic material surrounding the glomerular capillaries in salmon (*Oncorhynchus* spp.).

2.2.1.3 *Flavobacterium branchiophilum*

F. branchiophilum was first described in North America in 1926 and has since been detected in Europe and Asia (Bakhtiyar et al. 2022; Davis 1926). *F. branchiophilum* is ubiquitous in freshwater environments (Loch and Faisal, 2017). However, it is not reported to be part of the normal microbiota in fish. It is the aetiological agent of bacterial gill disease (BGD) that has a tropism for the gill epithelium and does not normally invade deeper tissues or internal organs. However, severe infections can lead to high morbidity and mortality (up to 75%) because gill lesions lead to respiratory and osmoregulatory failure (Davis, 1926; Speare et al., 1991; Ostland et al., 1995a). Other flavobacteria, namely *F. succinicans* and *F. hydatis*, have also been recovered from the gills of fish displaying clinical signs of BGD (Strohl and Tait, 1978; Good et al., 2015). The disease occurs most frequently in spring and early summer when water temperatures rise to between 5 °C and 19 °C (Bullock et al., 1994; Ostland et al., 1995a). Salmonids are most susceptible to infection with *F. branchiophilum*. This bacterium has also been reported to cause disease in walleye (*Stizostedion vitreum*), rohu (*Labeo rohita*), catla (*Catla catla*), silver carp (*Hypophthalmichthys molitrix*), and common shiners (*Luxilus cornutus*) (Smith, 2019).

BGD can affect fish of all ages, but mainly affects juvenile fish. Infection leads to hyperplasia of epithelial and goblet cells with clubbing and fusion of the gill lamellae and filaments, as well as hydropic degeneration and necrosis. In addition, infiltration of neutrophils and mononuclear leukocytes, congestion, telangiectasiae,

and numerous filamentous bacteria on the surface of epithelial cells can be observed (Ostland et al., 1990; Ferguson et al., 1991; Speare et al., 1991).

2.2.2 Other fish-pathogenic flavobacteria

Lesser-known flavobacteria have been recovered from external lesions and/or from internal organs of diseased fish, which often show clinical signs and gross lesions similar to those caused by well-known flavobacterial pathogens. However, the pathogenicity, gross, and histopathological lesions associated with these lesser-known flavobacteria isolated from diseased fish have not been well studied (Loch and Faisal, 2015; Wahli and Madsen, 2018; Heckman et al., 2023; Lee et al., 2023).

Loch and Faisal (Loch, 2012; Loch and Faisal, 2016) induced an experimental infection of Chinook salmon (*Oncorhynchus tshawytscha*) with *F. tructae* (synonyme *F. spartansii*) and described gross lesions, such as pale, swollen gills with multifocal haemorrhage, congestion at the base of the fins, perioral skin ulcerations, and extensive ulceration and necrosis of the caudal peduncle and caudal fin, penetrating deep into the underlying muscle. Notable internal gross lesions included an enlarged and friable spleen, haemorrhage in the skeletal muscle tissue, adipose tissue, the heart ventricle, brain, swim bladder, gonads, and kidneys, retrobulbar haemorrhage, enlarged and pale liver, haemorrhagic enteritis, ascites, and oedematous, swollen kidneys. Histopathological lesions included severe proliferative branchitis with fusion of gill lamellae and oedema, necrotising dermatitis, cellulitis and myositis with haemorrhage and infiltration with lymphocytes and macrophages, multifocal necrosis in the kidney and liver, degeneration and necrosis of the renal tubules, lymphocytic hepatitis and pancreatitis, splenic congestion and oedema, and multifocal oedema in the cerebellar cortex and brainstem.

Mallik et al. (2023) used *F. tructae* isolates obtained from lesions on the caudal peduncle of naturally infected rainbow trout for experimental infection of rainbow trout fingerlings, leading to disease progression and nearly 50% cumulative

mortality. Moribund rainbow trout from natural infections showed different gross lesions, including dark skin pigmentation, deep skin ulcerations, and eroded caudal fins, as well as mild lesions in the gills, liver, and kidneys. In the challenged group, fin, skin, and muscle necrosis, black patches on the dorsal body surface, lesions on the caudal peduncle, haemorrhage around the vent, and mild haemorrhage in the gills, liver, and kidneys were observed. Histopathological examination of naturally infected rainbow trout revealed gill hypertrophy and hyperplasia, degenerative lesions and necrosis in the skeletal muscles and internal organs, including the liver, kidney, and spleen, spleen congestion and exudate in the kidney tubules and Bowman's spaces. In experimentally infected fish, similar lesions were observed in the gills, liver, kidneys, muscles, and spleen. Additionally, vacuolation was observed at the tips of the gill lamellae, the liver showed congestion and necrotic bile ducts, and massive tubular necrosis and decreased hematopoietic tissue were observed in the kidneys.

Carson et al. (1993) isolated *F. johnsoniae* from diseased juvenile barramundi (*Lates calcarifer*) displaying gross lesions, such as extensive erosions and superficial ulcerations of the skin, predominantly of the caudal part of the body, and in some cases affecting the pectoral fins and lower jaw. Histopathological investigation revealed complete loss of epithelium with bacteria attached to the scales, oedema of underlying dermis and musculature, and mild, predominantly mononuclear cell infiltration in the subdermal connective tissue. In addition, severe hyaline droplet degeneration of kidney tubules was observed, while no significant lesions were detected in other examined organs, including gills. Soltani et al. (1994) successfully conducted an experimental infection of juvenile barramundi using this isolate. *F. johnsoniae*-like bacteria were also found to be associated with external lesions on the gills, jaws, skin, and fins in salmonids (Rintamäki-Kinnunen et al., 1997), including rainbow trout (Flemming et al., 2007; Suebsing and Kim, 2012).

Loch and Faisal (Loch and Faisal, 2014) suggested that isolates most similar to *F. aquidurense*, *F. pectinovorum,* and *F. anhuiense* based on 16S rRNA gene sequencing may represent fish pathogenic bacteria. They performed experimental

infections of Chinook salmon, brook trout (*Salvelinus fontinalis*), and brown trout (*Salmo trutta*) with these isolates and described possible gross lesions. However, a histopathological investigation was not included. Commonly observed lesions in fish infected with any of the three isolates included gill pallor, spleen congestion, hepatic swelling, congestion or pallor, and kidney congestion and oedema. Fish naturally infected or challenged with *F. aquidurense* also exhibited gross lesions, such as exophthalmos and periocular haemorrhage, mottled external coloration and deep ulcerations, swim bladder haemorrhage, and petechial haemorrhage in the brain. Gross lesions in fish challenged with *F. pectinovorum* included mottled external coloration, haemorrhage in the liver, and adhesions between the liver and body wall. Fish challenged with *F. anhuiense* also showed exophthalmos, deep skin ulcerations with haemorrhage, and swim bladder oedema. *F. aquidurense*, *F. pectinovorum,* and *F. anhuiense* were recovered from the liver, spleen, kidney, and brain of some of the challenged fish, confirming the presence of a systemic infection.

Lee et al. (2023) conducted an experimental infection of rainbow trout with *F. bernardetii* and *F. collinsii* isolates. *F. bernardetii* was isolated from the liver and kidney of diseased rainbow trout exhibiting skin hyperpigmentation, skin haemorrhage at the pectoral fin and abdominal area, exophthalmos, and ascites (Saticioglu et al., 2021a). *F. collinsii* was isolated from the spleen of one rainbow trout fingerling that had died without any disease signs. No clinical signs or mortalities were observed with *F. bernardetii*. *F. collinsii* exhibited very low virulence but was isolated from the internal organs of surviving fish, suggesting its ability to persist within the host and cause disease under favourable conditions. However, the research did not describe any gross lesions, except for caudal fin erosion that could be attributed to attacks from dominant fish, nor did it include a histopathological investigation (Lee et al., 2023).

2.3 PATHOGENESIS OF FLAVOBACTERIAL INFECTIONS

Despite the importance of flavobacterial infections, the course of infection, pathogenicity mechanisms, and the relationship between the bacterium and its

host are still not well understood, even among the well-known *Flavobacterium* species (Touchon et al., 2011; Li et al., 2017; Barbier et al., 2020; Zhu et al., 2022; Vaibarová and Čížek, 2024). There are considerable genetic differences and variations in virulence between different *Flavobacterium* species, as well as among strains within the same species (Ostland et al., 1995a; Klesius et al., 2008; Nicolas et al., 2008).

Comparative genomic studies have identified various virulence factors in flavobacteria (Abby et al., 2016; Chen et al., 2017; Ramesh and Souissi, 2018). The most common virulence mechanisms in Bacteroidota include gliding motility and the Type 9 Secretion System (T9SS), also known as the Por secretion system (PorSS), both of which are widespread among pathogenic flavobacteria and likely play a crucial role in pathogenesis (McBride and Nakane, 2015; Kayansamruaj et al., 2017; Declercq et al., 2021; Thunes et al., 2024). Genomic analyses have identified over 120 virulence-associated genes in flavobacteria. These genes encode proteins involved in immune evasion, inhibition of phagocytosis, bacterial adhesion, secretion systems, gliding motility, antibiotic resistance, and conjugative gene transfer. Some of the proteins function as proteases or toxins, including α-hemolysin, colibactin, and phytotoxins. Most flavobacteria possess genes for exopolysaccharides, capsule biosynthesis, heme acquisition, hydrolytic enzymes, the signalling nucleotide guanosine pentaphosphate involved in bacterial stress responses, and the AdeFGH efflux pump, which is essential for the secretion of biofilm components (Kumru et al., 2020). Flavobacteria express complex surface polysaccharides, including lipopolysaccharides, capsular polysaccharides, and exopolysaccharides that contribute to adhesion, biofilm formation, immune evasion, and inflammation (Raetz and Whitfield, 2002; Finlay and McFadden, 2006). The presence of a capsule has been confirmed in a few species, including *F. columnare* and *F. psychrophilum* (Decostere et al., 1999b; Møller et al., 2005).

The T9SS is found exclusively in Bacteroidota and is crucial for the virulence of flavobacteria (Sato et al., 2010; McBride and Zhu, 2013; Eckroat et al., 2021; Thunes et al., 2022; Lee et al., 2023). Functional T9SS is necessary for gliding motility, enzymatic activity, including secretion of peptidases, nucleases, lipases,

and glycoside hydrolases, haemolysis, heme uptake, adhesion, and biofilm formation in *F. psychrophilum* and *F. columnare* (Sato et al., 2010; Li et al., 2017; Barbier et al., 2020). In *F. columnare*, chondroitin AC lyase plays a crucial role in virulence and probably functions via T9SS because mutant strains lacking T9SS were unable to degrade chondroitin (Li et al., 2017). Experimental infections with wild-type and T9SS mutant *F. psychrophilum* and *F. columnare* strains demonstrated significantly reduced disease severity and no mortality in rainbow trout infected with the mutant strains (Li et al., 2017; Barbier et al., 2020). Bacterial quantification in infected fish showed that the mutant strains were either undetectable or present in much lower numbers in the host's internal organs (Barbier et al., 2020).

Flavobacteria are transmitted horizontally, with large numbers of bacteria being released into the water from the infected tissues of both live and dead fish (Fujihara and Nakatani, 1971; Madetoja et al., 2000; Madetoja et al., 2002; Pulkkinen et al., 2010; Starliper, 2011). Studies have clearly demonstrated that the skin mucus of rainbow trout is used as a nutrient source for the growth of *F. psychrophilum* and *F. columnare* (Shoemaker and LaFrentz, 2015; Papadopoulou et al., 2017). Both have a strong affinity for gills and skin (Kondo et al., 2002; Papadopoulou et al., 2017; Shoemaker et al., 2018), but adhesion alone is not sufficient to cause disease (Klesius et al., 2008; Papadopoulou et al., 2017). The exact portal of entry for *F. psychrophilum* and *F. columnare* remains unknown. It seems to depend on a breach in epithelium, whether of the skin or gills (Madetoja et al., 2000; Nematollahi et al., 2003a; Miwa and Nakayasu, 2005; Elkamel et al., 2010; Hoare et al., 2017; Cai, 2018). Miwa and Nakayasu (2005) investigated the pathogenesis in experimentally infected ayu and suggested that skin lesions were the main entry point for *F. psychrophilum* that invaded the dermis and spread through the connective tissue of the myosepta into the underlying muscle tissue. Lesions in other organs were only observed at later stages of the infection, probably due to dissemination through blood vessels. They concluded that infection through the gills or digestive system was unlikely.

2.3.1 *Flavobacterium psychrophilum*

F. psychrophilum isolates can be classified into three serotypes, namely Th, Fd, and FpT, based on reaction to specific antisera (Lorenzen et al., 1997), or into serotypes 0 (corresponding to FpT), 1 (corresponding to Fd), 2 (corresponding to Th), 3 (corresponding to FpT), and 4 (corresponding to FpT) based on serotyping by mPCR (Rochat et al., 2017). The FpT serotype is considered less virulent in rainbow trout (Sundell et al., 2019), whereas serotypes Fd and Th are commonly isolated during disease outbreaks (Madsen and Dalsgaard, 1999; Ilardi et al., 2023). The Th serotype is thought to be associated with higher virulence because it is the most frequently isolated serotype in rainbow trout outbreaks (Lorenzen et al., 1997; Ngo et al., 2017; Ilardi et al., 2023). Multilocus sequence typing (MLST) is commonly used for genetic typing, where isolates are divided into sequence types and clonal complexes based on seven loci (trpB, gyrB, dnaK, fumC, murG, tuf, atpA) (Jolley et al., 2018). In Europe, CC-ST10 is the most widespread clonal complex infecting rainbow trout, with ST2 being the most frequently observed sequence type within this group (Nilsen et al., 2014; Knupp et al., 2019). CC-ST10 isolates have been shown to be highly virulent (Sundell et al., 2019). The second largest clonal complex, CC-ST191, is also virulent in rainbow trout (Knupp et al., 2019). However, MLST alone cannot reliably predict virulence because isolates within the same sequence type may exhibit different virulence (Vaibarová and Čížek, 2024).

Nematollahi et al. (2003b) observed a negative correlation between the environmental temperature and bacterial attachment to the host. *F. psychrophilum* cells are surrounded by a thin polysaccharide layer that is thought to facilitate motility and adhesion to host cells (Holt 1987). This layer likely functions as an adhesin, enabling bacterial cells to glide across host tissues while remaining attached to the surface (Dalsgaard, 1993). Adhesion is associated with the colony morphology of the isolate, as smooth *F. psychrophilum* morphotypes are more adherent than rough. Gliding motility, proteolytic activity, and adherence capacity were shown to be important virulence factors (Sundell et al., 2019).

F. psychrophilum secretes extracellular enzymes that degrade key components of muscle and connective tissue, such as chondroitin sulphate, hyaluronic acid, elastin, collagen (types I, II, and/or IV), fibronectin, fibrinogen, casein, gelatine, laminin, actin, myosin, and/or haemoglobin (Bertolini et al. 1994; Bertolini and Rohovec 1992; Newton et al. 1997; Ostland et al. 2000; Otis 1984; Secades et al. 2001). This enzymatic activity contributes to the development of severe necrotic lesions that are commonly observed in infected fish (Cipriano and Holt, 2005; Bernardet and Bowman, 2006). *F. psychrophilum* produces cold-adapted metalloproteases that exhibit a wide range of hydrolytic activity and are active at temperatures as low as 4 °C (Secades et al. 2001; Secades et al. 2003), which is consistent with the low temperatures at which outbreaks of *F. psychrophilum* infections occur in salmonids (Bernardet and Bowman, 2006). It also produces haemolysins and can lyse rainbow trout erythrocytes, which may explain the anaemia in diseased fish (Lorenzen et al., 1997; Vaibarová and Čížek, 2024). However, the mechanisms leading to the haemorrhage have not yet been described (Zhu et al., 2022).

Recent studies have demonstrated that *F. psychrophilum* can enter different tissues via the bloodstream (Chai et al., 2021; Chinchilla et al., 2023). It has been suggested that *F. psychrophilum* colonises the spleen of fish asymptomatically and persists there until immunosuppression allows its proliferation and subsequent dissemination to other organs (Strepparava et al., 2014). Several studies indicate that macrophages (Nematollahi et al., 2005) and neutrophils (Avendaño-Herrera et al., 2020) play a crucial role in *F. psychrophilum* infections in trout. Viable bacteria have been detected in splenic macrophages, where they are probably protected from humoral immune responses, such as complement and lysozyme activity (Nematollahi et al., 2005). On the other hand, Semple et al. (2020) found that macrophage survival was most significantly reduced by live bacteria, while bacterial supernatant most strongly inhibited phagocytosis. This suggests that *F. psychrophilum* secretes virulence factors that help it evade the host immune system. Furthermore, increased expression of inflammatory cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNFα), and interleukin-10 (IL-10), was only observed in the presence of live bacteria and bacterial

supernatant. These findings confirm a link between the virulence of *F. psychrophilum* and its ability to evade phagocytosis. *F. psychrophilum* significantly suppresses the cellular and nonspecific humoral defense mechanisms, including lysozyme, complement component C3, and immunoglobulin (IgM) of infected fish. However, rainbow trout are capable of developing an effective adaptive immune response against *F. psychrophilum* (Siwicki et al., 2004; Deng et al., 2024).

2.3.2 *Flavobacterium columnare*

CD in rainbow trout and other salmonids is mostly caused by the species *F. columnare* (genetic group 1) and *F. davisii* sp. nov. (genetic group 3), while the species *F. covae* sp. nov. (genetic group 2) and *F. oreochromis* sp. nov. (genetic group 4) are associated with disease in channel catfish (*Ictalurus punctatus*) and tilapia (*Oreochromis* spp.), respectively (LaFrentz et al., 2022). Experimental infection studies have shown that *F. columnare* isolates are highly virulent in rainbow trout while *F. davisii* sp. nov. isolates are moderately virulent (Evenhuis and LaFrentz, 2016). These two species, in particular, exhibited growth at colder temperatures, which could explain this association with cold-water fish species (LaFrentz et al., 2022). Even before the reclassification of *F. columnare* into four distinct species, studies reported that the isolates exhibit high genetic heterogeneity while sharing homologous phenotypic characteristics and appearing phenotypically indistinguishable (Figueiredo et al., 2005; Dong et al., 2015).

The exact mechanisms by which *F. columnare* causes pathological lesions and disease are still unclear. The bacterium exhibits gliding motility, and it is assumed that fish skin mucus stimulates its chemotaxis (Klesius et al., 2008). High virulence strains attach more readily to gill tissue, but it is not clear whether adherence capacity alone is sufficient to cause systemic disease (Decostere et al., 1999a). Bader et al. (2005) developed a mutant strain of *F. columnare* that is incapable of adhesion and found that mortality was delayed and reduced by 75%. The bacterial capsule likely contributes to adhesion because highly virulent strains usually have a thicker, denser capsule (Pate and Ordal, 1967; Decostere et al., 1999b). Sun et

al. (2012) reported a significant increase in the expression of the rhamnose-binding lectin (RBL) gene during early infection. Treatment of fish with RBL ligands protected them from CD, likely due to competitive inhibition of *F. columnare* binding to the host RBL (Beck et al., 2012). Environmental factors also have an influence on bacterial adhesion, with high levels of divalent ions, nitrites, and organic matter, as well as low salinity and higher water temperatures promoting adhesion (Decostere et al., 1999c; Altinok and Grizzle, 2001). Biofilm formation, regulated by quorum sensing, is also thought to play a key role in pathogenesis (Staroscik and Nelson, 2008; Declercq et al., 2021).

To invade and damage the skin, muscles, or gills, *F. columnare* secretes extracellular enzymes, such as chondroitin AC lyase, which degrades connective tissue polysaccharides, such as chondroitin sulfate A and C, and hyaluronic acid (Dalsgaard, 1993). Kunttu et al. (2011) found that both gliding motility and secretion of chondroitin AC lyase are necessary for virulence. All isolates studied can degrade gelatine and casein, and some are also capable of degrading elastin (Newton et al., 1997). In addition, certain *F. columnare* strains release bactericidal substances that eliminate competing bacteria in their environment (Anacker and Ordal, 1959).

Some studies suggest that *F. columnare* can partially evade the immune defence by inhibiting the alternative complement activation pathway through the secretion of sialic acid (Ourth and Bachinski, 1987). Notably, histopathological examination of infected tissues typically reveals little to no inflammatory response, possibly due to bacterial-induced programmed cell death of host immune cells, particularly phagocytes (do Vale et al., 2003; Declercq et al., 2013a). Additionally, *F. columnare* likely suppresses immune responses by downregulating the NF-κB signaling pathway, a key regulator of innate immunity, through toxin secretion (Sun et al., 2012).

2.3.3 *Flavobacterium branchiophilum*

Limited information is available regarding the phenotypic and genotypic diversity of *F. branchiophilum* and the pathogenesis of BGD (Skulska, 2014). *F. branchiophilum* is highly contagious because it can adhere to fish gills in less than an hour (Ferguson et al., 1991; Ostland et al., 1995a). Touchon et al. (2011) analyzed the genome of *F. branchiophilum* strain FL-15, revealing 20 different adhesin genes. Their analysis also showed that *F. branchiophilum* employs surprisingly different pathogenicity mechanisms compared to related *Flavobacterium* species, such as *F. psychrophilum*. It carries a gene for a preprotein that shares 51% similarity with the heat-labile toxin (LTA) of enterotoxigenic *E. coli* (Touchon et al., 2011), which stimulates adenylate cyclase activity, leading to excessive water and electrolyte loss through host intestinal epithelial cells. This suggests that the *F. branchiophilum* toxin may have a similar mechanism of action, potentially disrupting the osmoregulatory function of gill epithelial cells (Vanden Broeck et al., 2007). Additionally, *F. branchiophilum* possesses a gene encoding a preprotein with 47% similarity to streptopain (Touchon et al., 2011). Streptopain is an important virulence factor in streptococci, playing a role in bacterial colonization, invasion, and inhibition of wound healing (Kapur et al., 1993).

Extracellular proteases likely play an important role in virulence because the bacterium can degrade gelatine and casein (Wakabayashi et al., 1989). Additionally, bacterial extracellular products, including protease, phosphatase, and phosphoamidase, induced gill lesions in juvenile rainbow trout similar to those observed in natural infections with *F. branchiophilum* (Ototake and Wakabayashi, 1985). Genome analysis identified genes encoding different proteases, including metallopeptidases and serine peptidases, as well as various transporters for the uptake of peptides and amino acids. These are likely important for protein degradation and the uptake of nutrients during host tissue colonization (Touchon et al., 2011).

Although *F. branchiophilum* is not capable of gliding motility (Ostland et al., 1994), its genome contains all the necessary *gld* genes and some *spr* genes. It also possesses the complete set of genes required for the effective functioning of the

T9SS (Touchon et al., 2011). The metabolism of *F. branchiophilum* is similar to that of other flavobacteria but uniquely includes a gene cluster for phenyl acetate degradation, a pathway associated with virulence due to toxic intermediates (Teufel et al., 2010; Touchon et al., 2011). *F. branchiophilum* also carries genes for glycoside hydrolases and loci for polysaccharide and pectin utilization, suggesting that this bacterium may be able to metabolize mucopolysaccharides in the gill mucus of fish. The genome contains various stress response genes, including those involved in detoxification, DNA damage repair, and antibiotic resistance (Touchon et al., 2011). Like *F. psychrophilum*, *F. branchiophilum* exhibits a high degree of recombination and horizontal gene transfer (McDaniel et al., 2010; Touchon et al., 2011).

2.4 DIAGNOSTIC METHODS

The diagnosis of flavobacterial infections is based on history, clinical signs, gross pathology, histopathology, and isolation of bacteria on growth media and identification of the causative agent (Loch and Faisal, 2017). Several phenotypic characteristics, serological, and molecular assays have been used to differentiate between flavobacterial species and/or strains (Loch and Faisal, 2015, 2017).

Culture-independent techniques have also been used for the detection of well-known flavobacteria in clinical or environmental samples (Bernardet and Bowman, 2006). There are a variety of polymerase chain reaction (PCR) tests that predominantly use 16S rRNA or *gyrB*-targeted species-specific primers for *F. psychrophilum* (Toyama et al., 1994; Bader and Shotts, 1998), *F. columnare* (Bader and Shotts, 1998; Bader et al., 2003; Mabrok et al., 2020), and *F. branchiophilum* (Toyama et al., 1996). Some studies have also developed genus-specific primers mainly targeting the 16S rRNA gene (Strepparava et al., 2012a; Adamek et al., 2013, 2018). Immunohistochemistry or fluorescent *in situ* hybridization (Strepparava et al., 2012a) can also be used to label bacteria in fixed tissue samples.

2.4.1 Culture

Numerous *Flavobacterium* species can grow on several commercially available nutrient-rich growth media, such as nutrient agar or trypticase soy agar. However, identification of fish pathogenic flavobacteria is often delayed or impeded because they are fastidious to culture, slow-growing, and frequently overgrown by saprophytic species (Bernardet and Bowman, 2006). They require specialized nutrient-poor media, such as Anacker and Ordal's (AO) (also called “cytophaga medium”) (Anacker and Ordal, 1955), Modified Shieh’s (MS) (Song et al., 1988; Decostere et al., 1997), Hsu-Shotts (Bullock et al., 1986; Loch, 2012), tryptone yeast extract salts (Holt, 1987; Saticioglu et al., 2021a), Reasoner's 2A medium (Reasoner et al., 1979; Pérez-Sancho et al., 2017), and others. MS and AO medium are most commonly used for general cultivation because they enable the growth of most pathogenic *Flavobacterium* species. Optimal incubation temperatures vary widely among species, but most fish pathogens grow best at 15–25 °C (Bernardet and Bowman, 2006). To prevent overgrowth of the pathogen by less fastidious saprophytic bacteria, especially when sampling from external lesions, selective media based on the intrinsic resistance of *F. columnare* and *F. psychrophilum* to certain antibiotics, such as tobramycin, neomycin, vancomycin and polymyxin B, can be used (Hawke and Thune, 1992; Decostere et al., 1997; Kumagai et al., 2004; Loch and Faisal, 2015).

Because flavobacterial species require different growth conditions, such as temperature, incubation time, and composition of the growth media, several different media and incubation conditions are required for successful primary isolation (Loch and Faisal, 2018). Cultivation of *F. branchiophilum* is particularly challenging because it is the most fastidious of all known pathogenic *Flavobacterium* species. While isolation may be achieved using AO agar (Bernardet and Bowman, 2006; Starliper, 2011), it is rarely attempted due to the fastidious nature of this bacterium (Starliper, 2011).

Colonies of *Flavobacterium* species on solid media are usually circular, convex, smooth and shiny, with entire or undulate margins, or flat and spreading with

uneven, rhizoid or filamentous margins, as in *F. columnare* strains, which usually adhere strongly to agar. They are usually yellow to orange due to the production of carotenoid and/or flexirubin pigments, but non-pigmented strains also exist (Bernardet and Bowman, 2006, 2015). Colony morphology can help to differentiate between species, although it should be noted that different strains of some species may have different colony types even under similar incubation conditions (Lee and Heo, 1998). The only *Flavobacterium* species that is described to emit a somewhat characteristic odor, often described as “sickening, distinct and fruity”, is *F. columnare* (Shamsudin and Plumb, 1996). In aging liquid culture, some *Flavobacterium* species, including *F. psychrophilum*, *F. columnare,* and *F. branchiophilum*, form spherical degenerative structures called “spheroplasts” (Reichenbach, 1989; Bernardet and Bowman, 2006). Additionally, in the absence of a host, *F. columnare* can adopt a less virulent form with altered colony morphology, likely as an energy-saving adaptation (Arias et al., 2012; Kunttu et al., 2012).

The morphology of flavobacteria is highly diverse, even within a species. *F. psychrophilum* isolates can form two colony morphology types, i.e. rough and smooth (Sundell et al., 2013). In several studies, genetically different *F. psychrophilum* isolates were found coexisting in the same fish farm and in the same fish individual. Both phase variants may be genetically indistinguishable or have different genetic profiles. In addition, resistant and susceptible strains of both morphotypes are often isolated in individual disease outbreaks. Such genotypic and phenotypic heterogeneity poses a challenge for disease identification and control in farm environments (Bruun et al., 2000; Madetoja et al., 2002; Chen et al., 2008; Del Cerro et al., 2010; Henríquez-Núñez et al., 2012; Sundell et al., 2013; Ngo et al., 2017).

Cryopreservation at –70 °C or less within a routine growth medium, such as AO broth, with the addition of 10–30% (v/v) glycerol (Bernardet and Bowman, 2006) or on commercially available porous glass beads (Microbank Pro-Lab Diagnostics, Bromborough, Merseyside, UK) (personal observation) can be used for long-term

storage of flavobacteria. Lyophilisation or storage in liquid nitrogen may also be used (Bernardet and Bowman, 2006).

2.4.2 Identification of isolates

The distinctive appearance of the colonies and the filamentous morphology of most *Flavobacterium* species provide a direct means of selecting putative *Flavobacterium* isolates. Further identification relies on phenotypic and molecular characteristics (Bernardet and Bowman, 2006; Loch and Faisal, 2015). Traditionally, the identification and differentiation of flavobacteria relied on lengthy and laborious phenotypic and chemotaxonomic (i.e., whole-cell protein profiles and fatty acid analysis) investigations (Bernardet et al., 2002; Bernardet and Bowman, 2006). Commercial biochemical identification systems, such as API20E, API 20NE, API ZYM, API ID 32E, API 32GN, API 50CH (bioMérieux, Marcy l'Etoile, France), and/or BIOLOG GENIII MicroPlates (Biolog, Hayward, CA) can also be used at temperatures close to the optimum for the *Flavobacterium* species tested (Bernardet and Bowman, 2006; Delalay et al., 2018; Saticioglu et al., 2021b). More advanced molecular techniques, such as DNA–DNA hybridization, sequence analysis of 16S rRNA and *gyrB* genes, and WGS, now provide faster, more accurate, and more comprehensive methods for identification of *Flavobacterium* spp. isolates (Bernardet et al., 2002; Bernardet and Bowman, 2006). Of note, phenotyping was found to be the least accurate methodology (Gélinas et al., 2024). In addition, there are few diagnostic reagents specific for the lesser-known fish-associated flavobacteria, making their identification more challenging (Loch and Faisal, 2015).

Sequencing of the 16S rRNA gene is the most commonly used method for the identification of flavobacteria. However, it lacks the precision in the identification of closely related species (Kumru et al., 2020; Heckman et al., 2023; Gélinas et al., 2024). *Flavobacterium* species often share high levels of 16S rRNA gene sequence similarity (Yi and Chun, 2006; Loch and Faisal, 2015). Traditionally, the cut-off indicating speciation in the 16S rRNA gene is 97% pairwise identity (Edgar, 2018). However, a higher threshold of 99% sequence identity has been proposed

for the identification of flavobacteria to the species level, while isolates with < 99% identity are identified to the genus level (Dong et al., 2016; Edgar, 2018; Mwega et al., 2019). Even at this higher threshold, many isolates exhibit higher (≥ 99%) 16S rRNA gene similarity to the type species and can still be classified as new or distinct species by subsequent polyphasic analysis (Verner-Jeffreys et al., 2017; Loch and Faisal, 2018; Heckman et al., 2023).

The *gyrB* gene has a faster evolutionary rate than the 16S rRNA gene, resulting in greater genetic heterogeneity and improved species-level discrimination for bacterial genotyping (Galloway-Peña et al., 2014; Reichley et al., 2017; Heckman et al., 2023). It has been used for genotyping of *Flavobacterium* species (Shah et al., 2012). However, this method has limitations in identifying certain species due to mismatches in the primer binding site, which can hinder amplification and lead to false-negative results. Bioinformatics analysis showed that only 70% of *Flavobacterium* species could be accurately identified using this approach. Due to the high diversity of *Flavobacterium* strains, it is unlikely that new primers could be designed to enable effective genotyping across the entire genus (Gélinas et al., 2024). In addition, the *gyrB* primers do not appear to be specific to the genus *Flavobacterium* and could theoretically hybridize with species from closely related genera (García-López et al., 2019).

Therefore, WGS-based taxonomy using average nucleotide identity (ANI) and molecular phylogeny is the most accurate approach to identify *Flavobacterium* species. Reconstruction of the entire genome provides insight into the entire genetic repertoire of a given strain (Gélinas et al., 2024). The introduction of WGS contributed significantly to the identification of genetic traits linked to virulence, immunogenicity, and AMR, advancing the development of improved strategies for disease treatment and prevention (Declercq et al., 2021).

Recent studies have shown that matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a reliable alternative approach for the accurate, rapid, and cost-effective identification of different *Flavobacterium* species and possibly for differentiation of strains (Pérez-Sancho et al., 2017;

Saticioglu et al., 2021a). Previous studies have shown that the MALDI-TOF MS technique is an effective tool for distinguishing closely related species that are often indistinguishable by 16S rRNA gene analysis (Fernández-Álvarez et al., 2016; Fernández-Álvarez et al., 2017; Fernández-Álvarez et al., 2018). Identification of well-known fish pathogens, namely *F. psychrophilum, F. branchiophilum, F. columnare, F. covae, F. davisii,* and *F. oreochromis*, is possible with high confidence using MALDI-TOF MS (Strepparava et al., 2012b; Pérez-Sancho et al., 2017; Fernández-Álvarez et al., 2018; LaFrentz et al., 2022). Accurate species-level identification of bacterial isolates using MALDI-TOF MS relies on the presence of the species mass spectrum profile (MSP) in the reference protein database. Some studies have shown good results in the accurate identification of lesser-known *Flavobacterium* species available in the database (Strepparava et al., 2012b; Saticioglu et al., 2021a; Saticioglu et al., 2021b; Zurbuchen et al., 2023). MALDI-TOF MS databases can be continuously updated, and further studies incorporating a larger number of spectra and strains for each fish-pathogenic species will better capture the variability of *Flavobacterium* spp. This will improve the identification of flavobacterial species during disease outbreaks, facilitate disease management, and reduce the risk of pathogen spread (Fernández-Álvarez et al., 2018).

2.5 TREATMENT AND ANTIMICROBIAL RESISTANCE

Disease control is mainly based on management, biosecurity measures – similar to those used against other pathogens – and antimicrobial treatment because no consistently effective commercial vaccine against *F. psychrophilum* or other flavobacteria is yet available (Brenden et al., 2023; Vaibarová and Čížek, 2024). The development of vaccines against flavobacterial diseases is challenging due to the high diversity of pathogenic fish species, insufficient knowledge of molecular pathogenesis, and the occurrence of mixed-species outbreaks (Heckman et al., 2023; Saticioglu et al., 2024). In addition, the rapid emergence of AMR in fish pathogens is concerning, especially given the limited number of antimicrobials approved for aquaculture (only two or three per country) (Declercq et al., 2013b). Therefore, isolation and identification of the pathogen and its AST are crucial for

successful treatment and to reduce the further development of AMR (Loch and Faisal, 2017). In addition, the treatment of flavobacterial diseases has a significant economic impact on fish aquaculture. For example, in Sweden, an estimated 50–60% of all antibiotics are used to treat infections caused by *F. psychrophilum* and *F. columnare* (Söderlund et al., 2018).

Until recently, the antimicrobial susceptibility of flavobacteria was evaluated with various phenotypic tests using different, non-standardised media and growth conditions. Furthermore, there were no criteria that would allow a standardised interpretation of the results and a comparison between existing studies (Miranda et al., 2016; Ngo et al., 2018).

In 2014, the CLSI published the first guideline VET04-A2 (CLSI, 2014) for standardised AST of aquatic bacterial pathogens, including *F. psychrophilum*. The standardised CLSI method has been used in a limited number of studies (Smith et al., 2016; Van Vliet et al., 2017; Ngo et al., 2018; Söderlund et al., 2018; Jarau et al., 2019; Saticioglu et al., 2019; Li et al., 2021; Vaibarová et al., 2024; Zamparo et al., 2024). In addition, four of these studies used Kronvall's normalised resistance interpretation (NRI) method (Kronvall, 2010) to calculate the provisional epidemiological cut-off values for wild type (CO_{WT}) and provided their raw minimum inhibitory concentration (MIC) data, allowing interpretation and comparison of their results (Smith et al., 2016; Van Vliet et al., 2017; Ngo et al., 2018; Saticioglu et al., 2019).

In 2020, the CLSI published the latest standardised guidelines for AST and the first internationally recognised epidemiological cut-off values (ECVs) for the assessment of MICs for *F. psychrophilum* and *F. columnare* (CLSI, 2020a, 2020b). ECVs for erythromycin (ERY), florfenicol (FLO), oxytetracycline (OXY), enrofloxacin (ENR), flumequine, and oxolinic acid (OXO) are available for *F. psychrophilum* and *F. columnare*, whereas an ECV for ampicillin (AMP) is currently available only for *F. columnare* (CLSI, 2020b). These ECVs facilitate the differentiation of WT and NWT isolates when a standardized AST method is

applied. However, AST and interpretation of results for other *Flavobacterium* species remain challenging.

2.5.1 Phenotypic characterization of antimicrobial resistance profiles

Several studies have reported reduced susceptibility of *F. psychrophilum* isolates to multiple antimicrobials widely used in aquaculture (Henríquez-Núñez et al., 2012; Miranda et al., 2016; Smith et al., 2016; Van Vliet et al., 2017; Ngo et al., 2018). In addition, the results of some studies, including those from neighbouring countries, suggest that most flavobacteria are multidrug resistant (Varga et al., 2016; Mwega et al., 2020; Heckman et al., 2023). Several studies have investigated the susceptibility profiles of *F. psychrophilum* and *F. columnare*. However, data on the AMR of other flavobacterial species are scarce (Heckman et al., 2023). Notably, meaningful comparisons between studies are only possible if standardized and harmonized methods and growth media are used for AST.

A limited number of studies used the standardised broth microdilution protocol for AST of *F. psychrophilum* (Smith et al., 2016; Van Vliet et al., 2017; Ngo et al., 2018; Söderlund et al., 2018; Jarau et al., 2019; Saticioglu et al., 2019; Li et al., 2021; Vaibarová et al., 2024; Zamparo et al., 2024). In studies using the broth microdilution method for AST of *F. psychrophilum*, variable results were observed, albeit most studies reported low MICs for FLO (0–15.7% of NWT isolates), low to moderate MICs for AMP and gentamicin (GEN) (0–30% of NWT isolates), and ERY (16.7–20.0% of NWT isolates), and moderate to high MICs for trimethoprim/sulfamethoxazole (TRS), OXY (58–90% of NWT isolates), and quinolones (70–88% of NWT isolates), namely OXO, flumequine and ENR (Hesami et al., 2010; Henríquez-Núñez et al., 2012; Miranda et al., 2016; Smith et al., 2016; Van Vliet et al., 2017; Ngo et al., 2018; Saticioglu et al., 2019; Li et al., 2021; Vaibarová et al., 2024). Only two studies reported a high frequency (52.8% and 92.5%) of NWT isolates for FLO (Hesami et al., 2010; Henríquez-Núñez et al., 2012). FLO has become the new drug of choice for the treatment of *F. psychrophilum* infections in most countries, following a progressive decline in the efficacy of alternative drugs for the treatment of this severe disease, such as

OXY, aminopenicillins, and quinolones (Barnes and Brown, 2011; Ngo et al., 2018; Saticioglu et al., 2019).

Even fewer studies have used the standardised broth microdilution protocol for AST of *F. columnare* (de Abreu Reis Ferreira et al., 2022; LaFrentz et al., 2022). LaFrentz et al. (2022), who reclassified *F. columnare* into four distinct species, also performed an AST and concluded that no relevant differences were found between the four species. All isolates were WT for amoxicillin, ERY, FLO, and OXY. However, all four species were NWT for the quinolone ENR, and some *F. covae* isolates were NWT for ERY. In addition, MICs up to ≤ 2 µg/ml and > 2 µg/ml were observed for GEN and TRS, respectively. Similarly, de Abreu Reis Ferreira et al. (2022) reported that *F. oreochromis* was WT for all antimicrobials tested, except for ENR. Declercq et al. (2013b) analyzed 100 *F. columnare* isolates from different fish species using the broth microdilution method. All isolates were WT for FLO, and most were WT for ERY and AMP. However, 10–16% of isolates were NWT for quinolones (ENR, flumequine, and OXO), and 11% were NWT for OXY. Additionally, MICs of up to ≤ 2 µg/ml and ≤ 4 µg/ml were observed for GEN and TRS, respectively.

Studies on the antimicrobial susceptibility of *F. branchiophilum* are scarce. Two studies investigated the susceptibility of *F. branchiophilum* to penicillin G using the disc diffusion method on AO agar and concluded that all isolates were susceptible (Ostland et al., 1994; Ko and Heo, 1997). Adamek et al. (2018) performed AST using the disc diffusion method and reported that the isolates were susceptible to FLO (Adamek et al., 2018). However, the lack of a standardised method for AST and ECVs for this species limits the validity of the conclusions drawn in these studies.

Carson et al. (1993) investigated the antimicrobial susceptibility of *F. johnsoniae* and found different results for the isolates tested. However, OXO and acriflavine were identified as the most effective inhibitors of growth, while trimethoprim, polymyxin B, and sulfamethazine showed no apparent inhibitory effect.

Two studies investigated the antimicrobial susceptibility of several lesser-known flavobacteria using the broth microdilution method (Mwega et al., 2020; Heckman et al., 2023). Heckman et al. (2023) compared the antimicrobial susceptibility profiles of three representative isolates from each of the nine major phylogenetic clades (A–I) and found different susceptibility patterns. All clades had at least one tested isolate with an MIC above the established *F. psychrophilum* ECV for OXY. Additionally, four *Flavobacterium* clades (B, G–I) consistently exhibited high MICs for 11 of 18 antimicrobials tested, including FLO, TRS, ENR, GEN, ERY, and amoxicillin. It has been reported that *F. hydatis*, *F. plurextorum*, and *F. chilense* harbour the highest number of AMR genes among the 86 *Flavobacterium* genomes analyzed (Kumru et al., 2020). Notably, at least one isolate from each clade had MICs at or above the concentration required for resistance for OXY and FLO in *F. psychrophilum*, suggesting potential resistance. In addition, isolates from these clades exceeded the testing range for TRS (Heckman et al., 2023). Mwega et al. (2020) performed AST of 67 Flavobacteriaceae isolates, including species from the genera *Flavobacterium* and *Chryseobacterium*, although the latter has been reclassified outside this family. However, the research presented combined results for both genera, without providing data for individual species. The authors concluded that the isolates were predominantly susceptible to ENR, moderately susceptible to TRS and FLO, and all isolates exhibited reduced susceptibility to AMP, GEN, ERY, OXY, and ceftiofur.

2.5.2 Genotypic characterization of antimicrobial resistance profiles

WGS provides an alternative approach to predict the AMR profile based on the detection of acquired AMR genes and resistance-associated mutations, but requires comprehensive and accurate AMR gene databases. When defining WT and NWT populations, both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the CLSI refer to the genotype of isolates with regard to the presence of acquired AMR genes or AMR-associated mutations (Silley, 2012). However, AMR genes and AMR-associated mutations are not yet considered in the definition of WT and NWT populations in *Flavobacterium* spp.

because the mechanisms underlying AMR are still largely unclear and require further investigation (Saticioglu et al., 2019).

There are only a few studies that allow a comparison of the genotypic and phenotypic AMR profiles of *F. psychrophilum* and *F. columnare* (Izumi and Aranishi, 2004; Del Cerro et al., 2010; Henríquez-Núñez et al., 2012; Shah et al., 2012; Saticioglu et al., 2019; Abd El-Tawab et al., 2020), and even fewer studies used WGS to determine their genotypic AMR profiles (Söderlund et al., 2018; Chokmangmeepisarn et al., 2021; Declercq et al., 2021; Park et al., 2021). Several studies investigated the genome characteristics of *F. psychrophilum* and *F. columnare* (Wu et al., 2015; Castillo et al., 2016; Castillo et al., 2021; Kayansamruaj et al., 2017; Kumru et al., 2017; Söderlund et al., 2018; Duchaud et al., 2018; Mata et al., 2018; Chokmangmeepisarn et al., 2021; Declercq et al., 2021; Park et al., 2021; Saticioglu et al., 2024), while only one such research is available for *F. branchiophilum* (Touchon et al., 2011). Previous studies described the presence of AMR genes, such as *floR*, *sul2,* and various *tet* genes in the *F. psychrophilum* genomes, which were not associated with phenotypic resistance to FLO, sulfonamides, and tetracyclines, respectively (Söderlund et al., 2018; Saticioglu et al., 2019). However, some studies have identified the presence of AMR genes, such as *cfxA*, *ermF,* and *tetQ*, which may be involved in reduced susceptibility to cefoxitin, ERY, and tetracycline, respectively (Alvarez et al., 2004). In addition, the *tetA* gene was found in an *F. columnare* isolate displaying acquired phenotypic resistance to OXY (Declercq et al., 2021).

Flavobacteria often exhibit multidrug-resistant phenotypes that may be associated with intrinsic mechanisms (Mwega et al., 2020; Heckman et al., 2023), such as efflux pumps (Clark et al., 2009; Chen et al., 2017; Mata et al., 2018; Králová et al., 2021), low permeability of the outer membrane (Nikaido, 1994), expression of extended-spectrum beta-lactamases (Chen et al., 2017), mutations in DNA gyrase and topoisomerase genes (Shah et al., 2012), and biofilm formation (Sundell and Wiklund, 2011). The presence of T83A or T83V substitution in GyrA is associated with quinolone resistance in *F. psychrophilum* and *F. columnare* (Izumi and

Aranishi, 2004; Shah et al., 2012; Mata et al., 2018; Söderlund et al., 2018; Saticioglu et al., 2019; Chokmangmeepisarn et al., 2021; Declercq et al., 2021).

Verner-Jeffreys et al. (2017) analysed 377 genomes of the Flavobacteriaceae family (phylum Bacteroidota) and suggested that the transmission of AMR genes commonly associated with gram-negative bacteria, particularly from the phylum Pseudomonadota, may be limited or impossible due to genetic incompatibility between these evolutionarily distant bacterial groups. This could mean that the mechanisms leading to reduced susceptibility to antimicrobials are different in flavobacteria than in other bacterial fish pathogens and that the known AMR genes in Pseudomonadota are generally not found in Flavobacteriaceae. Possible barriers to gene transfer and functional expression of AMR genes need to be further investigated (Verner-Jeffreys et al., 2017; Saticioglu et al., 2019). Several studies highlight the importance of efflux pumps, in particular the resistance-nodulation-division (RND) family and the major facilitator superfamily (MFS), in multidrug resistance of flavobacteria (Clark et al., 2009; Chen et al., 2017; Zhang et al., 2017; Mata et al., 2018; Castillo et al., 2021; Chokmangmeepisarn et al., 2021; Declercq et al., 2021; Park et al., 2021).

2.6 PREVENTION OF FLAVOBACTERIAL DISEASES AND INFLUENCE OF WATER QUALITY ON THEIR OCCURRENCE

Flavobacteria are transmitted horizontally between fish. The main sources of infection are infected and dead fish, which release large numbers of bacterial cells into the surrounding water. Flavobacteria can also be transmitted via eggs by colonising their surface. In addition, *F. psychrophilum* can be transmitted vertically, which facilitates its spread through international trade in fish eggs (Taylor, 2004).

Outbreak prevention is a challenge because flavobacteria are ubiquitous in the environment and difficult to eradicate from fish farms and fish eggs. Measures, such as improved management and hygiene practices, can help contain the problem, although complete elimination cannot be guaranteed (Starliper, 2011; Loch and Faisal, 2018). In addition, many flavobacteria form biofilms, making them

difficult to eliminate even with the use of disinfectants, such as iodophor (Loch and Faisal, 2018). Therefore, fish are constantly exposed to flavobacteria in the environment, with outbreaks likely to occur when fish are under stress or when immunologically naïve juvenile fish are exposed to the pathogen (Söderlund et al., 2018). Farmed fish are particularly susceptible to disease, and maintaining optimal husbandry conditions, such as low rearing densities, good hygiene, and water quality, adequate water flows, and appropriate nutrition, is an important preventive measure (Suomalainen et al., 2005; Loch and Faisal, 2017). In addition, co-infections with ectoparasites can increase the susceptibility of fish to bacterial pathogens by causing microscopic lesions and inducing stress (Bandilla et al., 2006).

The role of environmental factors in the initiation and pathogenesis of flavobacterial diseases is not yet well understood (Straus et al., 2015; Elmore, 2016). Some studies suggest that predisposing factors, including low water temperature (below 20 °C), elevated nitrite concentrations, and pH between 6 and 8, may increase the risk of infection with *F. psychrophilum* (Soltani and Burke, 1995; Nematollahi et al., 2003b; Elmore, 2016). Additionally, water pH between 7 and 7.5, decreased dissolved oxygen, and increased water temperature, concentration of ammonia and nitrite, concentration of divalent cations (water hardness), and organic matter may be the environmental stimuli that contribute to the initiation of infection with *F. columnare* (Decostere et al., 1999c; Eissa et al., 2010; Farmer et al., 2011; Verma et al., 2011; Straus et al., 2015). Some studies observed that *F. branchiophilum* infections often occur in association with reduced water flow, reduced dissolved oxygen, increased concentrations of ammonia, rising water temperature, and water turbidity due to sediment disturbance during heavy rainfall (Bullock, 1990; Good et al., 2008; Starliper, 2011). Liu et al. (2019) concluded that pathogenic *Flavobacterium* spp. exhibit a strong negative correlation with water temperature and a positive correlation with ammonium concentration and dissolved oxygen.

In the aquatic environment, both the host and the pathogen are strongly influenced by changes in water conditions. Therefore, maintaining optimal water quality in fish

farms is crucial to ensure fish health and well-being. The recommended commonly measured parameters for rainbow trout growth include a temperature of 13–19 °C (Schurmann et al., 1991), dissolved oxygen of 7–13 mg/l (Pedersen, 1987; Caldwell and Hinshaw, 1994), pH of 7–8, ammonia below 0.0125 mg/l, nitrite below 0.000012 mg/l, and nitrate below 0.025 mg/l (Molony, 2001). However, rainbow trout can adapt to temperatures between 0 and 22 °C, dissolved oxygen as low as 5 mg/l, and pH between 5 and 9 (EFSA, 2008). Notably, the optimum range of water quality parameters also depends on the life stage of the fish (Noble et al., 2020).

3 MATERIALS AND METHODS

3.1 FISH SAMPLING AND WATER QUALITY MEASUREMENTS

Rainbow trout from four Slovene fish farms were included in this research, with the farms labelled F1 to F4 to ensure data anonymity. The research has been approved by the Ethical Committee for experiments on animals and the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection, Ministry of Agriculture, Forestry and Food (approval number: U34401-10/2022/6). The fish farmers signed an Owner's informed consent form (Appendix 1) before the start of the research. An overview of the research is shown in Figure 1.

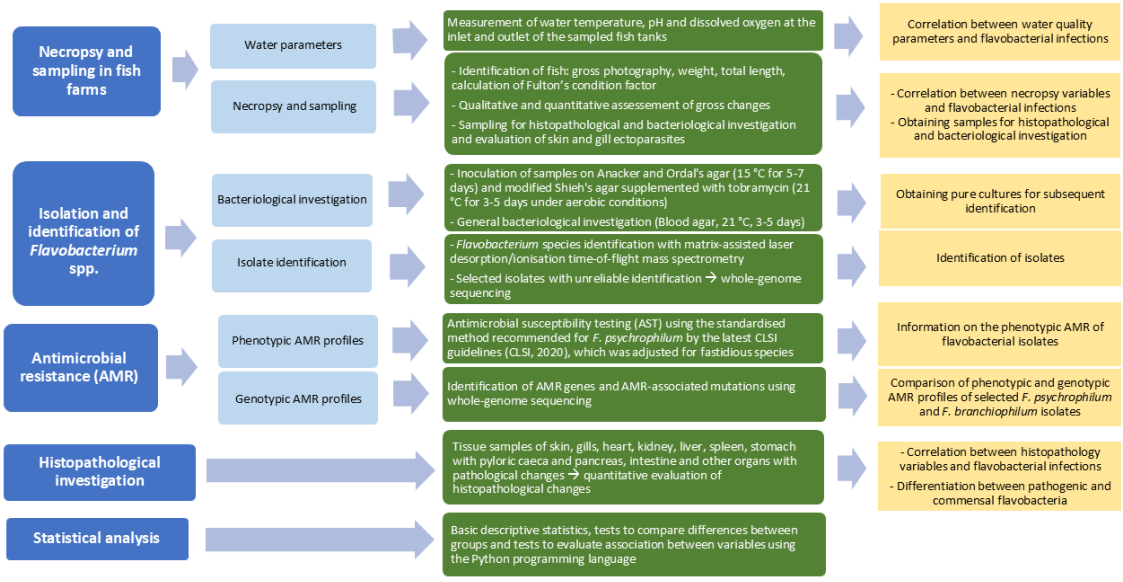


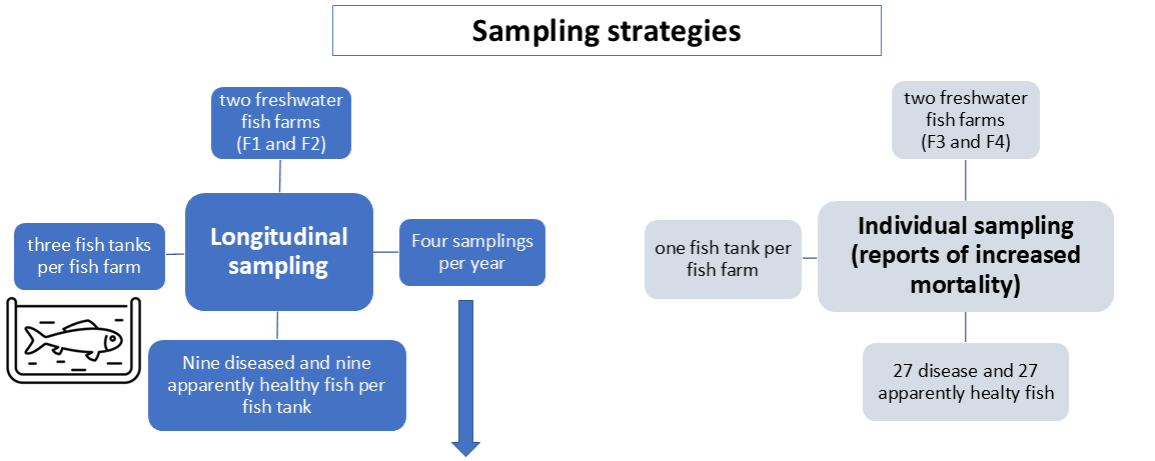
Figure 1: Study overview – a list of the methods used, along with a description of the purpose of each method

Two sampling strategies were used in the research: longitudinal and individual (Figure 2). To investigate the spatiotemporal dynamics of flavobacteria and the influence of annual changes in water temperature, dissolved oxygen, and pH on the occurrence of flavobacterial diseases, longitudinal (LONG) sampling was performed at two fish farms (F1 and F2) from October 2022 to August 2023 in four

seasons: autumn, winter, spring, and summer. Each sampling included nine diseased and nine apparently healthy fish from three separate tanks, resulting in a total of 27 diseased and 27 apparently healthy fish per sampling. During the LONG sampling, 432 rainbow trout were collected.

To investigate possible outbreaks of flavobacteriosis and the biodiversity of pathogenic flavobacteria, two individual (IND) samplings were conducted after fish farmers reported increased fish mortality, as defined by Article 23 of the Rules on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals (Official Gazette of the Republic of Slovenia, No. 6/14, 10/19 and 16/19). The first sampling was carried out in March 2023 at a third fish farm (F3), and the second in July 2024 at a fourth farm (F4). At each sampling, 27 diseased and 27 apparently healthy fish were selected from a single fish tank. In the two IND samplings, 108 rainbow trout were collected.

A total of 540 rainbow trout of different life stages were sampled during this research.



AUTUMN 2022		WINTER 2023		SPRING 2023		SUMMER 2023	
LONG1	LONG2	LONG3	LONG4	LONG5	LONG6	LONG7	LONG8
F1	F2	F1	F2	F1	F2	F1	F2

Figure 2: Sampling strategies including longitudinal (LONG) and individual (IND) samplings

For each sampling, all fish tanks on the farm were assessed, and the tanks with the most visibly diseased fish were selected for sampling. The fish were classified as diseased if they showed clinical signs, such as lethargy, anorexia, swimming near the water surface and opercular flaring, swimming abnormalities, as well as gross lesions, such as a distended abdomen, skin, and/or fin haemorrhage, erosions and ulcerations, skin discoloration (hypo- or hyperpigmentation), and exophthalmos. The fish were classified as apparently healthy if they exhibited normal behaviour and were without evident gross lesions.

Information on fish farms and factors that may affect fish health was obtained using a questionnaire for fish farmers (Appendix 2). General information on the fish farms from the questionnaire is given in Table 1. There were no hydrological connections between the sampled fish farms.

Table 1: General information on the fish farms (F)

FISH FARM	F1	F2	F3	F4
Production system	parallel and flow-through	parallel and flow-through	parallel and flow-through	parallel
Fish tanks	concrete and plastic	concrete	concrete	concrete
Water source	spring	river	river	stream
Upstream fish farms	no	yes	yes	no
Notifiable diseases	free	infectious hematopoietic necrosis virus	infectious hematopoietic necrosis virus	free

The sampled fish had never been vaccinated against any pathogens and had not been treated with antimicrobials in the month before sampling. Additionally, they had not been exposed to husbandry stressors in the two weeks preceding sampling. All fish were fed commercial Biomar fish feed, except for F3, where this information was not reported. For LONG samplings, no diseases were confirmed by a veterinarian, nor were any changes in mortality, behaviour, or appearance observed by the fish farmers in the month before sampling. An exception was noted at F2 in the autumn of 2022 and summer of 2023 when mildly increased mortality was reported. In IND samplings at F3 and F4, severely increased mortality was observed, prompting contact with a veterinarian. At F4, fish were treated with formalin a few days before sampling.

At each sampling, measurements of water temperature, pH, and dissolved oxygen were recorded at both the inlet and outlet of each sampled fish tank. Measurements were taken using the WTW™ Multi-parameter portable digital meter MultiLine® 3420 SET B, order no. 2FD46B (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany), with a WTW™ optical IDS dissolved oxygen sensor FDO® 925-3 (Xylem Analytics, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) and WTW™ IDS pH combined electrode SenTix® 940 (Xylem Analytics, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany).

3.2 NECROPSY AND SAMPLING

Necropsy and sampling were carried out in the fish farms. Fish were euthanized individually with an overdose of tricaine methane sulfonate (MS-222) (PHARMAQ AS, Overhalla, Norway) buffered with sodium bicarbonate ($NaHCO_3$) in a 1:2 ratio to reduce mucosal irritation and immediately necropsied. At necropsy, each fish was photographed, and its weight (g) and total length (cm) were measured to calculate the Fulton's condition factor (K) using the formula “$K = 100 \times \text{weight/total length}^3$”. The weight of the fish was measured using an electronic balance (G&G GmbH E3000YA).

During necropsy, selected gross lesions were assessed semiquantitatively according to the adapted method developed by Adams et al. (1993) and Heath et al. (2004). The score assigned to each organ ranged from 0 to 3. The necropsy fish index was calculated as the sum of all individual scores, with a possible range from 0 to 30 (Table 2).

For identification of ectoparasites and/or *Flavobacterium*-like bacteria, gill and skin scrapings were smeared onto slides, air-dried, and manually stained with Giemsa according to the protocol of the Institute of Pathology, Wild Animals, Fish and Bees (IPDRČ). A microscopic evaluation was then performed to determine the parasite infestation rate using a semiquantitative scoring system: 0 for no parasite infestation, 1 for mild infestation (1–10 parasites), 2 for moderate infestation (11–

20 parasites), and 3 for severe infestation (> 20 parasites). Due to the small size of amoebae and *Ichthyobodo necator*, precise quantification was challenging. Therefore, infestation was assessed subjectively (mild, moderate, and severe) (Heath et al., 2004).

Table 2: Description of organ variables and allocated score values for the assessment of gross lesions according to the adapted method developed by Adams et al. (1993) and Heath et al. (2004)

ORGAN	**VARIABLE**	**SCORE VALUE**
SKIN	Normal	0
	Mild aberrations	1
	Moderate aberrations	2
	Severe aberrations	3
FINS	No active erosion	0
	Previous erosion healed; light active erosion with no haemorrhage or secondary infection evident	1
	Moderate active erosion with some haemorrhage	2
	Severe active erosion with haemorrhage or secondary infection evident	3
EYES	Normal	0
	Any aberrations	3
OPERCULES	No shortening; gills completely covered	0
	Mild shortening	1
	Moderate shortening	2
	Severe shortening; considerable portion of the gills exposed	3
GILLS	Normal	0
	Any aberrations	3
LIVER	Normal	0
	Any aberrations	3
KIDNEY	Normal	0
	Any aberrations	3
SPLEEN	Normal	0
	Any aberrations	3
STOMACH	Normal	0
	Mild inflammation or other aberrations	1
	Moderate inflammation or other aberrations	2
	Severe inflammation or other aberrations	3
INTESTINE	Normal	0
	Mild inflammation or other aberrations	1
	Moderate inflammation or other aberrations	2
	Severe inflammation or other aberrations	3

3.3 HISTOPATHOLOGICAL INVESTIGATION

For histopathological investigation, samples of gills, skin, heart, liver, spleen, kidney, stomach with pyloric caeca and pancreas, intestine, and other organs with pathological lesions were taken at necropsy, except for the brain and eyes due to the lengthy sampling process. The samples were immediately fixed in 10% neutral buffered formalin for 24 to 48 hours at room temperature and then routinely embedded in paraffin. Four-µm thick tissue sections were deparaffinised and stained with HE. In selected cases, additional Gram staining was performed according to the IPDRČ protocol. Stained sections were examined with a light microscope. The presence of other potential pathogens observed in the histological slides was evaluated subjectively and categorized as mild, moderate, or severe.

Selected histopathological lesions were evaluated semiquantitatively according to the adapted method developed by Bernet et al. (1999), Saraiva et al. (2015), and Steinbach et al. (2016) (Table 3). According to this method, the relevance of a lesion depends on its importance factor:
(1) minimal pathological importance (the lesion is easily reversible when exposure to stressors ends),
(2) moderate pathological importance (the lesion is reversible in most cases when the stressor is removed), and
(3) marked pathological importance (the lesion is generally irreversible and leads to partial or complete loss of organ function).
Every lesion was assigned a score ranging from zero to six, depending on the degree and extent of the lesion:
(0) unchanged organ/tissue,
(1–2) mild occurrence,
(3–4) moderate occurrence, and
(5–6) severe occurrence or a diffuse lesion.

The organ index, along with circulatory, regressive, progressive, and inflammatory reaction indices, were calculated for each organ according to Bernet et al. (1999).

These indices represent the sum of the multiplied importance factors and scores for each lesion of the corresponding reaction pattern, or the total sum of all lesions observed in the examined organ. The possible range of index values is listed in Table 3 alongside each organ and reaction pattern.

Table 3: Evaluated histopathological lesions and allocated importance factors (IF) according to the adapted method developed by Bernet et al. (1999), Saraiva et al. (2015), and Steinbach et al. (2016)

ORGAN AND REACTION PATTERN (The possible range of index values)	**TISSUE**	**LESION**	**IF**
GILLS (0–432)			
Circulatory (0–72)	Lamellae	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Aneurysm	1
		Thrombus	2
	Gill arch	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Aneurysm	1
		Thrombus	2
Regressive (0–144)	Epithelium	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Supporting tissue	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Gill arch	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
Progressive (0–108)	Epithelium	Hypertrophy of epithelial cells	1
		Hyperplasia of epithelial cells	2
		Hyperplasia of goblet cells	1
		Hyperplasia of chloride cells	1
		Lamellar fusion	1
		Filament fusion	2
	Supporting tissue	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2

Table 3 continued

Table 3 continued

	Gill arch	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
Inflammatory (0–108)	Filaments/lamellae	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Gill arch	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
SKIN (0–474)			
Circulatory (0–60)	Dermis	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
	Muscle/subcutis	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–174)	Epidermis	Erosion	1
		Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Basement membrane	Defect (ulceration)	2
	Dermis	Loss of scales/bone resorption	2
		Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Muscle	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
Progressive (0–84)	Epidermis	Hypertrophy of epithelial cells	1
		Hyperplasia of epithelial cells	2
		Hyperplasia of goblet cells	1
	Dermis	Hypertrophy	1
		Hyperplasia	2

Table 3 continued

Table 3 continued

		Fibrosis	2
	Muscle	Hypertrophy	1
		Hyperplasia/regeneration	2
		Fibrosis	2
Inflammatory (0–156)	Epidermis	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
	Dermis	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Muscle/subcutis	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
HEART (0–444)			
Circulatory (0–60)	Epicardium	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
	Myocardium	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–144)	Epicardium	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Myocardium	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Endocardium	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
Progressive (0–78)	Epicardium	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
	Myocardium	Hypertrophy	1

Table 3 continued

Table 3 continued

		Hyperplasia/regeneration	2
		Fibrosis	2
	Endocardium	Hypertrophy	1
		Hyperplasia	2
Inflammatory (0–162)	Epicardium	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Myocardium	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Endocardium	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Activation of RES	1
SPLEEN (0–294)			
Circulatory (0–30)		Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–102)	Capsule	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Parenchyma	Activation of MMCs	1
		Cytoplasm deposits/infiltration	1
		Atrophy/lymphopaenia	2
		Degeneration	2
		Necrosis	3
Progressive (0–48)	Capsule	Hypertrophy	1
		Hyperplasia	2
	Parenchyma	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
Inflammatory (0–114)	Capsule	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1

Table 3 continued

Table 3 continued

	Parenchyma	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Activation of RES	1
		Exudate	1
KIDNEY (0–438)			
Circulatory (0–30)		Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–162)	Haematopoietic tissue	Activation of MMCs	1
		Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Tubule	Cytoplasm deposits/infiltration	1
		Lumen deposits	2
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Glomerulus	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
Progressive (0–90)	Haematopoietic tissue	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
	Tubule	Hypertrophy	1
		Hyperplasia/regeneration	2
		Fibrosis	2
	Glomerulus	Hypertrophy	1
		Hyperplasia/regeneration	2
		Fibrosis	2
Inflammatory (0–156)	Haematopoietic tissue	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Tubule	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2

Table 3 continued

Table 3 continued

		Infiltration EGCs	2
	Glomerulus	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
LIVER (0–300)			
Circulatory (0–30)		Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–108)	Liver tissue	Activation of MMCs	1
		Cytoplasm deposits/infiltration	1
		Decreased vacuolation	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Bile duct	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
Progressive (0–60)	Liver tissue	Hypertrophy	1
		Hyperplasia/regeneration	2
		Fibrosis	2
	Bile duct	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
Inflammatory (0–102)	Liver tissue	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Bile duct	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
PANCREAS (0–162)			
Circulatory (0–30)		Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–48)		Cytoplasm deposits/infiltration	1
		Atrophy	2

Table 3 continued

Table 3 continued

		Degeneration	2
		Necrosis	3
Progressive (0–30)		Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
Inflammatory (0–54)		Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
STOMACH AND PYLORIC CAECA (0–564)			
Circulatory (0–60)	Lamina propria	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
	Other layers	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–204)	Epithelium	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
		Ulceration	2
	Glands	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Lamina propria	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Other layers	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
Progressive (0–96)	Epithelium	Hypertrophy	1
		Hyperplasia	2
	Glands	Hypertrophy	1
		Hyperplasia	2
	Lamina propria	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2

Table 3 continued

Table 3 continued

	Other layers	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
Inflammatory (0–204)	Epithelium	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
	Glands	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
	Lamina propria	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Other layers	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
INTESTINE (0–450)			
Circulatory (0–60)	Lamina propria	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
	Other layers	Haemorrhage	1
		Hyperaemia	1
		Intercellular oedema	1
		Thrombus	2
Regressive (0–156)	Epithelium	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
		Ulceration	2
	Lamina propria	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3
	Other layers	Cytoplasm deposits/infiltration	1
		Atrophy	2
		Degeneration	2
		Necrosis	3

Table 3 continued

Table 3 continued

Progressive (0–78)	Epithelium	Hypertrophy	1
		Hyperplasia	2
	Lamina propria	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
	Other layers	Hypertrophy	1
		Hyperplasia	2
		Fibrosis	2
Inflammatory (0–156)	Epithelium	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
	Lamina propria	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1
	Other layers	Infiltration lymphocytes	2
		Infiltration macrophages	2
		Infiltration neutrophils	2
		Infiltration EGCs	2
		Exudate	1

EGCs, eosinophilic granular cells; RES, reticuloendothelial system; MMCs, melano-macrophage centres.

3.4 BACTERIOLOGICAL INVESTIGATION

Sampling for bacteriological investigation was performed during necropsy. The dissecting instruments and external surfaces of the fish were disinfected with 70% ethanol before handling the internal organs and after necropsy of each fish.

For the isolation of flavobacteria, AO agar (Anacker and Ordal, 1955) and MS agar supplemented with tobramycin (Song et al., 1988; Decostere et al., 1997) were used. Gill and skin samples, as well as samples of spleen, kidney, and liver, were taken with sterile disposable loops and inoculated separately onto both agar plates. The plates with AO agar were incubated aerobically for 5–7 days at 15 °C, and plates with MS agar for 3–5 days at 21 °C. Bacterial growth was recorded every day. For general bacteriological investigation, samples of spleen, kidney,

and liver were inoculated onto blood agar and incubated aerobically for 3–5 days at 21 °C.

To obtain pure cultures, subculturing was performed on MS agar for 3 days at 21 °C. More fastidious species, such as *F. psychrophilum* and *F. branchiophilum*, were subcultured on AO agar for 3 days at 15 °C.

3.5 IDENTIFICATION OF ISOLATES

3.5.1 Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

Isolates were identified using MALDI-TOF MS. Single colonies exhibiting distinct morphology and colour were analysed with the Bruker Microflex LT MS (Bruker Daltonics, USA) using the standard protein extraction method with α-cyano-4-hydroxycinnamic acid as matrix according to the manufacturer's instructions. MSPs generated from proteins with masses between 2,000 and 20,000 Daltons were visualised and compared using Bruker flexControl 3.4.207 software and Bruker MBT Compass HT (Version 5.1.550) database. Isolates were identified with high confidence if the best match log(score) was ≥ 2 and the second-best match log(score) was < 2. Isolates that did not meet these criteria were classified as *Flavobacterium* spp. Isolates were stored at –70 °C in Microbank cryovials (Pro-lab Diagnostics, Austin, Tex.) according to the manufacturer's instructions.

MSPs of selected *Flavobacterium* spp. identified by WGS and subjected to AST were added to our in-house *Flavobacterium* MALDI-TOF MS database and analysed. The selected species included *F. branchiarum, F. bernardetii*, *F. collinsii*, *F. celericrescens*, *F. difficile*, *F. erciyesense, F. hercynium*, *F. oncorhynchi*, *F. piscis*, *F. tiangeerense,* and *F. tructae,* as well as several putative new species, FX2, FX3, FX5, FX9, FX10, FX15, FX18, FX23, FX27, FX32, and FX36. Their MSPs were compared with those of *F. psychrophilum*, *F. branchiophilum,* and some other commonly isolated *Flavobacterium* species available in the Bruker reference database (Version 5.1.550). The MALDI Biotyper

software (MALDI Biotyper Compass Explorer 3.1., Bruker Daltonics) was used to create a dendrogram with standard parameters, where the distance values were relative and normalised to a maximum of 1000.

3.5.2 Whole-genome sequencing and bioinformatics analysis

Isolates with unreliable MALDI-TOF MS identification (n = 81), particularly those isolated from internal organs or in greater numbers from skin or gills, as well as those showing frequently observed colony morphologies and/or MALDI-TOF MS results, were selected for WGS (Appendix 3). In addition, to determine the genotypic AMR profiles, a subset of seven *F. psychrophilum* isolates representing different resistotypes based on phenotypic AST, and two *F. branchiophilum* isolates from different fish farms were selected for WGS. Six different resistotypes were identified in *F. psychrophilum*, and one isolate for each was selected for WGS, except for resistotype 6, for which two representative isolates were selected (Table 13). In total, 90 isolates underwent WGS.

Total DNA was extracted from pure cultures using the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD, USA). DNA libraries were prepared with the Illumina TruSeq DNA Nano Library Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed on the NextSeq 500 System using 2 × 150 bp chemistry (Illumina, San Diego, CA, USA) to a minimum coverage of 150×. Raw reads were assembled with Shovill version 1.18 (https://github.com/tseemann/shovill) using the --trim option and SPAdes version 3.13.1 (Bankevich et al., 2012) as the underlying assembler with default parameters.

AMR genes were identified using ResFinder version 4.1 (Bortolaia et al., 2020) and CARD's Resistance Gene Identifier (RGI) version 6.0.3 (Alcock et al., 2020), both with default parameters. To determine the presence of previously described missense mutations in the DNA gyrase subunit A gene (*gyrA*) conferring quinolone resistance, *gyrA* genes were extracted from the assemblies and aligned with the previously published *gyrA* genes from *F. psychrophilum* and *E. coli* (Izumi and

Aranishi, 2004), after which they were translated into amino acid sequences using Geneious Prime version 2022.1.1 (BioMatters Ltd.).

MLST was performed using the Sequence Query tool implemented in the PubMLST database for *F. psychrophilum* (Jolley et al., 2018). Molecular serotype of *F. psychrophilum* isolates was predicted based on the presence or absence of three previously described serovar-associated genes (Rochat et al., 2017), as determined by BLASTn.

The most closely related validly described *Flavobacterium* species were identified based on the nucleotide identity of the 16S rRNA and *gyrB* gene sequences. For this purpose, the 16S rRNA and *gyrB* gene sequences of the studied strains were first extracted from the assemblies. Best hits were then identified using the EzBioCloud 16S identification service (Yoon et al., 2017) for the 16S rRNA gene and the NCBI BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI nr/nt database for the gyrB gene. The most closely related validly described *Flavobacterium* species were then included in the calculation of pairwise ANI values based on the BLAST algorithm using Pyani v0.2.11 (Pritchard et al., 2016). A threshold of 95% ANI was used for species delineation (Kim et al., 2014).

3.6 ANTIMICROBIAL SUSCEPTIBILITY TESTING

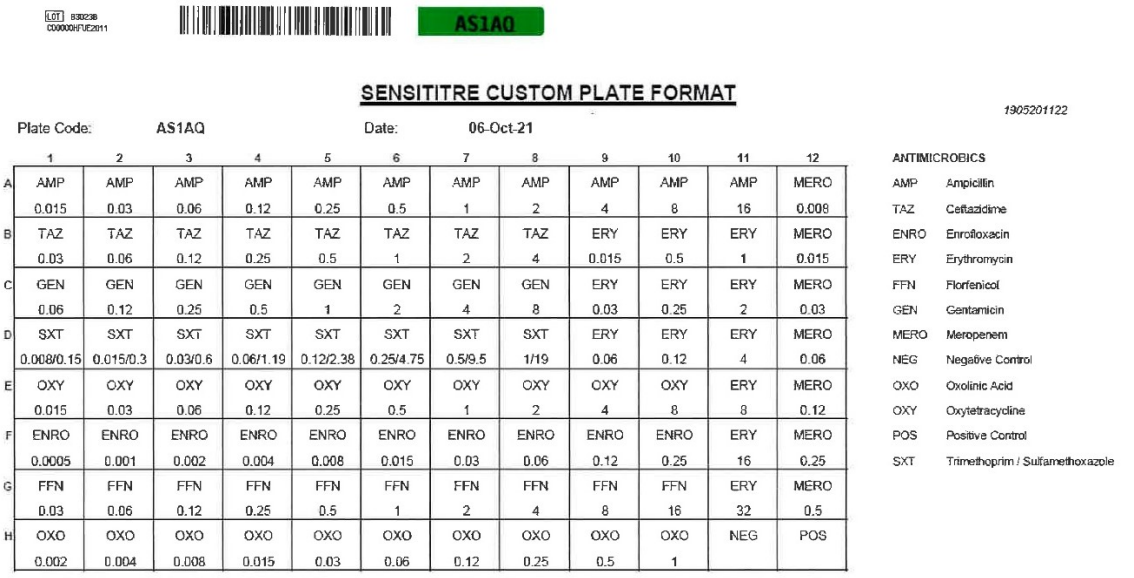
The phenotypic AMR profiles were investigated for representative *F. psychrophilum* and *F. branchiophilum* isolates from each positive fish tank at every sampling point. Where possible, two isolates from diseased fish and one isolate from apparently healthy fish were selected per positive fish tank. A total of 54 representative *F. psychrophilum* isolates were obtained from the gills, skin, and/or internal organs (spleen, kidney, and liver) of rainbow trout sampled at four fish farms. In addition, eight representative *F. branchiophilum* isolates were recovered from the gills of rainbow trout from farms F1 and F2 in the summer of 2023 (Appendix 3).

AST was also performed for 15 isolates representing 11 lesser-known species, namely two isolates of *F. branchiarum, F. bernardetii*, *F. collinsii,* and *F. celericrescens,* and one isolate of *F. difficile*, *F. erciyesense, F. hercynium*, *F. oncorhynchi*, *F. piscis*, *F. tiangeerense,* and *F. tructae*, all confirmed by WGS. Additionally, several putative new species (n = 41) were identified by WGS, and AST was performed for those with at least two representative isolates (29 isolates representing 11 putative new species), namely FX2 (3 isolates), FX3 (3 isolates), FX5 (3 isolates), FX9 (2 isolates), FX10 (2 isolates), FX15 (6 isolates), FX18 (2 isolates), FX23 (2 isolates), FX27 (2 isolates), FX32 (2 isolates), and FX36 (2 isolates) (Appendix 3).

A total of 106 *Flavobacterium* isolates representing 24 different species underwent AST.

3.6.1 Phenotypic AST of *Flavobacterium psychrophilum*

AST was performed using the broth microdilution method recommended for *F. psychrophilum* in the CLSI guideline VET03 (CLSI, 2020a). The MICs for 10 antimicrobials were determined using the Sensititre custom AS1AQ plates (Trek Diagnostic Systems) (Figure 3). These were 96-well dry-form test plates containing twofold serial dilutions of the following antimicrobials: AMP 0.015–16 µg/ml (beta-lactam, penicillin subclass), ceftazidime (CTZ) 0.03–4 µg/ml (beta-lactam, third-generation cephalosporin subclass), ENR 0.0005–0.25 µg/ml (fluoroquinolone), ERY 0.015–32 µg/ml (macrolide), FLO 0.03–16 µg/ml (amphenicol), GEN 0.06–8 µg/ml (aminoglycoside), meropenem (MER) 0.008–0.5 µg/ml (beta-lactam, carbapenem subclass), OXO 0.002–1 µg/ml (quinolone), OXY 0.015–8 µg/ml (tetracycline), and TRS 0.008/0.15–1/19 µg/ml (folate pathway inhibitors).



SENSITITRE CUSTOM PLATE FORMAT

Plate Code: AS1AQ Date: 06-Oct-21 1905201122

	1	2	3	4	5	6	7	8	9	10	11	12
A	AMP 0.015	AMP 0.03	AMP 0.06	AMP 0.12	AMP 0.25	AMP 0.5	AMP 1	AMP 2	AMP 4	AMP 8	AMP 16	MERO 0.008
B	TAZ 0.03	TAZ 0.06	TAZ 0.12	TAZ 0.25	TAZ 0.5	TAZ 1	TAZ 2	TAZ 4	ERY 0.015	ERY 0.5	ERY 1	MERO 0.015
C	GEN 0.06	GEN 0.12	GEN 0.25	GEN 0.5	GEN 1	GEN 2	GEN 4	GEN 8	ERY 0.03	ERY 0.25	ERY 2	MERO 0.03
D	SXT 0.008/0.15	SXT 0.015/0.3	SXT 0.03/0.6	SXT 0.06/1.19	SXT 0.12/2.38	SXT 0.25/4.75	SXT 0.5/9.5	SXT 1/19	ERY 0.06	ERY 0.12	ERY 4	MERO 0.06
E	OXY 0.015	OXY 0.03	OXY 0.06	OXY 0.12	OXY 0.25	OXY 0.5	OXY 1	OXY 2	OXY 4	OXY 8	ERY 8	MERO 0.12
F	ENRO 0.0005	ENRO 0.001	ENRO 0.002	ENRO 0.004	ENRO 0.008	ENRO 0.015	ENRO 0.03	ENRO 0.06	ENRO 0.12	ENRO 0.25	ERY 16	MERO 0.25
G	FFN 0.03	FFN 0.06	FFN 0.12	FFN 0.25	FFN 0.5	FFN 1	FFN 2	FFN 4	FFN 8	FFN 16	ERY 32	MERO 0.5
H	OXO 0.002	OXO 0.004	OXO 0.008	OXO 0.015	OXO 0.03	OXO 0.06	OXO 0.12	OXO 0.25	OXO 0.5	OXO 1	NEG	POS

ANTIMICROBICS	
AMP	Ampicillin
TAZ	Ceftazidime
ENRO	Enrofloxacin
ERY	Erythromycin
FFN	Florfenicol
GEN	Gentamicin
MERO	Meropenem
NEG	Negative Control
OXO	Oxolinic Acid
OXY	Oxytetracycline
POS	Positive Control
SXT	Trimethoprim / Sulfamethoxazole

Figure 3: The layout of the Sensititre custom AS1AQ microtitre plate (Trek Diagnostic Systems)

Escherichia coli strain ATCC 25922 was used as a quality control strain as recommended in the CLSI guideline VET03 (CLSI, 2020a). In addition, the reference strain *F. psychrophilum* ATCC 49418^T isolated in 1989 was included for comparison.

Sensititre autoinoculator (Trek Diagnostic Systems) was used to add the inoculated diluted cation-adjusted Mueller-Hinton broth (DCAMHB) to 96-well plates. The inoculated plates were covered with plastic adhesive seals, incubated at 18 °C, and read after 96 hours. The ECVs for ENR, ERY, FLO, OXO, and OXY against *F. psychrophilum* and the acceptable ranges for the *E. coli* quality control strain are given in CLSI guideline VET04 (CLSI, 2020b).

3.6.2 Phenotypic AST of *Flavobacterium branchiophilum*

The MICs for 10 antimicrobials were determined using the Sensititre custom AS1AQ plates (Trek Diagnostic Systems) as for *F. psychrophilum*. No growth was observed during the initial phenotypic testing of *F. branchiophilum* isolates for an AMR profile according to the CLSI guideline VET03 (CLSI, 2020a) recommended for *F. psychrophilum*. Several adjustments of this method were attempted, and growth was achieved by adding 50 mg/l of calcium, magnesium, and potassium

cations (Skulska, 2014) to the sterile distilled water used to dilute the commercial CAMHB (DCAMHB+C). Additionally, better growth was observed when the initial bacterial suspensions were prepared in CAMHB instead of sterile saline, and at the upper end of the 0.5 McFarland standard. A Sensititre autoinoculator (Trek Diagnostic Systems) was used to add the inoculated DCAMHB+C to 96-well plates. To evaluate the growth of *F. branchiophilum* in DCAMHB+C, we also performed the broth microdilution method using the non-standardised AO broth, which is known to support the growth of this bacterium. The inoculated plates were covered with plastic adhesive seals and incubated at 18 °C for 96 hours.

The following adjustments were also tested to promote the growth of *F. branchiophilum* but produced no growth: (*i*) supplementation of full-strength CAMHB and DCAMHB with 5% fetal bovine serum (Gieseker et al., 2016), (*ii*) supplementation of full-strength CAMHB and DCAMHB with 0.11 g of yeast extract (Bernardet and Bowman, 2006), and (*iii*) full-strength CAMHB (Hesami et al., 2010).

3.6.3 Phenotypic AST of lesser-known *Flavobacterium* species

The MICs for 10 antimicrobials were determined using Sensititre custom AS1AQ plates (Trek Diagnostic Systems), following the method recommended for *F. psychrophilum* (CLSI, 2020a). Similar to *F. branchiophilum*, no growth was observed for some isolates (n = 13) during the initial AST testing, conducted according to the CLSI guideline VET03 (CLSI, 2020a). This included isolates of species *F. bernardetii, F. celericrescens*, *F. difficile,* and *F. tiangeerense,* and isolates of putative new species FX2, FX3, FX5, FX15, and FX18 (Appendix 6). For these isolates, we repeated the AST using the adjusted method we developed for *F. branchiophilum*, which resulted in good growth.

3.6.4 Determination of wild type (WT) and non-wild type (NWT) phenotypes

We use the term WT for fully susceptible isolates and NWT for isolates with reduced susceptibility (Silley, 2012). For *F. psychrophilum* isolates, WT and NWT

populations were defined based on the ECVs for ENR, ERY, FLO, OXO, and OXY as provided in the CLSI guideline VET04 (CLSI, 2020b). We also used Kronvall's NRI method (Kronvall, 2010) to calculate the provisional CO_{WT} values based on the MIC data obtained in this research. The CO_{WT} values were calculated for those antimicrobials for which no more than three of 54 isolates had MICs below or above the tested range and for which the data set generated standard deviations of the normalised distribution lower than 1.2 $\log_2$ µg/ml. In the calculations, the off-scale results were assigned MICs immediately below or above the tested range (Smith et al., 2016; Ngo et al., 2018; Saticioglu et al., 2019). The NRI method was used with the permission of the patent holder Bioscand AB (Täby, Sweden; European patent No. 1383913, US Patent No. 7,465,559). The automatic Microsoft Excel spreadsheet used to perform the NRI calculations was accessed online (http://www.bioscand.se/nri/) (courtesy of P. Smith, W. Finnegan, and G. Kronvall).

Determination of WT and NWT phenotypes for *F. branchiophilum* and other flavobacteria was not possible due to the lack of a standardised AST method and established ECVs. In addition, the NRI method could not be applied due to the limited number of isolates tested. However, some studies have used the ECVs established *F. psychrophilum* (ECV_FP) to interpret the MICs for other *Flavobacterium* species (Heckman et al., 2023), and the same approach was applied in this research.

3.7 STATISTICAL ANALYSIS

Given the estimated difference in the prevalence of flavobacterial isolation in healthy and diseased fish (40%; 20% in apparently healthy fish and 60% in diseased fish), a sample size of 54 fish per sampling was calculated at a 95% confidence level and 80% statistical power to detect differences between groups. In LONG sampling, fish were sampled from three tanks (9 apparently healthy and 9 diseased fish per tank), whereas in IND sampling, fish were sampled from a single tank (27 apparently healthy and 27 diseased fish per tank) to ensure greater statistical power.

Statistical analyses were performed using the Python programming language (Van Rossum and Drake, 2009), with core statistical modelling implemented using the statsmodels package (Seabold and Perktold, 2010). Data visualisation was performed with Matplotlib (Hunter, 2007). The protocol shown in Figure 4 was used for group comparisons. The normality of the continuous variables was first assessed with the Shapiro-Wilk test (Shapiro and Wilk, 1965). Associations between categorical variables were evaluated using the chi-squared test (Pearson, 1900), and relationships between continuous variables were quantified using Spearman's rank correlation (Spearman, 1904) and Pearson's product-moment correlation (Pearson, 1895). Since all continuous measures were non-normally distributed ($p < 0.05$), the Mann-Whitney U test (Mann and Whitney, 1947) was used for two-group comparisons and the Kruskal-Wallis H test (Kruskal and Wallis, 1952) for multi-group comparisons. To account for the clustering of multiple *p*-values, these were combined using Fisher's method for meta-analysis (Fisher, 1925). The logistic regression models were estimated using the maximum likelihood method. For multivariate comparisons, we employed generalised estimating equations (GEE) to model the correlation structure of the clustered observations (Liang and Zeger, 1986). To assess the similarity among the individual fish, hierarchical clustering was performed using the Jaccard distance, with clusters constructed using the average linkage method. Clustering was conducted separately according to the presence or absence of isolated *Flavobacterium* species and the detection of other fish pathogens in the assessed individuals (Ward, 1963).

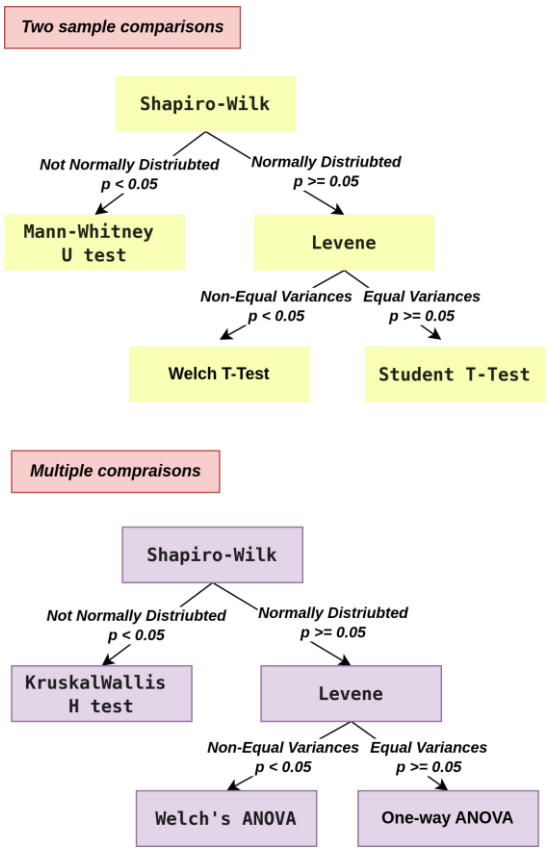


Figure 4: Considered statistical protocol

We evaluated the statistical test power for the analysis of necropsy and histopathological variables for each bacteria-comparison pair by estimating power values for small, medium, and large effect sizes, and adequacy was classified based on whether sample sizes were sufficient to reliably detect at least medium-sized effects (≥ 0.8 power) (Cohen, 1988). Several species were isolated from enough fish to allow statistical analysis of histopathological lesions, including *F. psychrophilum*, *F. hercynium, F. hydatis, F. oncorhynchi, F. pectinovorum,* and *F. saccharophilum* with good adequacy and *F. branchiophilum, F. hibernum, F. piscis,* and *F. frigidimaris* with moderate adequacy. However, the analysis was only performed for *F. psychrophilum*, *F. branchiophilum*, *F. hercynium*, *F. hydatis,* and *F frigidimaris* because the other species mentioned have very similar MSPs and their identification using MALDI-TOF MS is not highly reliable. For the other lesser-known flavobacteria, the statistical power was limited or inadequate to support the statistical analysis due to the small number of isolates identified to the species level.

For the statistical analysis of necropsy and histopathology data, fish that were systemically infected with other pathogens were excluded to avoid misattributing lesions caused by these agents to flavobacterial disease. Fish that tested positive (POS) for *F. psychrophilum* were also excluded when analysing the results for *F. branchiophilum*. Additionally, fish that tested POS for *F. psychrophilum* or *F. branchiophilum* were excluded when analysing the results for the lesser-known *Flavobacterium* spp.

Flavobacteria were isolated from the diseased and apparently healthy fish, and gross lesions were observed in both. For the statistical analyses, we defined a necropsy fish index threshold of ≥ 5.5 to distinguish between truly diseased and healthy individuals (Figure 5). The threshold was determined by comparing necropsy fish index values in two ways: (1) between diseased and apparently healthy fish (Welch t-test, t = - 24.517, df = 437.38, $p < 0.001$), and (2) between fish that tested POS for *F. psychrophilum* and negative apparently healthy (NAH) fish, excluding fish that tested POS for other pathogens (Two Sample t-test, t = 11.087, df = 296, $p < 0.001$). In both cases, the calculated threshold was the same.

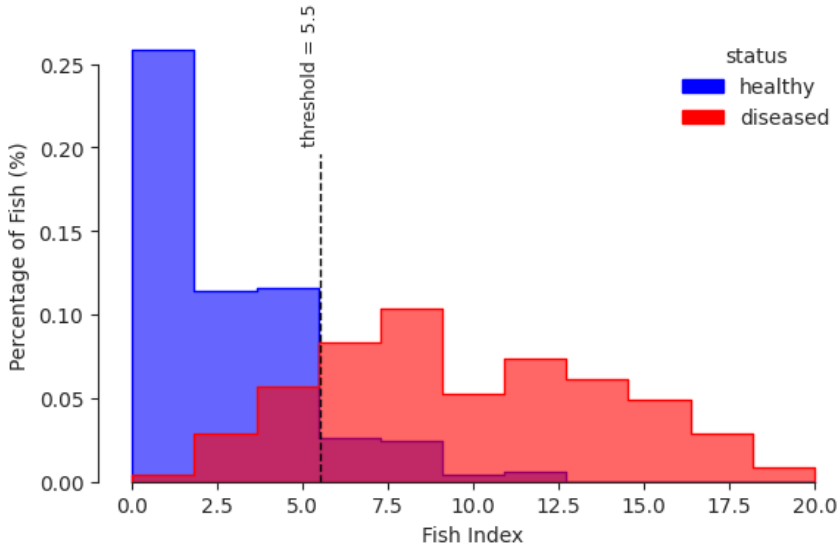


Figure 5: Necropsy fish index of diseased and apparently healthy fish and the determined threshold

To evaluate the association between the isolation of *Flavobacterium* spp. and the presence of pathological lesions, the necropsy and histopathology data of fish that tested POS for the investigated *Flavobacterium* sp. were analysed in two ways: (1) fish that were POS in at least one organ sampled for bacteriology (per fish for all organs), and (2) fish that were POS in the same organ assessed for pathological lesions (per organ for the organs sampled for bacteriology). In both cases, only fish with a necropsy index ≥ 5.5 were included. These were compared with fish that were negative for the investigated *Flavobacterium* sp., had a necropsy index < 5.5, and were AH. All fish that tested POS for other systemic pathogens were always excluded. This classification was chosen to differentiate infected (INF) fish from negative healthy (NH) fish based on combined bacteriological and pathological criteria. Health-based categorisation of fish and associated inclusion criteria are listed in Table 4.

Table 4: Health-based categorisation of fish and associated inclusion criteria

CATEGORY	CRITERIA
diseased fish	status diseased
apparently healthy fish	status healthy
positive (POS) fish	positive isolation
negative apparently healthy (NAH) fish	negative isolation + status healthy
infected (INF) fish	positive isolation + fish index ≥ 5.5
negative healthy (NH) fish	negative isolation + fish index < 5.5 + status healthy

4 RESULTS

4.1 SAMPLED FISH

In general, the sampled fish ranged from approximately 4 to 33 months of age, with weights between 3 and 650 g, total lengths from 6.5 to 40 cm, and K from 0.5 to 2.07. The mean K of sampled fish per fish farm was 1.09 in F1, 0.94 in F2, 0.98 in F3, and 0.91 in F4. Mann-Whitney U tests showed that apparently healthy fish had significantly ($p < 0.05$) greater body weight (median 69 g versus 49.5 g; $p = 0.004$), total length (median 19.0 cm versus 17.7 cm; $p = 0.013$) and K (median 1.00 versus 0.94; $p < 0.0012$) than diseased fish.

In the LONG sampling, fish from F1 were between 4 and 13 months old, weighing between 3 and 305.5 g, and measuring from 6.5 to 31.5 cm in length. Fish from F2 were 17 to 33 months old, with weights ranging from 11 to 650 g and lengths from 10.5 to 40 cm. The mean K was 1.05 in F1 and 0.97 in F2 in autumn, 1.07 in F1 and 0.93 in F2 in winter, 1.08 in F1 and 1.05 in F2 in spring, and 1.16 in F1 and 0.81 in F2 in summer. Information on the mean and range of body weight and total length, as well as the mean K of diseased and apparently healthy fish from each sampled fish tank, is shown in Table 5.

Table 5: Information on the mean and range of body weight (W; g) and total length (L; cm), as well as the mean Fulton's condition factor (K) of diseased (D) and apparently healthy (H) fish from each fish tank (T) sampled during longitudinal sampling (LONG) in fish farms F1 and F2

SEASON			AUTUMN		WINTER		SPRING		SUMMER	
SAMPLING			LONG1		LONG3		LONG5		LONG7	
STATUS			D	H	D	H	D	H	D	H
F1	T1	W	5.78	10.56	40.07	76.69	116.22	185.06	101.66	230.17
			4–8	6–16	10–59	32.5–113.5	80.5–146	100.5–300	25.5–300.5	165.5–305.5
		L	8.41	10.04	15.33	19.00	21.62	25.22	19.61	26.56
			7.5–9.1	8.6–11.6	10.5–18.3	15.5–22	19–24	20–31.5	13–26.5	23.5–29
		K	0.97	1.02	1.08	1.07	1.14	1.12	1.11	1.22
	T2	W	6.78	9.89	43.56	81.56	38.06	85.00	32.17	88.50
			4–10	8–12	12–90.5	38–111.5	17.5–81.5	71.5–105	23–48.5	55–124.5
		L	8.49	9.70	15.13	19.28	15.78	20.28	14.17	19.33

Table 5 continued

Table 5 continued

F1	T2		6.7–9.9	9.1–10.1	11–19.3	15–22	13–19.8	19–21.5	11.5–16.5	16.5–21.5
		K	1.12	1.08	1.05	1.12	0.89	1.02	1.14	1.20
	T3	W	4.78	8.33	36.50	51.67	71.50	173.89	19.67	21.72
			3–8	6–11	8–86	32.5–70	18.5–112.5	98–267	10–27.5	16.5–27.5
		L	7.41	9.42	14.59	16.67	18.40	24.44	11.89	12.39
			6.5–8.6	8.4–8.6	9.3–20	15–18	13–23	21–29	10.5–13	11.5–14
		K	1.13	0.98	1.03	1.09	1.11	1.18	1.14	1.14
SEASON			AUTUMN		WINTER		SPRING		SUMMER	
SAMPLING			LONG2		LONG4		LONG6		LONG8	
STATUS			D	H	D	H	D	H	D	H
F2	T1	W	186.00	353.67	215.06	265.61	123.39	90.81	120.17	141.72
			146–301	127–576	96–382	167.5–410	42.5–263	43–150.5	43.5–235	73.5–195
		L	28.41	31.14	29.11	29.72	21.96	22.63	24.67	25.67
			25.3–32.5	24.5–37	24.5–32	27–34.5	17–31	16.5–27.5	19–30.5	20.5–29
		K	0.80	1.09	0.84	1.00	1.25	0.81	0.72	0.82
	T2	W	194.67	204.25	124.56	58.00	58.83	47.28	138.17	87.67
			109–320	121–348	13.5–339.5	36–75.5	27.5–94	18.5–94	21–650	61.5–162
		L	27.14	27.13	21.50	18.44	17.69	16.72	23.22	21.94
			22.6–30.7	24.5–31	11–29.5	15.5–20.5	13.8–21.5	12.5–21	14.5–40	19–27
		K	0.93	0.98	1.01	0.90	1.02	0.94	0.74	0.81
	T3	W	229.11	456.44	134.00	38.39	90.89	94.26	37.22	55.06
			162–372	318–648	11–285	16.5–72.5	52.5–121.5	13.8–147.5	14.5–62	38–90.5
		L	29.33	34.67	23.00	15.44	22.00	22.22	16.00	18.00
			25–35	31.5–37.5	11–32	11–21	18–25	10.5–25.5	12–19	16–20.5
		K	0.90	1.08	0.88	0.97	0.83	1.40	0.87	0.92

In the IND sampling, fish from F3 were 20 months old, weighed between 30.5 and 90.5 g, with lengths ranging from 14.5 to 21 cm. Fish from F4 were 7 months old, weighed from 3.5 to 10 g, and measured from 7.5 to 10 cm in length. Information on the mean and range of body weight and total length, as well as the mean K of diseased and apparently healthy fish from each IND sampling, is shown in Table 6.

Table 6: Information on the mean and range of body weight (W; g) and total length (L; cm), as well as the mean Fulton's condition factor (K) of diseased (D) and apparently healthy (H) fish from individual sampling (IND) in fish farms F3 and F4

IND		D	H
F3	W	56.19	57.52
		30.5–90.5	36.5–81.5
	L	17.82	17.86
		14.5–21	15.5–21
	K	0.96	1.00
F4	W	6.07	6.83
		4–9.5	3.5–10
	L	8.72	9.04
		7.5–10	8–10
	K	0.91	0.91

Kruskal-Wallis tests showed that the K varied significantly depending on the season ($p < 0.001$) and was highest on average in winter (1.04 ± 0.18) and autumn (1.04 ± 0.12) and lowest in spring (0.98 ± 0.19) and summer (0.99 ± 0.19). Dunn post-hoc comparisons (Bonferroni-adjusted) showed that winter and autumn differed significantly from spring and summer ($p \leq 0.001$), while winter and autumn, as well as spring and summer, showed no differences.

K varied significantly between fish farms as it averaged lowest at F2 (0.89 ± 0.17), was similarly low at F4 (0.91 ± 0.11), intermediate at F3 (0.98 ± 0.09), and highest at F1 (1.09 ± 0.19, n = 215). A Kruskal–Wallis test confirmed a strong overall farm effect ($H = 139.463$, $p < 0.001$). Dunn post-hoc comparisons (Holm-adjusted) showed F1 differed significantly from every other farm ($p \leq 0.0013$); F2 and F4 did not differ from each other ($p = 0.5933$), but both were lower than F3 ($p \leq 0.0281$).

4.2 DIVERSITY OF FLAVOBACTERIA IN SLOVENE FARMED RAINBOW TROUT

At least one *Flavobacterium* species was isolated from one or more organs of 89.3% (482/540) of all sampled fish, predominantly from the gills (70.2% of POS fish) and skin (81.1%), and to a lesser extent from internal organs (16.9%). Of the isolates from internal organs, 74.5% were identified as *F. psychrophilum*. A single *Flavobacterium* species was isolated from 35.9% (194/540) of the fish, while two or more different isolates were obtained from 53.3% (288/540) of the sampled fish.

Multiple different isolates per fish were recovered from the gills and skin only, except in the liver of three fish. However, histopathological investigation revealed no lesions in the livers of these fish. A total of 2652 isolates belonging to the genus *Flavobacterium* were identified by MALDI-TOF MS, of which 2105 were preserved in storage. Notably, 75.98% (2015/2652) of the identified isolates could not be classified to the species level during this research.

The colony morphology of flavobacteria varied greatly, ranging in colour from very light yellow (almost translucent) to dark orange, with some colonies exhibiting an iridescent sheen, colony size from small (< 1 mm) to large (> 5 mm), shape from round with entire margins to irregular with mildly to severely rhizoid margins, as well as the surface presenting as shiny, matte or, rarely, mucoid. Notably, colony morphology differed considerably even between isolates of the same species. Isolates of *F. psychrophilum* formed light to dark yellow, round colonies with entire or mildly to moderately rhizoid margins, and a shiny or shiny-matte surface. In contrast, the colony morphology of *F. branchiophilum* isolates was more uniform, with yellow to dark yellow, slightly iridescent, round colonies with entire margins, and a shiny-matte surface. Representative examples of *F. psychrophilum* and *F. branchiophilum* primary cultures are shown in Figure 6 and Figure 7, respectively.

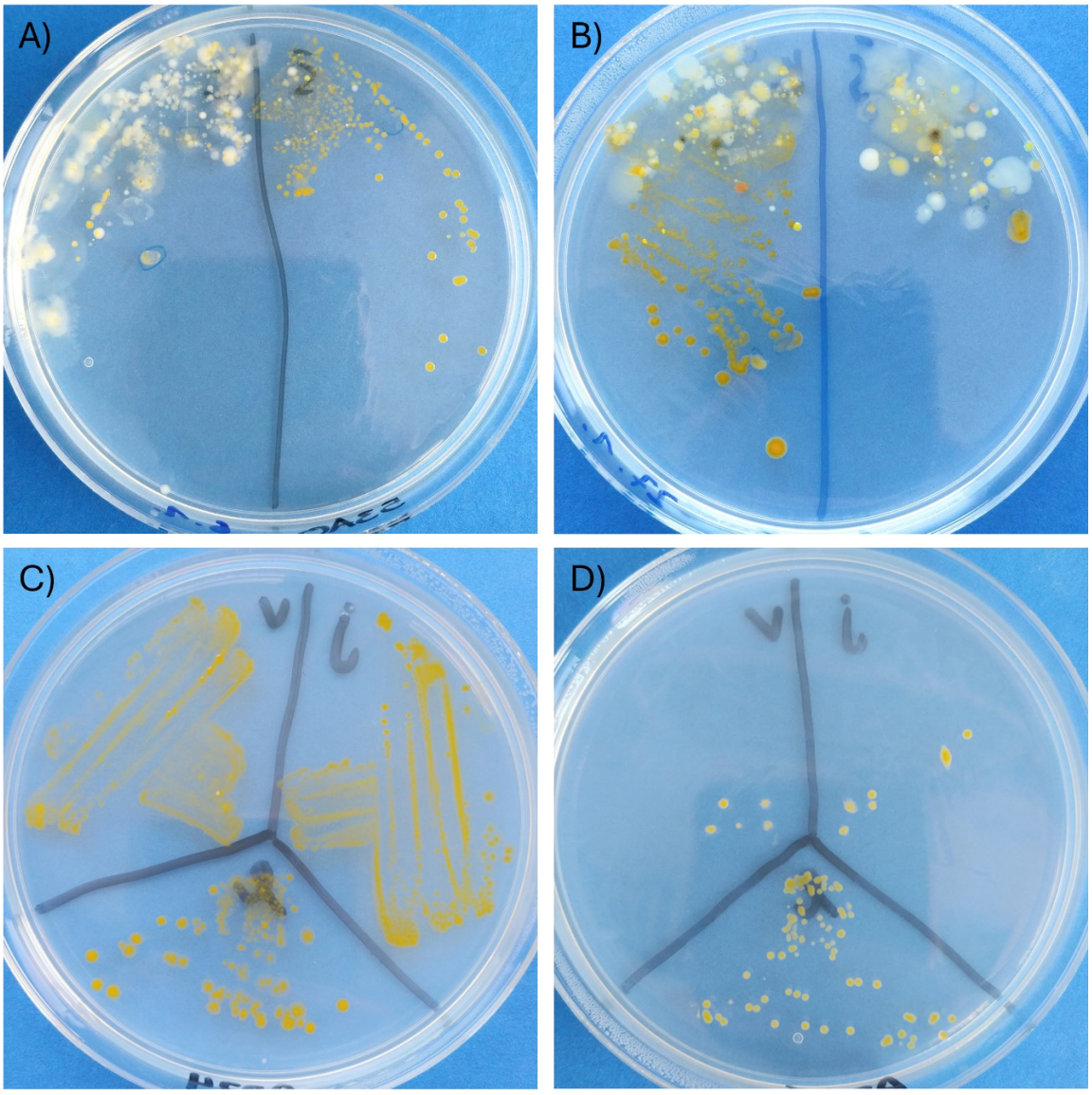


Figure 6: Primary isolation of *Flavobacterium psychrophilum* on Anacker and Ordal's (AO) agar from gills (A), skin (B), and internal organs (C and D); note the different colony morphologies

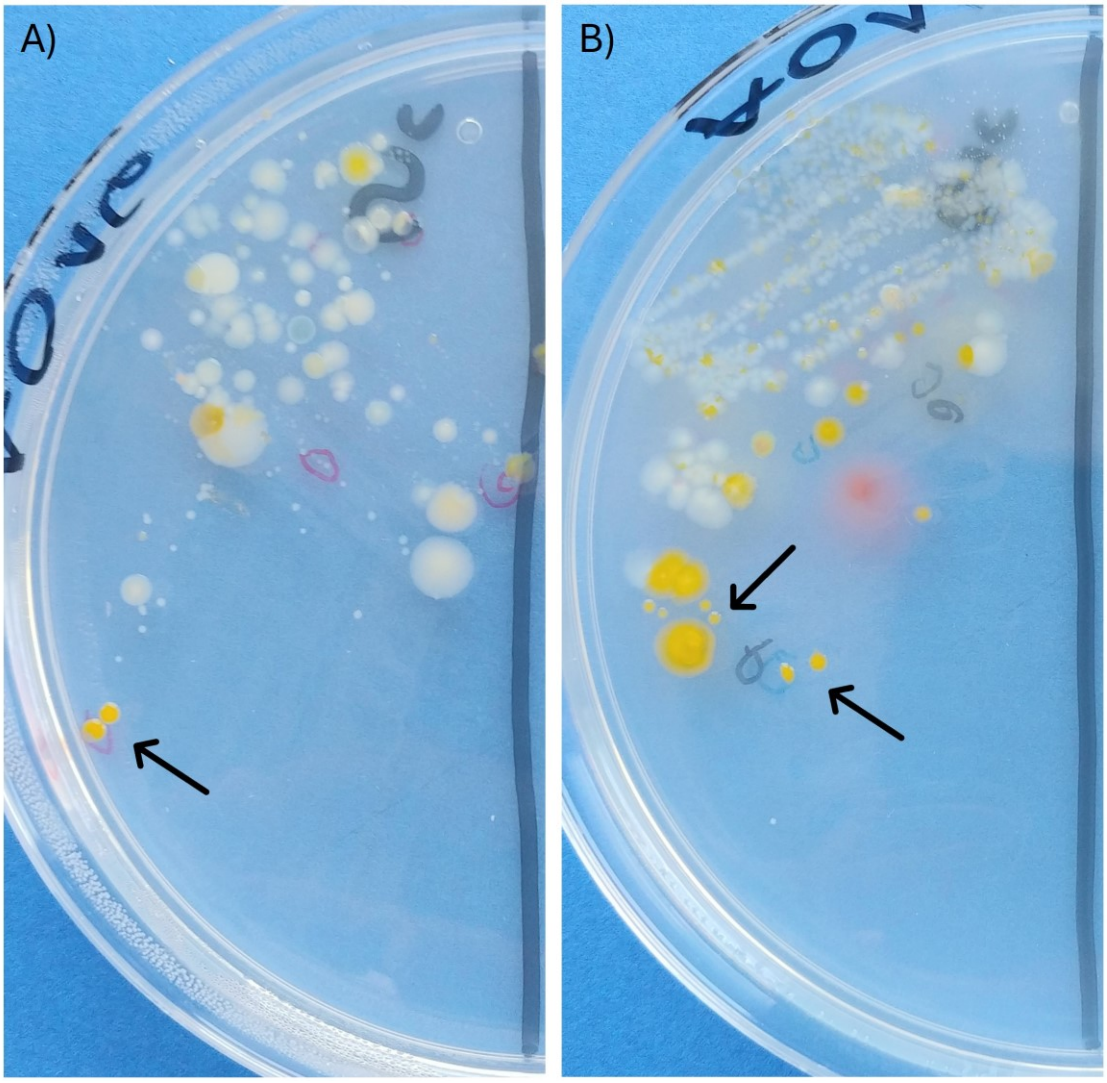


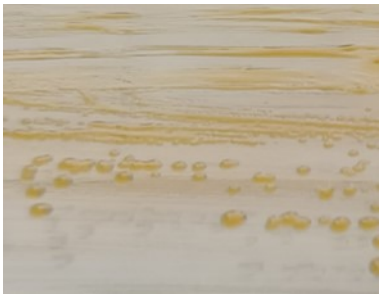
Figure 7: Primary isolation of *Flavobacterium branchiophilum* (arrows) on Anacker and Ordal's (AO) agar from gills; multiple similar larger yellow colonies in figure A) represent putative new species FX10

On the other hand, many flavobacterial species exhibited very similar or indistinguishable colony morphology. Therefore, identification based on colony appearance alone was not reliable. In addition, colony morphology also strongly depended on the growth agar used and the age of the culture. *F. psychrophilum* grew on both AO agar and MS agar, whereas *F. branchiophilum* grew only on AO agar. To illustrate the range of colony morphologies in other isolated *Flavobacterium* spp. images of colonies identified by WGS and subjected to AST are shown in Figure 8.

A)



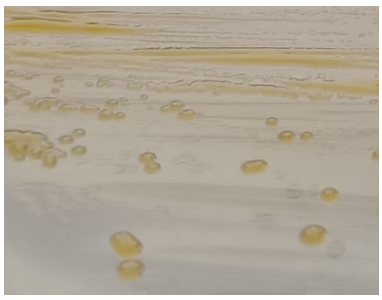
B)



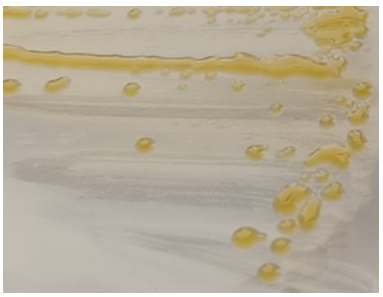
C)



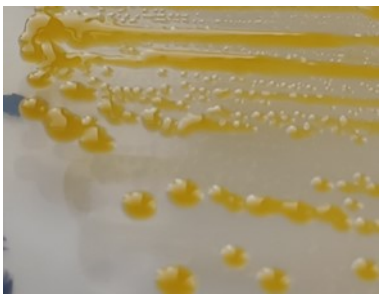
D)



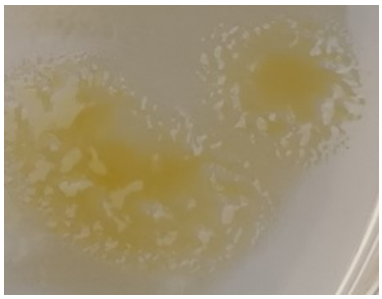
E)



F)



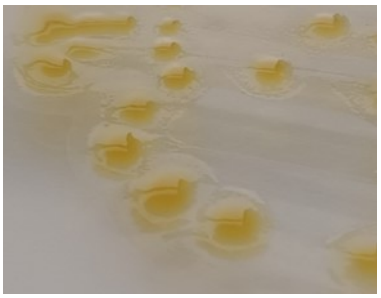
G)



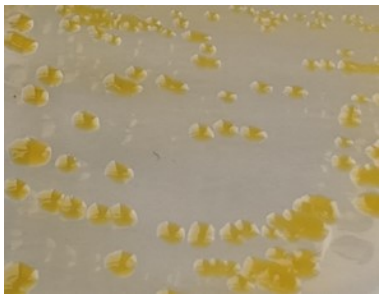
H)



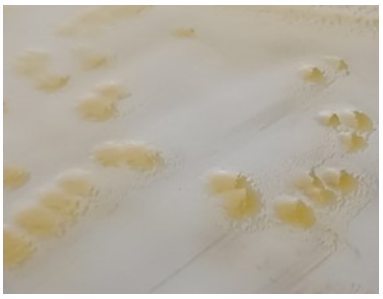
I)



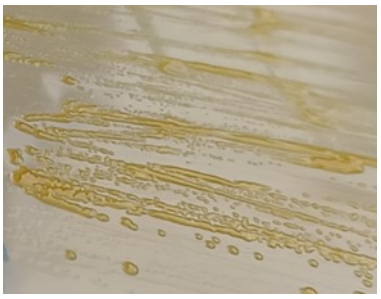
J)



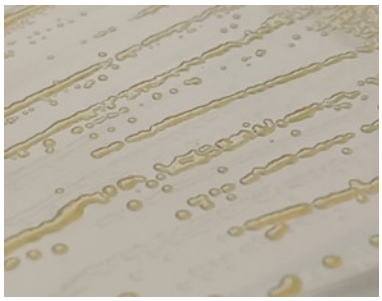
K)



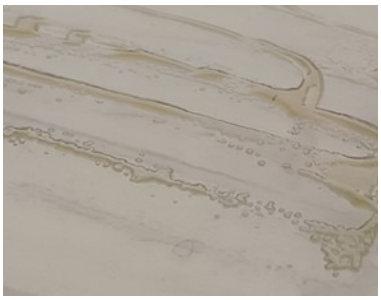
L)



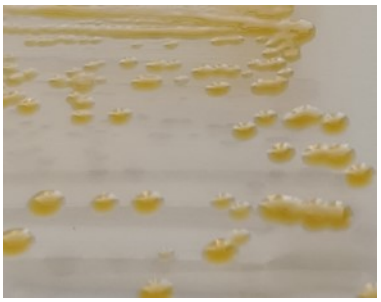
M)



N)



O)



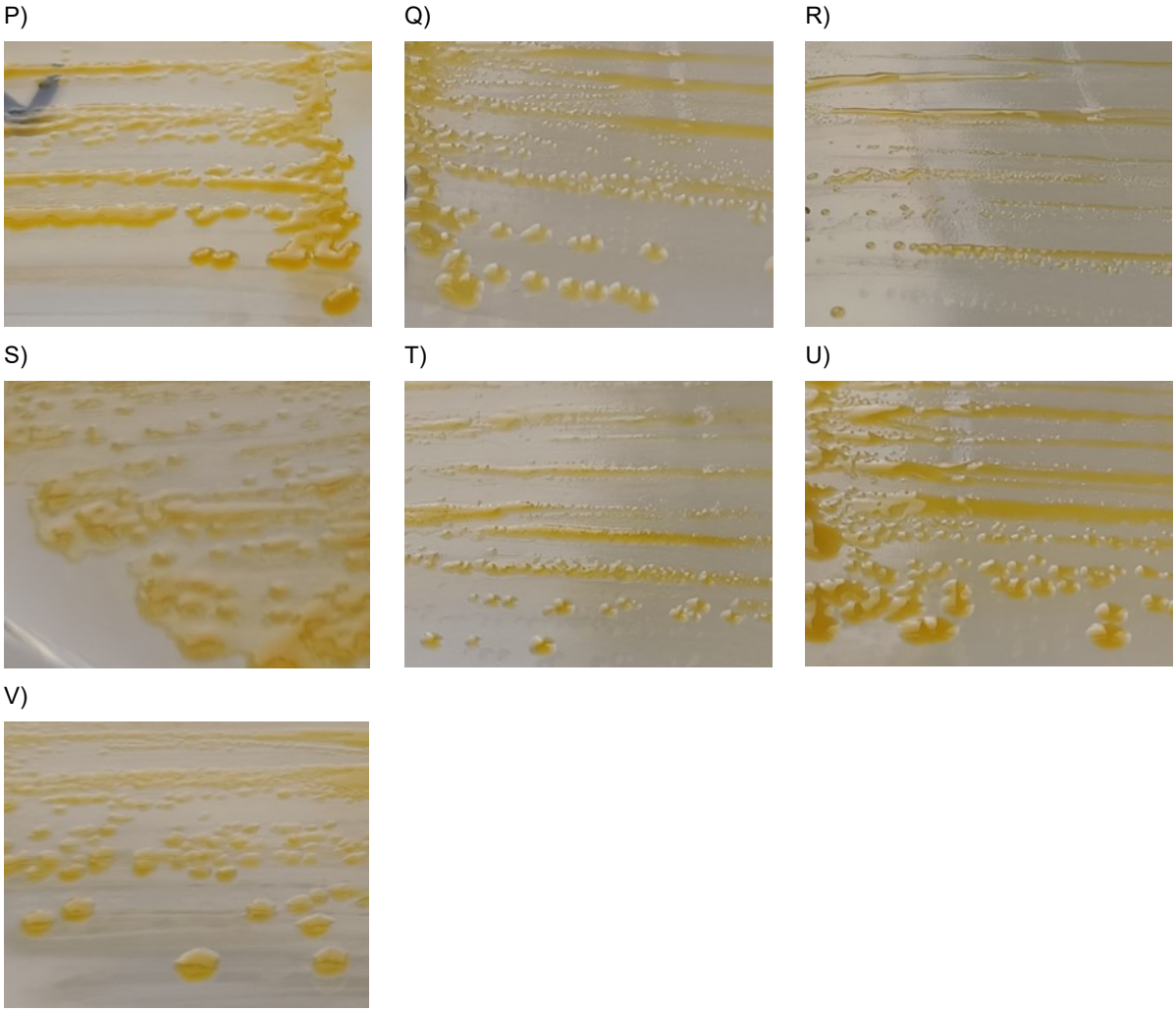


Figure 8: Colony morphology of *Flavobacterium* spp. identified by whole-genome sequencing and subjected to antimicrobial susceptibility testing, namely a) *F. branchiarum* (F324), b) *F. bernardetii* (F609), c) *F. collinsii* (F1673), d) *F. celericrescens* (F1959), e) *F. difficile* (F1633), f) *F. erciyesense* (F562), g) *F. hercynium* (F2050), h) *F. oncorhynchi* (F1942), i) *F. piscis* (F1228), j) *F. tiangeerense* (F1485), k) *F. tructae* (F1900), l) FX2 (F334), m) FX3 (F1048), n) FX5 (F1140), o) FX9 (F945), p) FX10 (F1511), q) FX15 (F2167), r) FX18 (F626), s) FX23 (F1259), t) FX27 (F1527), u) FX32 (F1715) and v) FX36 (F2035)

4.2.1 Well-known flavobacteria

Out of 540 rainbow trout sampled, 128 fish (23.7%) tested POS for *F. psychrophilum,* and 11 fish (2.04%) tested POS for *F. branchiophilum*, which were identified with high confidence using MALDI-TOF MS.

F. psychrophilum was isolated from diseased (99/128; 77.3%) and apparently healthy (29/128; 22.7%) fish from all organs sampled, across all farms studied, and during all seasons (Figure 9). A Pearson chi-square test showed that diseased fish had about 4.8 times higher odds of testing POS for *F. psychrophilum* than apparently healthy fish ($p < 0.0001$).

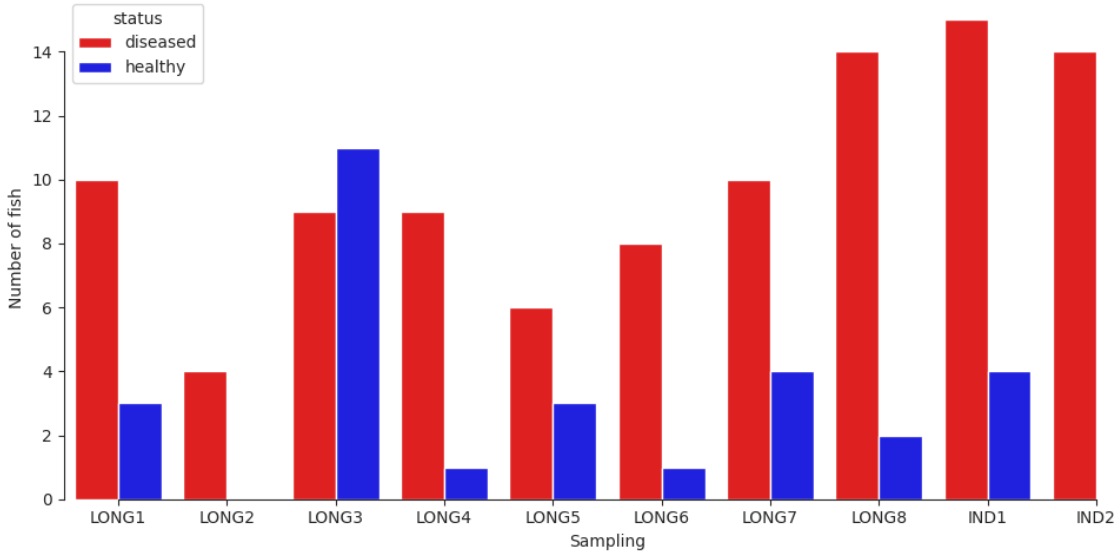


Figure 9: Number of diseased (red) and apparently healthy fish (blue) testing positive for *Flavobacterium psychrophilum* per sampling (LONG, longitudinal sampling; IND, individual sampling)

The Pearson chi-squared test revealed significant differences in the percentage of *F. psychrophilum*-POS fish between the samplings ($\chi^2 = 22.282$, $p = 0.008$). Namely, samplings LONG3 ($p < 0.05$) and IND1 ($p < 0.05$) had significantly higher positivity rates, while LONG2 ($p < 0.01$) had a significantly lower rate than the rest. In contrast, no significant differences were found in *F. psychrophilum* infection rates between samplings ($\chi^2 = 16.534$, $p = 0.0565$). However, sampling IND1 had a significantly higher infection rate than the others ($p < 0.05$). On the other hand, the Pearson chi-squared test revealed no significant differences in the percentage of *F. psychrophilum*-POS fish between fish farms. However, significant variation in infection rates across farms ($\chi^2(df = 3) = 9.17$, $p = 0.027$) was found. To account for the lack of independence among fish sampled within the same event, both a GEE model and a cluster-robust generalised linear model were applied and showed consistent results. Fish from F3 had significantly higher odds for infection

compared to those from F1 and F2, with an odds ratio of approximately 2.2 ($p < 0.001$). Similarly, fish from L4 had roughly 1.8 times higher odds of infection than those from F1 and F2 (*p*-value was approximately 0.005–0.008, depending on the clustering adjustment method). Fish from F1 did not differ significantly from F2 in infection risk (odds ratio approximately 0.9, *p*-value approximately 0.68). Infection prevalence was lowest at F1 and F2 (about 14%), whereas F3 and F4 exhibited significantly higher rates (about 24–28%) of infection. The percentage of fish that were POS or INF with *F. psychrophilum* per sampling is shown in Figure 10.

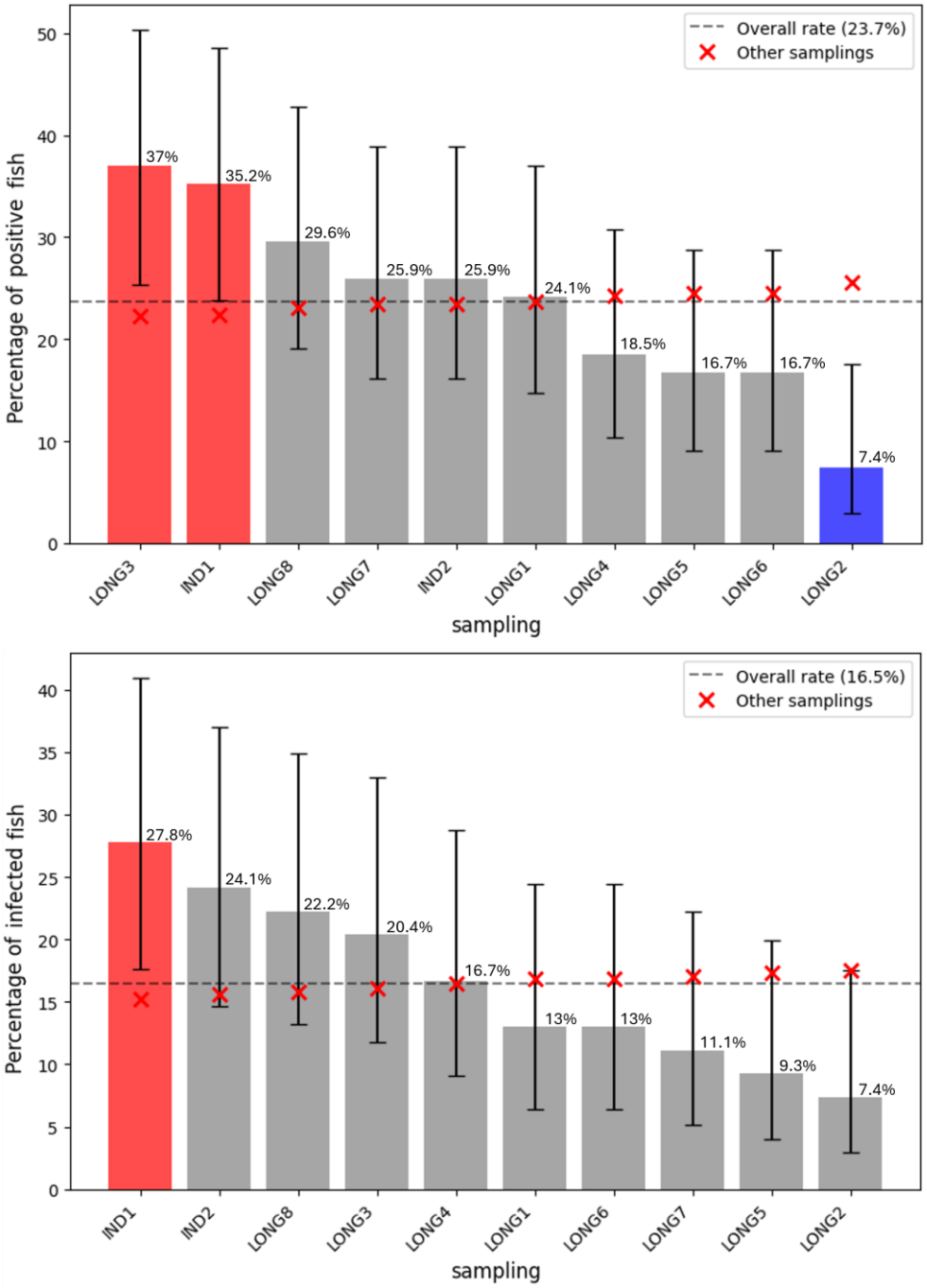


Figure 10: Percentage of fish positive and infected with *Flavobacterium psychrophilum* per sampling (LONG, longitudinal sampling; IND, individual sampling); black full lines indicate standard deviations; grey dashed line and red X indicate overall rate across samplings and an average rate across other samplings, respectively; colour of bars reflects significance based on Pearson chi-squared test, namely red for significantly higher, blue for significantly lower, and grey for no significant difference

F. psychrophilum was isolated from various combinations of sampled organs, namely from all sampled organs (5/128, 3.9%), from the skin and/or gills and at

least one internal organ (26/128, 20.3%), from the internal organs only (35/128, 27.3%) or from the gills and/or skin only (62/128, 48.4%), resulting in 41 isolates from gills, 66 from skin, 40 from kidney, 39 from liver, and 35 from spleen (Table 7, Figure 11).

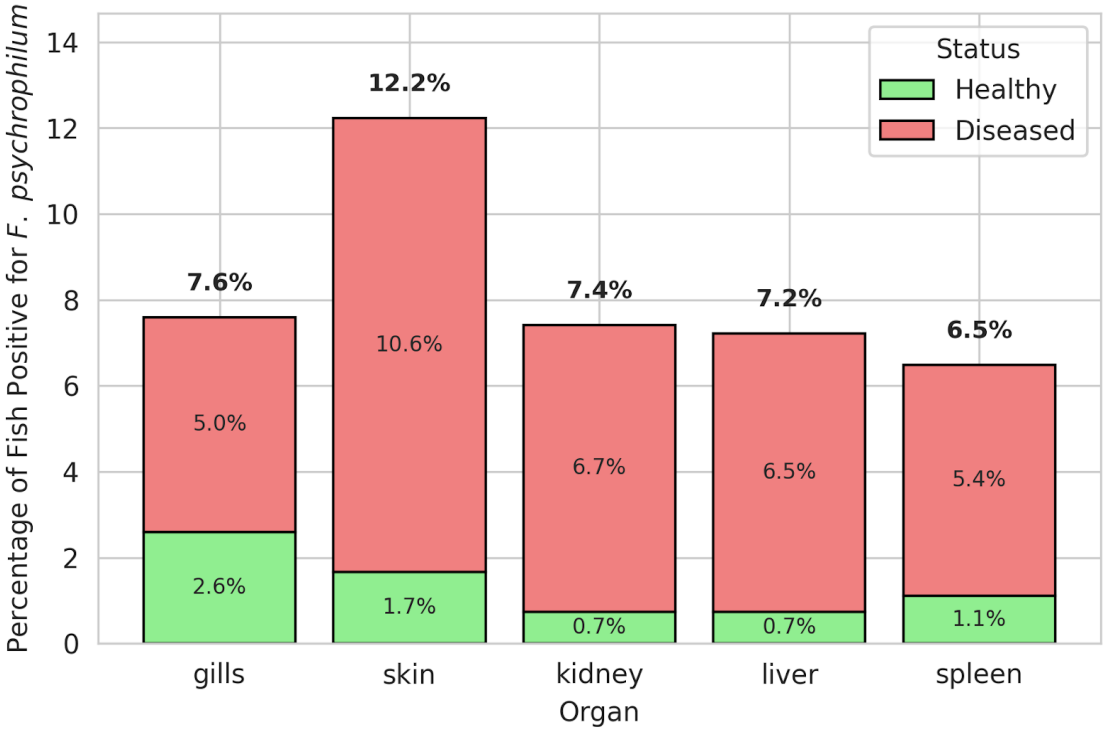


Figure 11: Percentage of diseased and apparently healthy fish testing positive for *F. psychrophilum* per organ.

F. branchiophilum was isolated only from the gills of diseased (8/11; 72.73%) and apparently healthy (3/11; 27.27%) fish sampled during the summer at both F1 and F2. Four of these fish tested POS for both pathogens. However, both *F. psychrophilum* and *F. branchiophilum* were isolated from the gills of only one fish. The number of fish and organs POS for *F. psychrophilum* and *F. branchiophilum* per sampling, fish farm, and fish tank are given in Table 7.

No species belonging to the *F. columnare* group were detected.

Table 7: Number of diseased (D) and apparently healthy (H) fish and organs positive for *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* per sampling (LONG, longitudinal sampling; IND, individual sampling), fish farm (F), and fish tank

SAMPLING	FISH TANK	No. FISH		SKIN		GILLS		KIDNEY		LIVER		SPLEEN	
		D	H	D	H	D	H	D	H	D	H	D	H
F. psychrophilum													
LONG1 – F1 AUTUMN	1	5	0	2	0	0	0	1	0	1	0	2	0
	2	2	3	1	2	1	1	1	0	1	0	0	0
	3	3	0	0	0	1	0	1	0	2	0	0	0
	No.	10	3	3	2	2	1	3	0	4	0	2	0
LONG2 – F2 AUTUMN	1	1	0	0	0	0	0	1	0	0	0	0	0
	2	3	0	2	0	1	0	2	0	1	0	1	0
	3	0	0	0	0	0	0	0	0	0	0	0	0
	No.	4	0	2	0	1	0	3	0	1	0	1	0
LONG3 – F1 WINTER	1	6	5	1	3	6	2	2	0	1	0	3	0
	2	1	2	0	0	1	1	1	1	1	2	1	1
	3	2	4	0	0	1	1	1	3	1	1	1	3
	No.	9	11	1	3	8	4	4	4	3	3	5	4
LONG4 – F2 WINTER	1	1	0	1	0	0	0	0	0	0	0	0	0
	2	4	1	4	1	0	0	0	0	0	0	0	0
	3	4	0	3	0	0	0	0	0	1	0	2	0
	No.	9	1	8	1	0	0	0	0	1	0	2	0
LONG5 – F1 SPRING	1	2	0	2	0	0	0	0	0	0	0	0	0
	2	1	1	1	0	0	1	1	0	0	0	0	0
	3	3	2	2	1	1	1	1	0	1	0	0	0
	No.	6	3	5	1	1	2	2	0	1	0	0	0
LONG6 – F2 SPRING	1	2	0	1	0	1	0	2	0	1	0	2	0
	2	3	0	3	0	0	0	3	0	3	0	3	0
	3	3	1	2	0	0	0	2	0	2	1	2	0
	No.	8	1	6	0	1	0	7	0	6	1	7	0
LONG7 – F1 SUMMER	1	3	3	3	2	0	1	0	0	0	0	0	0
	2	1	1	0	0	0	1	1	0	1	0	0	0
	3	6	0	6	0	1	0	1	0	1	0	1	0
	No.	10	4	9	2	1	2	2	0	2	0	1	0
LONG8 – F2 SUMMER	1	8	1	8	0	2	1	1	0	1	0	0	0
	2	6	1	5	0	4	1	2	0	2	0	4	0
	3	0	0	0	0	0	0	0	0	0	0	0	0
	No.	14	2	13	0	6	2	3	0	3	0	4	0
TOTAL F1	No.	35	21	18	8	12	9	11	4	10	3	8	4
TOTAL F2	No.	35	4	29	1	8	2	13	0	11	1	14	0
IND1 – F3	No.	15	4	8	0	7	3	3	0	3	0	3	2
IND2 – F4	No.	14	0	2	0	0	0	9	0	11	0	4	0
TOTAL		99	29	57	9	27	14	36	4	35	4	29	6
		128		66		41		40		39		35	

Table 7 continued

Table 7 continued

SAMPLING	FISH TANK	No. FISH		SKIN		GILLS		KIDNEY		LIVER		SPLEEN	
		D	H	D	H	D	H	D	H	D	H	D	H
F. branchiophilum													
LONG7 – F1 SUMMER	1	1	0	0	0	1	0	0	0	0	0	0	0
	2	1	0	0	0	1	0	0	0	0	0	0	0
	3	3	1	0	0	3	1	0	0	0	0	0	0
	No.	5	1	0	0	5	1	0	0	0	0	0	0
LONG8 – F2 SUMMER	1	1	0	0	0	1	0	0	0	0	0	0	0
	2	1	1	0	0	1	1	0	0	0	0	0	0
	3	1	1	0	0	1	1	0	0	0	0	0	0
	No.	3	2	0	0	3	2	0	0	0	0	0	0
TOTAL		8	3	0	0	8	3	0	0	0	0	0	0
		11		0		11		0		0		0	

A total of seven selected *F. psychrophilum* and two *F. branchiophilum* isolates underwent WGS analysis. *F. psychrophilum* isolates were assigned to three serotypes, namely Type-0 (4/7 isolates), Type-2 (2/7 isolates), and Type-3 (1/7 isolates). MLST further classified the isolates into CC-ST10 (2/7 isolates), CC-ST01 (1/7 isolates), and several newly identified sequence types (4/7 isolates). The isolate metadata of the reconstructed *F. psychrophilum* and *F. branchiophilum* genomes is given in Table 8.

Table 8: Whole-genome sequencing data on seven *Flavobacterium psychrophilum* and two *Flavobacterium branchiophilum* isolates, including sequence read archive (SRA) run accession number, total genome size (GS), number of contigs (No.), N_{50}, GC content (GC), sequence type based on multilocus sequence typing (MLST ST), MLST allele profile, and molecular serotype

Isolate	*Flavobacterium* species	SRA run accession number	GS [Mbp]	No. (> 500 bp)	N_{50} [bp]	GC [%]	MLST ST	MLST allele profile	Molecular serotype
F88	*F. psychrophilum*	SRR28415848	2.76	73	157870	32.54	ST385 (new)	1, 2, 1, 1, 1, 1, 2	Type-2
F1186	*F. psychrophilum*	SRR28415853	2.74	75	133336	32.35	ST3 (CC-ST10)	2, 2, 2, 2, 2, 3, 2	Type-0
F1187	*F. psychrophilum*	SRR28415847	2.7	76	131395	32.48	ST108 (CC-ST01)	1, 1, 2, 1, 1, 1, 41	Type-3
F1516	*F. psychrophilum*	SRR28415850	2.98	142	110910	32.29	ST401 (new)	3, 2, 3, 103, 2, 4, 3	Type-0
F1519	*F. psychrophilum*	SRR28415849	2.76	83	172390	32.32	ST92 (CC-ST10)	2, 2, 2, 2, 2, 3, 41	Type-2

Table 8 continued

Table 8 continued

F1719	*F. psychrophilum*	SRR28415852	2.99	153	79657	32.32	ST402 (new)	3, 22, 3, 43, 2, 3, 3	Type-0
F621	*F. psychrophilum*	SRR28415851	3.02	165	74217	32.33	ST400 (new)	3, 22, 3, 86, 3, 2, 3	Type-0
F1628	*F. branchiophilum*	SRR28415854	3.58	82	136724	32.75	NA	NA	NA
F1727	*F. branchiophilum*	SRR28415855	3.59	81	128306	32.74	NA	NA	NA

NA, not available

4.2.2 Lesser-known flavobacteria

Several lesser-known flavobacteria (22 species) were identified with high confidence using MALDI-TOF MS (best match log score value ≥ 2.0, second best match log score value < 2), namely *F. aquatile*, *F. aquidurense*, *F. araucananum*, *F. banpakuense*, *F. chungangense*, *F. chungbukense*, *F. cutihirudinis*, *F. frigidimaris*, *F. glaciei*, *F. hercynium*, *F. hibernum*, *F. hydatis*, *F. limicola*, *F. oncorhynchi*, *F. pectinovorum*, *F. piscis, F. plurextorum*, *F. psychrolimnae*, *F. reichenbachii*, *F. saccharophilum*, *F. terrigena,* and *F. tiangeerense*. The sampling information for these isolates, the number of POS fish, and the organ of isolation are given in Table 9.

The best matches for the lesser-known flavobacterial isolates that could not be reliably identified to the species level using MALDI-TOF MS (log score value < 2) included species mentioned above and several other species, namely *F. anhuiense, F. cauense, F. cheniae, F. cucumis, F. daejeonense, F. defluvii, F. degerlachei, F. denitrificans, F. flevense, F. frigidarium, F. gelidilacus, F. ginsengisoli, F. glycines, F. gossypii, F. granuli, F. johnsoniae, F. jumunjinense, F. micromati, F. nitrogenifigens, F. omnivorum, F. resistens, F. rivuli, F. saliperosum, F. sasangense, F. segetis, F. sinopsychrotolerans, F. suncheonense, F. urocaniciphilum, F. weaverense,* and *F. xinjiangense*.

Notably, the matching hints table in the Bruker MALDI Biotyper identification results includes comments on the highly similar MSPs observed among certain flavobacteria species, such as *F. oncorhynchi* and *F. plurextorum*, as well as *F. aquidurense*, *F. araucananum*, *F. hibernum*, *F. pectinovorum*, *F. piscis*,

F. psychroterrae, and *F. saccharophilum*. As a result, distinguishing between these species using MALDI-TOF MS is difficult.

Table 9: Information on the isolation of the lesser-known flavobacteria identified with high confidence using whole-genome sequencing (WGS) and/or matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), including the fish farm (F), fish tank (T), number of isolates from diseased (D) and apparently healthy (H) fish, organ of isolation and total number of positive fish (No.); putative new species are included if isolated from at least two fish

***Flavobacterium* spp.**	**No.**	**WGS***	**2nd MALDI-TOF MS (expanded database)**	**1st MALDI-TOF MS (Bruker database)**
F. aquatile	5	-	-	5 (F2 winter T2 **H1 spleen**; F1 spring T1 **H1 gills**, T2 **H1 gills**; F4 **D1 skin**, **H1 skin**)
F. aquidurense	8	-	-	8 (F1 spring T2 **D1 gills**, T3 **D1 skin**; F2 spring T3 **D1 gills**; F1 summer T2 **D2 gills**, T2 **H1 skin**, T3 **H2 gills**)
F. araucananum	3	-	-	3 (F2 spring T2 **H1 gills**; F3 **D1 skin**; F4 **H1 skin**)
F. banpakuense	1	-	-	1 (F2 spring T2 **H1 skin**)
F. bernardetii	2	2	-	-
F. branchiarum	3	2	1 (F1 winter T1 **H1 skin**)	-
F. celericrescens	2	2	-	-
F. chungangense	1	-	-	1 (F2 summer T1 **H1 skin**)
F. chungbukense	5	-	-	5 (F2 autumn T3 **D1 gills**, **D1 skin**, T1 **H1 skin**; F2 winter T1 **D1 gills and skin**; F2 spring T3 **D1 gills**)
F. collinsii	2	2	-	-
F. cupriresistens	1	1	-	-
F. cutihirudinis	1	-	-	1 (F2 autumn T3 **H1 skin**)
F. difficile	1	1	-	-
F. erciyesense	1	1	-	-
F. frigidimaris	13	-	-	13 (F1 winter T1 **H1 skin**, T2 **D1 skin**, T3 **D1 skin**; F2 spring T1 **D1 skin**, T2 **D1 gills and skin**, **D1 skin**, **H2 gills**, **H1 skin**, T3 **D1 gills**; F3 **H3 skin**)
F. glaciei	2	-	-	2 (F3 **D1 gills**, **D1 skin**)

Table 9 continued

Table 9 continued

F. hercynium	24	1	-	23 (F1 winter T1 **D1 skin**, **H1 skin**, T2 **H2 skin**, T3 **D4 skin**, **H1 skin**; F2 winter T1 **H1 skin**, T2 **D1 gills**, **H1 skin**; F1 spring T1 **H1 gills**; F2 spring T1 **D1 gills**, **D1 skin**, **H1 skin**, T3 **D1 gills and skin**, **H1 skin**; F2 summer T1 **D1 skin**, **H1 gills**, T2 **D1 gills**, **D1 skin**; F4 **D1 skin**)
F. hibernum	16	-	-	16 (F2 autumn T2 **H1 skin**; F1 winter T1 **D1 gills**, **D1 skin**, T2 **H1 skin**, T3 **H1 skin**; F2 winter T3 **D1 gills**; F1 spring T1 **H2 skin**; F2 spring T1 **H1 gills**, T3 **D1 gills**, **H1 skin**; F3 **D1 gills**, **D1 skin**, **H2 skin**; F4 **D1 skin**)
F. hydatis	22	-	-	22 (F2 autumn T1 **H1 gills**; F1 winter T2 **D1 skin**; F2 winter T2 **D1 skin**, **H1 skin**, T3 **D1 gills**; F2 summer T1 **D1 gills**; F3 **D3 gills**, **D6 skin**, **D1 gills and skin**, **H6 skin**)
F. limicola	4	-	-	4 (F1 spring T1 **D1 gills**; F3 **D2 gills**, **H1 gills**)
F. oncorhynchi	37	1	-	36 (F2 autumn T1 **D1 gills**, **H1 gills**, **H1 skin**, T2 **D1 skin**, **H1 skin**, T3 **D2 skin**, **H2 skin**, **H1 gills and skin**; F2 winter T3 **D1 skin**, **H1 skin**; F2 spring T1 **H1 skin**; F1 summer T1 **D3 skin, D1 liver, H4 skin**, T2 **D1 skin**, **H4 skin**, **H1 gills**, T3 **D1 gills**, **H3 skin**, **H1 gills**; F2 summer T1 **D1 gills**, **H1 skin**, T3 **D1 skin**; F4 **D1 skin**)

Table 9 continued

Table 9 continued

F. pectinovorum	42	-	-	42 (F2 autumn T1 **H1 skin**, T3 **H1 skin**; F1 winter T1 **D2 skin**, **H2 skin**, T2 **D2 skin**, **H1 skin**, T3 **D1 skin**; F2 winter T1 **H1 skin**, T2 **D1 gills**, **D1 skin**, T3 **H2 skin**; F1 spring T1 **H1 skin**, T2 **D1 skin**, **H1 gills**, T3 **D1 gills**, **D1 gills and skin**, **H1 skin**; F2 spring T1 **D1 skin**, T2 **D1 gills**, **D1 skin**, **H1 gills**, **H1 skin**, T3 **D1 skin**, **D1 gills and skin**, **H2 gills**; F1 summer T2 **D1 gills**; F2 summer T1 **D1 skin**, T2 **D2 skin**; F3 **D2 skin**, **D1 gills**, **H4 skin**, **H1 gills**)
F. piscis	18	1	-	17 (F2 autumn T1 **H1 gills**; F1 winter T2 **D1 skin, H1 skin**; F1 spring T3 **H2 skin**; F2 spring T1 **D2 gills**, **D1 gills and skin**, **H1 gills**, **H1 skin**, T2 **D1 skin**, **H2 gills**, **H1 skin**, T3 **D1 gills**, **H1 gills**; F3 **D1 skin**)
F. plurextorum	1	-	-	1 (F2 summer T1 **D1 gills**)
F. psychrolimnae	7	-	-	7 (F1 spring T1 **H1 gills**; F3 **D2 gills**, **D2 skin**, **D1 gills and skin**; F4 **H1 skin**)
F. reichenbachii	7	-	-	7 (F2 autumn T1 **D1 gills**, T3 **D1 gills**; F2 winter T3 **H1 skin**; F2 spring T2 **D1 gills**, T3 **D1 gills**; F2 summer T1 **H1 gills**; F3 **H1 skin**)

Table 9 continued

Table 9 continued

F. saccharophilum	53	-	-	53 (F1 autumn T3 **D1 skin**; F2 autumn T1 **H1 gills**, T2 **D1 skin**, **H1 skin**, T3 **H1 skin**; F1 winter T2 **H1 skin**; F2 winter T1 **D1 gills**, **D1 skin**, T3 **H1 skin**; F1 spring T1 **D1 gills**, **H1 gills**, T2 **H1 gills**, **H1 skin**, T3 **D1 skin**, **H1 gills**; F2 spring T1 **D1 gills**, **D1 gills and skin**, **H2 skin**, **H1 gills**, T2 **D3 gills**, T3 **D2 skin**, **D1 gills**; F1 summer T1 **D2 gills**, **D1 skin**, T2 **H2 gills**, **H2 skin**, T3 **H1 gills**; F2 summer T1 **D1 gills**, T2 **D1 skin**; F3 **D3 gills**, **D2 skin**, **H8 skin**, **H1 gills**, **H1 gills and skin**; F4 **D1 gills**, **H1 gills**)
F. terrigena	1	-	-	1 (F2 summer T1 **D1 skin**)
F. tiangeerense	2	1	-	1 (F1 spring T2 **H1 skin**)
F. tructae	2	1	1 (F2 summer T2 **D1 gills**)	-
FX2	3	3	-	-
FX3	5	3	3 (F2 summer T2 **D2 gills and skin**; F4 **D1 gills**)	-
FX5	3	3	-	-
FX6	2	1	1 (F2 autumn T1 **D1 liver**)	-
FX9	5	2	3 (F1 winter T3 **D1 gills**; F1 summer T3 **D1 gills**; F3 **H1 gills**)	-
FX10	7	2	6 (F1 spring T1 **D1 skin**, **D1 gills**, **H1 gills**, T2 **D2 gills**; F1 summer T2 **D1 gills**)	-
FX15	11	6	5 (F1 winter T1 **H1 gills**; F4 **D2 gills**, **D1 gills and skin**, **H1 gills**)	-
FX18	3	2	2 (F3 **H2 gills**)	-
FX23	2	2	-	-
FX32	5	2	3 (F2 summer T1 **D2 gills and skin**; F4 **D1 skin**)	-

* The isolation sources of the isolates selected for WGS are provided in Appendix 3

WGS and ANI analysis of selected isolates with unreliable MALDI-TOF MS results (n = 81), particularly those isolated from internal organs or in greater numbers from skin or gills, as well as those with frequently observed colony morphologies and/or MALDI-TOF MS results, revealed that 16 isolates belonged to 12 known

Flavobacterium species (ANI ≥ 95%), namely *F. bernardetii* (n = 2), *F. branchiarum* (n = 2), *F. celericrescens* (n = 2), *F. collinsii* (n = 2), *F. cupriresistens*, *F. difficile*, *F. erciyesense*, *F. hercynium, F. oncorhynchi*, *F. piscis*, *F. tiangeerense,* and *F. tructae*. Remarkably, most isolates (n = 60) represented 41 putative new *Flavobacterium* species (ANI < 95%) (Table 10). Phylogenetically most closely related, validly described *Flavobacterium* species were included in the calculation of pairwise ANI values (Appendix 4). Additionally, five isolates were not flavobacteria, with four isolates belonging to the genus *Pedobacter* (MALDI-TOF MS best match log(score) < 2) and one isolate to the genus *Kaistella* (MALDI-TOF MS first and second match log(score) ≥ 2). The isolation sources of the isolates selected for WGS are provided in Appendix 3.

Table 10: Average nucleotide identity (ANI)-based species delineation of *Flavobacterium* isolates

Species/FX	**Isolate**	**Best 16S rRNA hit (% identity)**	**ANIb best hit (%)**	**Best *gyrB* hit (% identity)**	**ANIb best hit (%)**
F. bernardetii	F1040	*F. bernardetii* (99.93)	**95**	*F. haoranii* (85.26)/ *F. sediminilitoris* (83.66)	75/75
F. bernardetii	F609	*F. bernardetii* (99.93)	**95**	*F. haoranii* (85.52)/ *F. sediminilitoris* (84.02)	75/75
F. branchiarum	F324	*F. branchiarum* (99.89)	**96**	*F. cupriresistens* (86.64)/ *F. johnsoniae* (86.52)	78/77
F. branchiarum	F963	*F. branchiarum* (99.72)	**96**	*F. cupriresistens* (87.09)/ *F. johnsoniae* (86.79)	78/78
F. celericrescens	F1856	*F. celericrescens* (99.86)	**97**	*F. channae* (87.19)/ *F. cyclinae* (93.19)	82/82
F. celericrescens	F1959	*F. celericrescens* (99.86)	**97**	*F. channae* (87.50)/ *F. cyclinae* (93.57)	82/82
F. collinsii	F1548	*F. collinsii* (99.44)	**95**	*F. collinsii* (96.91)	95
F. collinsii	F1673	*F. collinsii* (99.44)	**95**	*F. collinsii* (96.91)	95
F. cupriresistens	F2097	*F. cupreum* (98.47)	82	*F. cupriresistens* (98.25)	**97**
F. difficile	F1633	*F. difficile* (99.36)	**95**	*F. channae* (83.57)/ *F. indicum* (84.24)	76/76
F. erciyesense	F562	*F. erciyesense* (99.85)	**95**	*F. eburneipallidum* (86.23)/*F. flavipigmentatum* (85.68)	76/76
F. hercynium	F2050	*F. hercynium* (99.51)	**94**	*F. bizetiae* (92.33)	81
F. oncorhynchi	F1942	*F. oncorhynchi* (99.65)	**95**	*F. plurextorum* (97.11)	92
F. piscis	F1228	*F. piscis* (99.85)	**97**	*F. bizetiae* (95.67)	87
F. tiangeerense	F1485	*F. tiangeerense* (99.23)	**95**	*F. flavipigmentatum* (86.37)/*F. lacustre* (85.66)	75/77
F. tructae	F1900	*F. tructae* (100.0)	**98**	*F. tructae* (98.76)	98

Table 10 continued

Table 10 continued

FX1	F1141	*F. bernardetii* (99.19)	87	*F. channae* (83.55)/ *F. haoranii* (84.96)	76/75
FX2	F334	*F. bernardetii* (99.19)	90	*F. channae* (85.14)/ *F. sediminilitoris* (84.14)	76/75
FX2	F1038	*F. bernardetii* (99.19)	90	*F. channae* (85.44)/ *F. sediminilitoris* (83.66)	76/75
FX2	F317	*F. bernardetii* (99.19)	90	*F. channae* (85.14)/ *F. sediminilitoris* (84.14)	76/75
FX3	F1048	*F. bernardetii* (99.63)	91	*F. haoranii* (84.87)/ *F. sediminilitoris* (83.86)	75/75
FX3	F1821	*F. bernardetii* (99.33)	90	*F. channae* (83.54)/ *F. haoranii* (84.77)	76/75
FX3	F1782	*F. bernardetii* (99.33)	90	*F. channae* (83.54)/ *F. haoranii* (84.77)	76/75
FX3	F1783	*F. bernardetii* (99.56)	90	*F. haoranii* (84.61)/ *F. sediminilitoris* (84.08)	75/75
FX4	F1100	*F. terrigena* (99.03)	86	*F. indicum* (85.13)/ *F. sediminilitoris* (83.12)	77/75
FX5	F1933	*F. terrigena* (99.03)	88	*F. haoranii* (84.64)/ *F. sediminilitoris* (83.06)	75/75
FX5	F1140	*F. terrigena* (99.03)	88	*F. haoranii* (84.64)/ *F. sediminilitoris* (83.06)	76/75
FX5	F1142	*F. terrigena* (99.03)	88	*F. haoranii* (84.64)/ *F. sediminilitoris* (83.06)	76/75
FX6	F1466	*F. succinicans* (99.37)	84	*F. ammonificans* (86.11)	76
FX7	F1987	*F. bernardetii* (99.41)	88	*F. channae* (85.10)/ *F. sediminilitoris* (84.09)	76/75
FX8	F1718	*F. bernardetii* (99.41)	90	*F. channae* (84.98)/ *F. sediminilitoris* (83.76)	76/75
FX9	F1616	*F. caseinilyticum* (99.26)	85	*F. eburneipallidum* (85.40)/ *F. lacustre* (88.53)	78/80
FX9	F945	*F. laiguense* (99.5)	78	*F. eburneipallidum* (85.40)/ *F. lacustre* (88.53)	78/80
FX10	F944	*F. bizetiae* (98.82)	75	*F. acetivorans* (85.63)/ *F. flavipigmentatum* (86.64)	76/75
FX10	F1511	*F. bizetiae* (98.53)	75	*F. acetivorans* (85.78)/ *F. flavipigmentatum* (86.64)	76/75
FX11	F1498	*F. frigoritolerans* (98.54)	76	*F. flavipigmentatum* (86.53)/ *F. lacustre* (85.77)	75/77
FX12	F62	*F. tyrosinilyticum* (97.83)	87	*F. ginsengisoli* (93.72)	87
FX13	F65	*F. restrictum* (97.84)	76	*F. aestivum* (85.59)/ *F. praedii* (85.78)	79/81
FX14	F77	*F. succinicans* (99.37)	84	*F. ammonificans* (85.96)	76
FX15	F2167	*F. succinicans* (98.61)	84	*F. ammonificans* (85.85)	76
FX15	F2043	*F. succinicans* (98.61)	84	*F. ammonificans* (85.91)	76
FX15	F1859	*F. succinicans* (98.82)	84	*F. ammonificans* (85.91)	76
FX15	F1692	*F. succinicans* (98.68)	84	*F. ammonificans* (85.80)	76
FX15	F106	*F. succinicans* (98.61)	84	*F. ammonificans* (85.91)	76
FX15	F1677	*F. succinicans* (98.61)	84	*F. ammonificans* (85.91)	76
FX16	F283	*F. aquatile* (99.30)	90	*F. aquatile* (96.49)	90
FX17	F616	*F. bizetiae* (98.80)	83	*F. pectinovorum* (94.49)	87

Table 10 continued

Table 10 continued

FX18	F626	*F. bernardetii* (99.33)	88	*F. haoranii* (84.83)/ *F. sediminilitoris* (84.30)	75/75
FX18	F1773	*F. bernardetii* (99.33)	89	*F. haoranii* (84.83)/ *F. sediminilitoris* (84.30)	75/75
FX19	F656	*F. glaciei* (99.15)	NA	*F. galactosidilyticum* (85.15)/ *F. lacustre* (88.43)	80/80
FX20	F666	*F. glaciei* (99.01)	NA	*F. eburneipallidum* (85.40)/ *F. lacustre* (89.25)	78/80
FX21	F799	*F. reichenbachii* (100.0)	81	*F. cupriresistens* (90.11)/ *F. panici* (89.59)	81/82
FX22	F1112	*F. psychroterrae* (99.41)	81	*F. bizetiae* (92.17)	81
FX23	F1213	*F. frigidimaris* (100.0)	82	*F. bizetiae* (90.96)	85
FX23	F1259	*F. frigidimaris* (100.0)	82	*F. bizetiae* (90.96)	85
FX24	F1252	*F. tiangeerense* (98.53)	75	*F. acetivorans* (85.46)	76
FX25	F1418	*F. aquidurense* (99.16)	85	*F. bizetiae* (95.52)	86
FX26	F1486	*F. saccharophilum* (99.90)	88	*F. pectinovorum* (94.02)	83
FX27	F1527	*F. piscis* (99.09)	81	*F. pectinovorum* (90.28)	81
FX27	F1593	*F. piscis* (99.09)	81	*F. pectinovorum* (90.28)	82
FX28	F1631	*F. caseinilyticum* (98.61)	86	*F. lacustre* (89.15)	80
FX29	F1678	*F. gawalongense* (99.33)	78	*F. flavipigmentatum* (86.97)/ *F. praedii* (85.60)	76/76
FX30	F1683	*F. cheonhonense* (100.0)	91	*F. rivulicola* (87.12)	80
FX31	F1698	*F. chungangense* (98.82)	NA	*F. bizetiae* (90.27)	81
FX32	F1715	*F. bizetiae* (98.3)	75	*F. flavipigmentatum* (87.01)/ *F. praedii* (85.55)	76/76
FX32	F2020	*F. bizetiae* (98.38)	76	*F. flavipigmentatum* (86.35)/*F. praedii* (85.50)	76/76
FX33	F1797	*F. succinicans* (98.12)	76	*F. praedii* (89.61)	83
FX34	F1849	*F. sinopsychrotolerans* (97.50)	79	*F. lacustre* (86.50)	82
FX35	F1902	*F. terrigena* (98.89)	83	*F. channae* (84.35)/ *F. cyclinae* (83.27)	76/76
FX36	F2035	*F. resistens* (98.75)	81	*F. pectinovorum* (90.68)	81
FX36	F1996	*F. resistens* (98.75)	81	*F. pectinovorum* (90.68)	81
FX37	F2009	*F. chungbukense* (98.40)	84	*F. luteolum* (96.19)	90
FX38	F2034	*F. pectinovorum* (98.89)	81	*F. collinsii* (96.39)	90
FX39	F2063	*F. aquatile* (99.30)	89	*F. aquatile* (95.85)	89
FX40	F2074	*F. circumlabens* (98.12)	80	*F. johnsoniae* (93.56)	83
FX41	F1757	*F. laiguense* (98.33)	82	*F. bizetiae* (87.13)/ *F. praedii* (88.93)	76/83

Some *Flavobacterium* species genomes contain two distinct copies of the *gyrB* gene; in such cases, best hits for both copies are included in the table and separated by a slash (/)

NA, no genome available

The species *F. bernardetii, F. branchiarum, F. celericrescens, F. collinsii, F. cupriresistens, F. difficile*, *F. erciyesense,* and *F. tructae* are not yet included in the MALDI-TOF MS Bruker reference database. To address this limitation, the

taxonomic information from the WGS analysis was added to our in-house *Flavobacterium* MALDI-TOF MS database by incorporating the MSPs of selected newly characterised isolates, including the known and putative new species identified in this study, the latter only included if represented by at least two isolates. Following the expansion of the database, a second MALDI-TOF MS analysis was performed to re-evaluate the initial identification results for selected previously unidentified isolates. The results of the second MALDI-TOF MS analysis for isolates with high confidence of identification, including sampling information, number of POS fish, and organ of isolation, are given in Table 9.

Further differentiation of selected newly characterised isolates from their close phylogenetic neighbours was performed using MALDI-TOF MS analysis. According to the dendrogram based on MSPs, the analysed *Flavobacterium* spp. grouped into two main clusters. One of these clusters was further divided into two larger subclusters and a smaller subcluster containing *F. psychrophilum* strains only. Isolates of *F. bernardetii, F. celericrescens, F. difficile,* FX2, FX3, FX5, FX18, and FX36 (F1996) clustered together with *F. branchiophilum* CIP 109950 and *F. terrigena* DSM 17934^T. Regarding the two subclusters closely related to *F. psychrophilum*, isolates of the species *F. branchiarum, F. collinsii, F. hercynium, F. oncorhynchi, F. piscis, F. tructae,* FX23, FX27, and FX36 (F2035) formed a subcluster together with *F. anhuiense* DSM 22555^T, *F. frigidimaris* DSM 15937^T, *F. hercynium* DSM 18292^T, *F. hibernum* DSM 12611^T, *F. hydatis* DSM 2063^T, *F. pectinovorum* DSM 6368^T, and *F. saccharophilum* DSM 1811^T, and the isolates of the species *F. erciyesense, F. tiangeerense,* FX9, FX10, FX11, FX15, and FX32 formed a subcluster together with *F. aquatile* DSM 1132^T, *F. flevense* DSM 1076^T, *F. frigidarium* DSM 17623^T, *F. limicola* DSM 15094^T, and *F. psychrolimnae* DSM 16141^T (Figure 12).

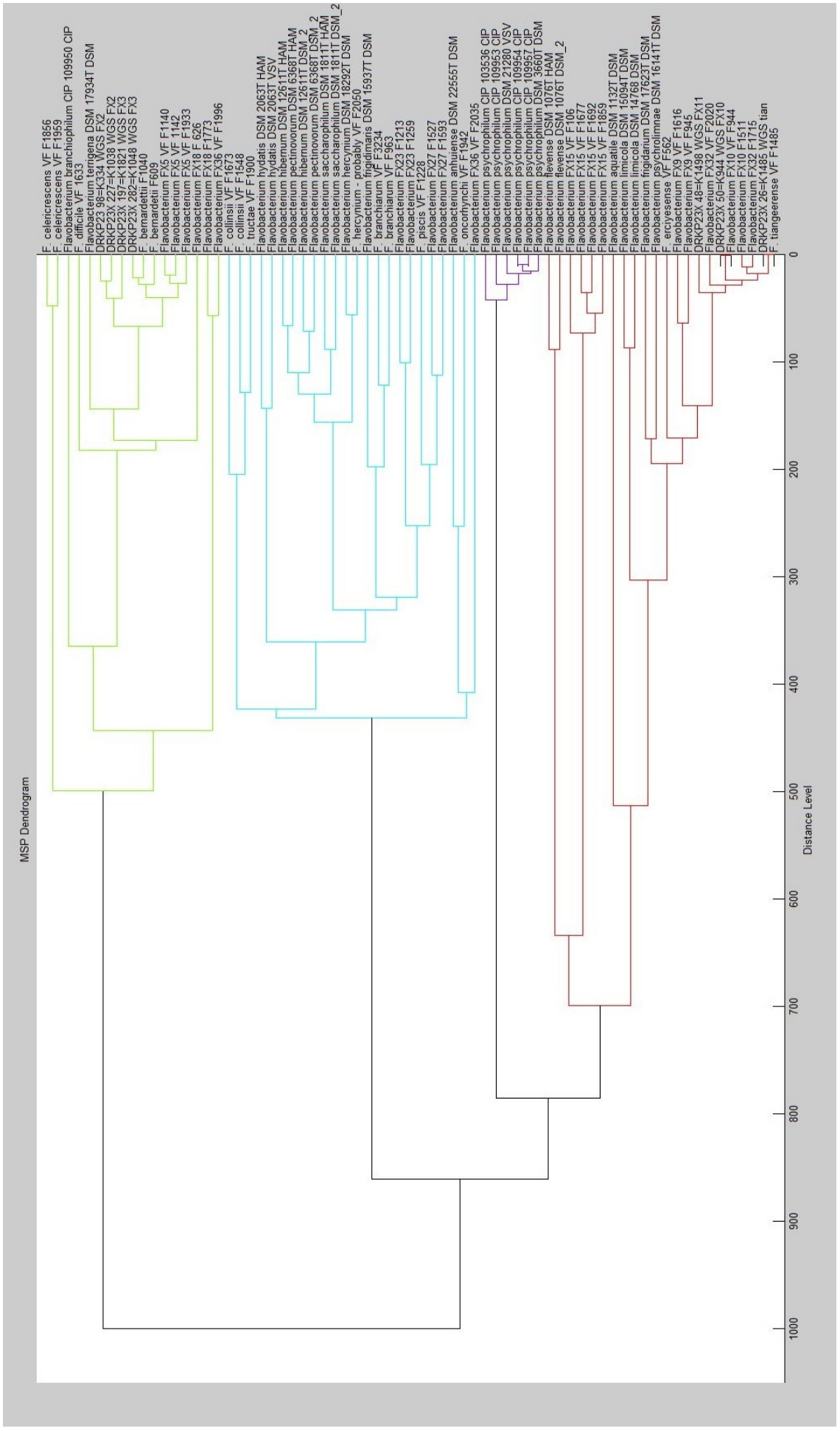

F. celericrescens VF F1856
F. celericrescens VF F1959
Flavobacterium branchiophilum CIP 109950 CIP
F. difficile VF 1633
Flavobacterium terrigena DSM 17934T DSM
DRKP23 98=K334 WGS FX2
DRKP23X 227=K1038 WGS FX2
DRKP23X 197=K1821 WGS FX3
DRKP23X 282=K1048 WGS FX3
F. bernardetii F1040
F. bernardetii F609
Flavobacterium FX5 VF F1140
Flavobacterium FX5 VF 1142
Flavobacterium FX5 VF F1933
Flavobacterium FX18 F626
Flavobacterium FX18 F1773
Flavobacterium FX36 VF F1996
F. collinsii VF F1673
F. collinsii VF F1548
F. collinsii VF F1900
F. fructae VF F1900
Flavobacterium hydatis DSM 2063T HAM
Flavobacterium hydatis DSM 2063T VSV
Flavobacterium hibernum DSM 12611T HAM
Flavobacterium hibernum DSM 12611T DSM_2
Flavobacterium pectinovorum DSM 6368T DSM_2
Flavobacterium saccharophilum DSM 1811T HAM
Flavobacterium saccharophilum DSM 1811T DSM_2
Flavobacterium hercynium DSM 18292T DSM
F. hercynium - probably VF F2050
Flavobacterium frigidimaris DSM 15937T DSM
F. branchiarum VF F3234
F. branchiarum VF F963
Flavobacterium FX23 F1213
Flavobacterium FX23 F1259
F. piscis VF F1228
Flavobacterium FX27 F1527
Flavobacterium FX27 F1593
Flavobacterium anhuiense DSM 22555T DSM
F. oncorhynchi VF F1942
F. oncorhynchi FX36 VF F2035
Flavobacterium psychrophilum CIP 103536 CIP
Flavobacterium psychrophilum CIP 109953 CIP
Flavobacterium psychrophilum DSM 21280 VSV
Flavobacterium psychrophilum CIP 109954 CIP
Flavobacterium psychrophilum CIP 109957 CIP
Flavobacterium psychrophilum DSM 3660T DSM
Flavobacterium flevense DSM 1076T HAM
Flavobacterium flevense DSM 1076T DSM_2
Flavobacterium FX15 VF F106
Flavobacterium FX15 VF F1677
Flavobacterium FX15 VF F1692
Flavobacterium FX15 VF F1859
Flavobacterium aquatile DSM 1132T DSM
Flavobacterium limicola DSM 15094T DSM
Flavobacterium limicola DSM 14768 DSM
Flavobacterium frigidarium DSM 17623T DSM
Flavobacterium psychrolimnae DSM 16141T DSM
F. erciyesense VF F562
Flavobacterium FX9 VF F1616
Flavobacterium FX9 VF F945
DRKP23X 48=K1498 WGS FX11
Flavobacterium FX32 VF F2020
DRKP23X 50=K944 WGS FX10
Flavobacterium FX10 VF F944
Flavobacterium FX10 F1511
Flavobacterium FX32 F1715
DRKP23X 26=K1485 WGS tian
F. tiangeerense VF F1485
MSP Dendrogram
Distance Level
0
100
200
300
400
500
600
700
800
900
1000

Figure 12: Dendrogram obtained by cluster analysis of MALDI-TOF MS spectra of selected *Flavobacterium* spp. identified using whole-genome sequencing

Several lesser-known *Flavobacterium* spp. were isolated from skin and/or gills in greater numbers, from individual to numerous morphologically similar colonies, namely *F. aquidurense, F. bernardetii, F. branchiarum, F. celericrescens, F. chungbukense, F. collinsii, F. difficile, F. hercynium, F. piscis, F. saccharophilum, F. tiangeerense,* and *F. tructae*, as well as from internal organs, namely *F. aquatile, F. bernardetii,* and *F. oncorhynchi*. Additionally, a total of 19 putative new *Flavobacterium* spp. from skin and/or gills and 13 from internal organs, as well as several yet unidentified species, were recovered. Examples of primary isolation of lesser-known species from skin and gills in greater numbers are shown in Figure 13.

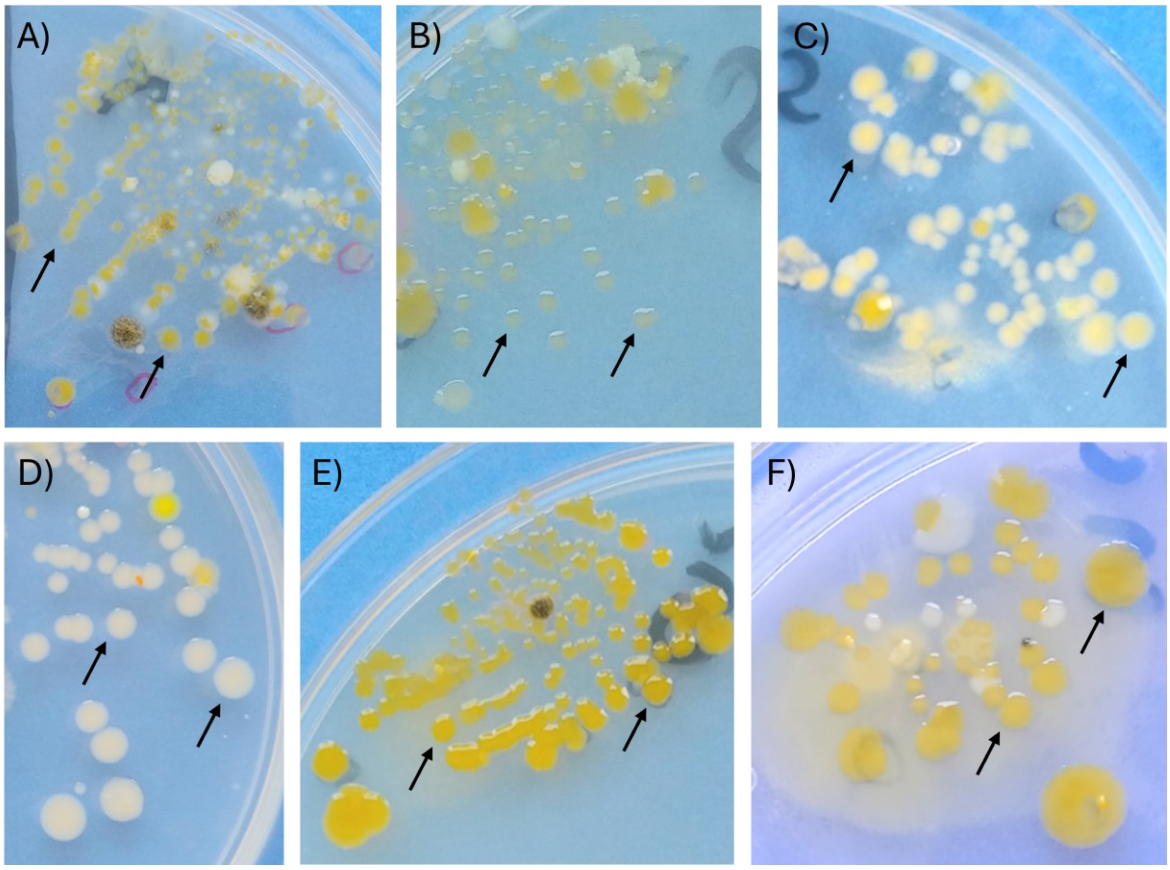


Figure 13: Primary isolation of A) FX3 (F1782) from the skin and B) *Flavobacterium difficile* (F1633), C) FX9 (F945), D) FX5 (F1140), E) FX10 (F1511) and F) FX15 (F2043) from the gills

We applied hierarchical clustering using Jaccard distance between fish based on *Flavobacterium* spp. isolated from more than 1% of individuals and generated clusters using average linkage (Figure 14). The results show that *Flavobacterium* spp., *F. psychrophilum,* and *F. saccharophilum* formed a tight cluster, with all three co-occurring in several fish. No significant association was found between the clustering patterns and metadata variables, such as health status, season, or sampling, based on both the mutual information and chi-squared analyses. Principal component analysis revealed that diverse *Flavobacterium* spp. were isolated at each sampling point (Figure 15).

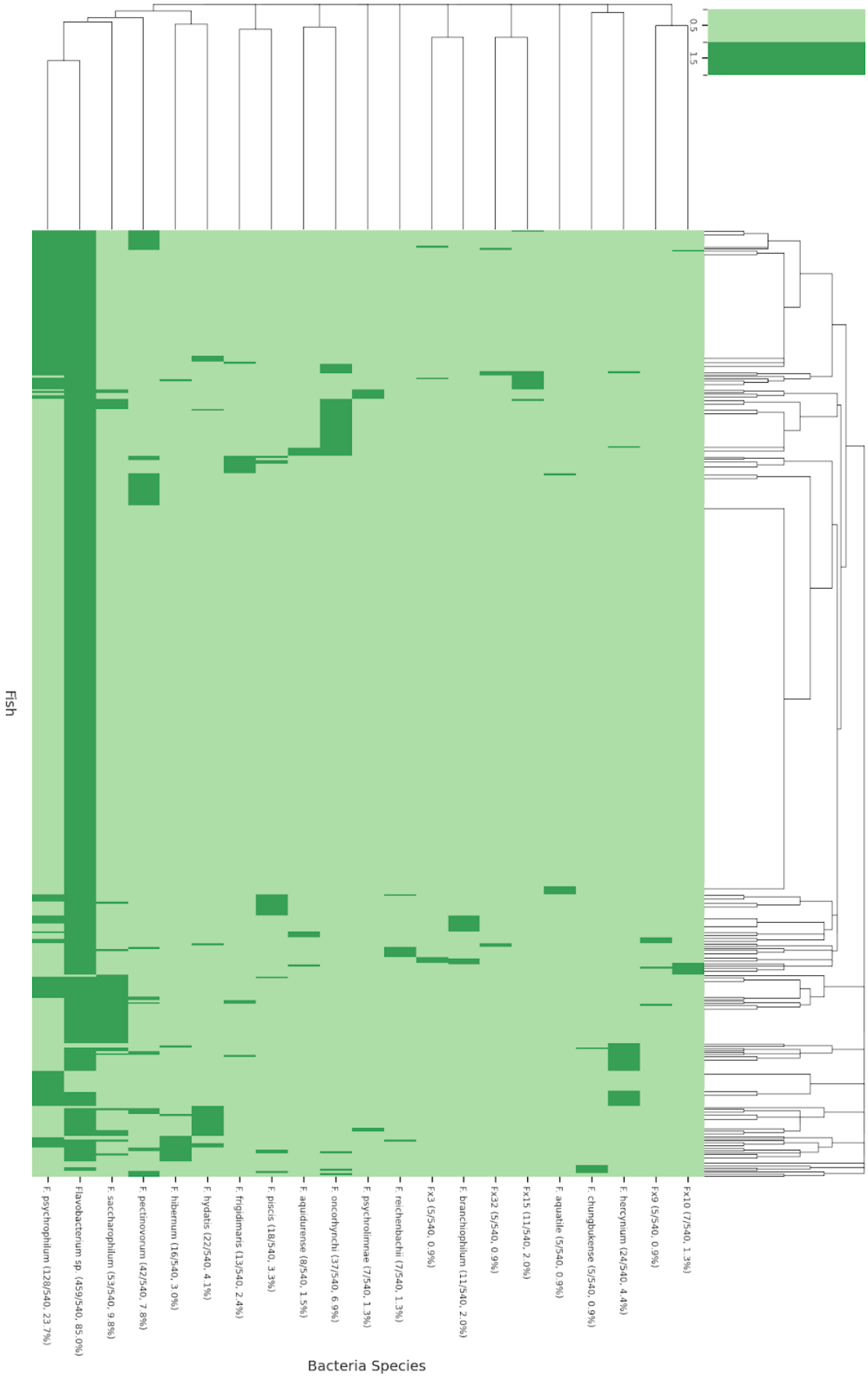

0.5
1.5
Fish
F. psychrophilum (128/540, 23.7%)
Flavobacterium sp. (459/540, 85.0%)
F. saccharophilum (53/540, 9.8%)
F. pectinovorum (42/540, 7.8%)
F. hibernum (16/540, 3.0%)
F. hydatis (22/540, 4.1%)
F. frigidimaris (13/540, 2.4%)
F. piscis (18/540, 3.3%)
F. aquidurense (8/540, 1.5%)
F. oncorhynchi (37/540, 6.9%)
F. psychrolimnae (7/540, 1.3%)
F. reichenbachii (7/540, 1.3%)
Fx3 (5/540, 0.9%)
F. branchiophilum (11/540, 2.0%)
Fx32 (5/540, 0.9%)
Fx15 (11/540, 2.0%)
F. aquatile (5/540, 0.9%)
F. chungbukense (5/540, 0.9%)
F. hercynium (24/540, 4.4%)
Fx9 (5/540, 0.9%)
Fx10 (7/540, 1.3%)
Bacteria Species

Figure 14: Hierarchical clustering of fish (n = 482; at least one *Flavobacterium* species isolated) based on flavobacterial species isolated in more than 1% of fish, totalling 20 unique species and *Flavobacterium* spp. group; the top dendrogram shows fish that cluster based on similar species profiles, and the left dendrogram shows species that cluster based on co-occurrence in the same fish

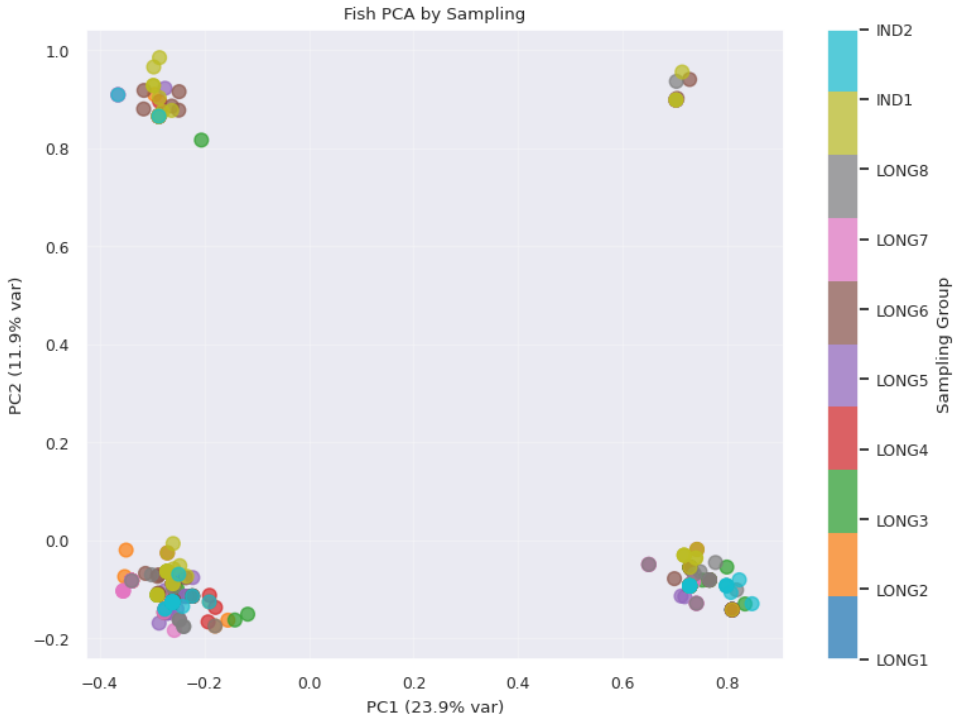


Figure 15: Principal-component analysis of individual fish based on binary presence profiles of the top 1% most prevalent *Flavobacterium* species. Fish are coloured by sampling, showing that heterogeneous flavobacterial species were isolated at each sampling

4.3 BIOMETRIC DATA, GROSS AND HISTOPATHOLOGICAL LESIONS IN FLAVOBACTERIAL DISEASES

4.3.1 Biometric data and gross lesions

4.3.1.1 *Flavobacterium psychrophilum*

F. psychrophilum-INF fish had significantly lower body weight (mean 62.4 g versus 100.79 g, p = 0.021) and total length (mean 16.75 cm versus 18.74 cm, p = 0.005) than NH fish, while the difference for K was not significant (mean 0.97 versus 1.00, p = 0.1125), as revealed by a Mann-Whitney U test per sampling, followed by a

Fisher meta-test over individual *p*-values. However, no significant difference was found when these variables were analysed per fish tank. The ranges of body weight, total length, and K for *F. psychrophilum*-INF and POS fish were 4–300.5 g and 4–650 g, 7–30.5 cm and 7–40 cm, and 0.59–2.07 and 0.5–2.07, respectively (Figure 16).

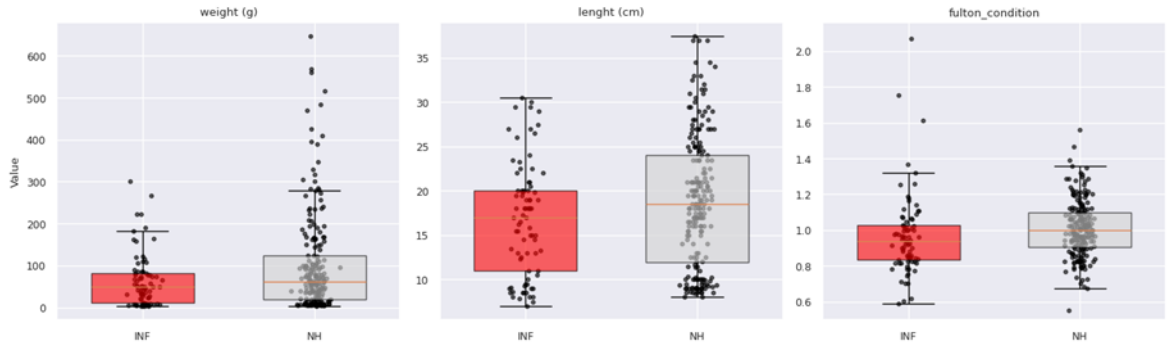


Figure 16: Boxplots showing weight, length, and Fulton's condition factor in fish infected (INF) with *Flavobacterium psychrophilum* and negative healthy (NH) fish

Infection with *F. psychrophilum* was associated with a significantly higher necropsy fish index ($p < 0.0001$) because INF fish showed necropsy indices roughly six times higher than NH fish (mean 11.13 versus 1.82), revealed by a Mann-Whitney U test per fish tank and subsequent Fisher meta-test over individual *p*-values (Table 11, Figure 17).

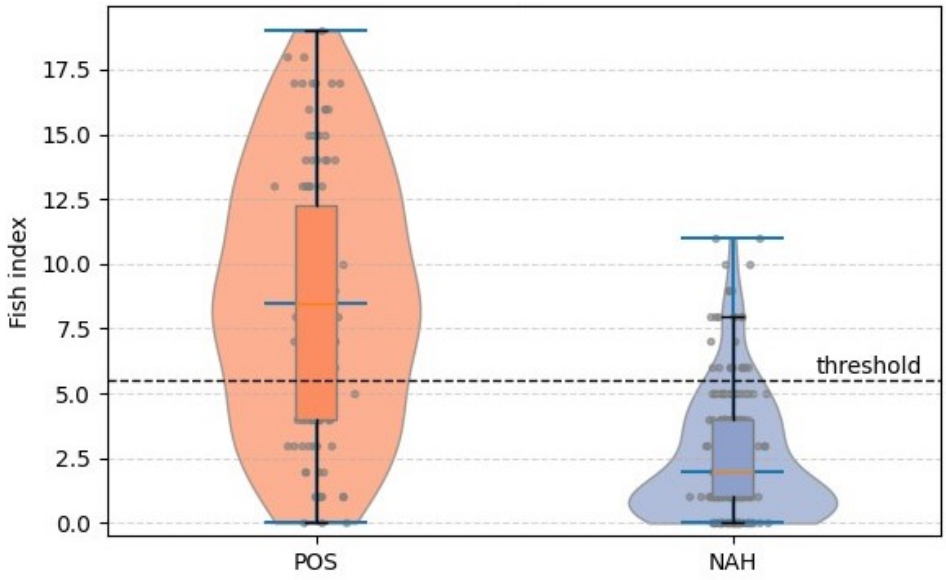


Figure 17: Violin boxplots showing necropsy fish indices in fish testing positive (POS) for *Flavobacterium psychrophilum* and negative apparently healthy (NAH) fish; infected (INF) fish are POS fish at or above the threshold, and negative healthy (NH) fish are NAH fish below the threshold

The score values of all organs except the opercula, stomach with pyloric caeca, and intestine were significantly higher in *F. psychrophilum*-INF fish. The *p*-values, means, medians, and standard deviations for all necropsy variables analysed are shown in Table 11.

Table 11: Necropsy variables associated with *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* infection, analysed per fish tank using the Mann–Whitney U test and Fisher's combined probability test, including mean, median, and standard deviation (sd) of each variable for infected (INF) fish and negative healthy (NH) fish; significant *p*-values ($p < 0.05$) are shown in red

NECROPSY VARIABLES	*F. psychrophilum*		*F. branchiophilum*
	Per fish INF/NH fish:	Per sampled organ INF/NH fish:	INF/NH fish:
Fulton's condition factor	*p* = 0.593 mean = 0.96/1.00 median = 0.94/1.00 sd = 0.22/0.16	/	*p* = 0.309 mean = 1.01/1.01 median = 0.90/1.01 sd = 0.36/0.16
Necropsy fish index	***p* < 0.001** mean = 11.13/1.82 median = 11.00/1.00 sd = 3.47/1.63	/	***p* = 0.016** mean = 9.43/1.87 median = 9.00/1.00 sd = 1.81/1.65
Skin score value	***p* < 0.001** mean = 1.17/0.53 median = 1.00/0.00 sd = 1.00/0.58	***p* < 0.001** mean = 1.62/0.53 median = 2.00/0.00 sd = 0.96/0.58	*p* = 0.735 mean = 0.86/0.54 median = 1.00/1.00 sd = 0.69/0.57
Fins score value	***p* = 0.010** mean = 0.94/0.37 median = 1.00/0.00 sd = 0.94/0.55	/	*p* = 0.251 mean = 1.43/0.39 median = 1.00/0.00 sd = 1.13/0.56

Table 11 continued

Table 11 continued

Eyes score value	***p* < 0.001** mean = 1.62/0.06 median = 3.00/0.00 sd = 1.51/0.41	/	*p* = 0.743 mean = 0.86/0.08 median = 0.00/0.00 sd = 1.46/0.48
Opercula score value	*p* = 1.000 mean = 0.12/0.09 median = 0.00/0.00 sd = 0.39/0.33	/	*p* = 0.727 mean = 0.57/0.08 median = 0.00/0.00 sd = 1.13/0.32
Gills score value	***p* < 0.001** mean = 1.77/0.11 median = 3.00/0.00 sd = 1.49/0.58	***p* < 0.001** mean = 1.70/0.11 median = 3.00/0.00 sd = 1.52/0.58	***p* = 0.006** mean = 2.14/0.10 median = 3.00/0.00 sd = 1.46/0.55
Spleen score value	***p* < 0.001** mean = 2.15/0.27 median = 3.00/0.00 sd = 1.36/0.86	***p* < 0.001** mean = 2.33/0.27 median = 3.00/0.00 sd = 1.27/0.86	***p* = 0.009** mean = 1.71/0.27 median = 3.00/0.00 sd = 1.60/0.86
Kidney score value	***p* = 0.009** mean = 0.96/0.03 median = 0.00/0.00 sd = 1.41/0.29	***p* = 0.001** mean = 1.24/0.03 median = 0.00/0.00 sd = 1.50/0.29	*p* = 0.463 mean = 0.43/0.04 median = 0.00/0.00 sd = 1.13/0.34
Liver score value	***p* < 0.001** mean = 2.12/0.33 median = 3.00/0.00 sd = 1.38/0.94	***p* < 0.001** mean = 2.03/0.33 median = 3.00/0.00 sd = 1.43/0.94	*p* = 0.069 mean = 1.29/0.34 median = 0.00/0.00 sd = 1.60/0.95
Stomach and pyloric caeca score value	*p* = 1.000 mean = 0.13/0.01 median = 0.00/0.00 sd = 0.57/0.21	/	*p* = 1.000 mean = 0.00/0.01 median = 0.00/0.00 sd = 0.00/0.20
Intestine score value	*p* = 0.993 mean = 0.18/0.01 median = 0.00/0.00 sd = 0.62/0.14	/	*p* = 0.840 mean = 0.14/0.02 median = 0.00/0.00 sd = 0.38/0.19

Various external and internal gross lesions were observed in *F. psychrophilum*-POS fish. The skin was frequently affected (90/128, 70.3%), with erosions and shallow to deep ulcerations up to 3 × 2.5 × 0.8 cm in size (22/128, 17.2%), in some cases with reddish and/or yellowish margins and haemorrhage in the underlying musculature (Figure 18A–D). The ulcerations were distributed over different body regions and most commonly affected the skin around the dorsal fin, caudal peduncle, or upper jaw (16/128, 12.5%) and to a lesser extent the lower jaw (8/128, 6.25%). In one case, a 1.5 × 1 cm perforative ulceration was observed in the abdominal wall behind the left pectoral fin, in which the liver was exposed and covered with fibrin (Figure 18C). Multifocal convex oedematous areas with scale loss, erosions, and haemorrhage were also observed, affecting up to 60% of the body surface (9/128, 7%). The fins, particularly the dorsal and caudal fins, were frequently eroded or ulcerated (75/128, 58.6%), in some cases with reddish or yellowish margins and/or exposed fin rays (Figure 19B). Occasionally, fish had severe ulceration of the dorsal fin and/or surrounding skin, resulting in a saddleback-like lesion (2/128, 1.6%) (Figure 19A), or complete loss of the caudal fin and deep ulceration of the caudal peduncle, exposing the vertebral column

(3/128, 2.3%) (Figure 18E). Mild to severe shortening of the opercula was observed (14/128, 10.9%) (Figure 18E). The fish also showed multifocal or extensive scale loss (42/128, 32.8%) (Figure 19A), hyperaemia of the ventral abdomen and fins (14/128, 10.9%), and/or petechiae on the ventral abdomen (6/128, 4.7%). Hyperpigmentation or hypopigmentation of the skin was common (Figure 18A, Figure 19B). Two fish had an S-shaped tail (Figure 18F). In the oral cavity, one fish had an ecchymosis on the hard palate, and another fish had petechiae on the tongue and hard palate. Ocular lesions (53/128, 41.4%) included corneal opacity (32/128, 25%), exophthalmos (20/128, 15.6%) (Figure 19C), haemorrhage (9/128, 7%), and/or rupture of the eye (14/128, 10.9%). The gills (55/128, 43%) were anaemic, light pink or greyish white (47/128, 36.7%) (Figure 19A) or mottled light and dark pink (9/128, 7%), had petechiae (16/128, 12.5%), and/or mild to severe clubbing of the filaments (10/128, 7.8%), with multifocal white nodules 1–3 mm in diameter (2/128, 1.6%).

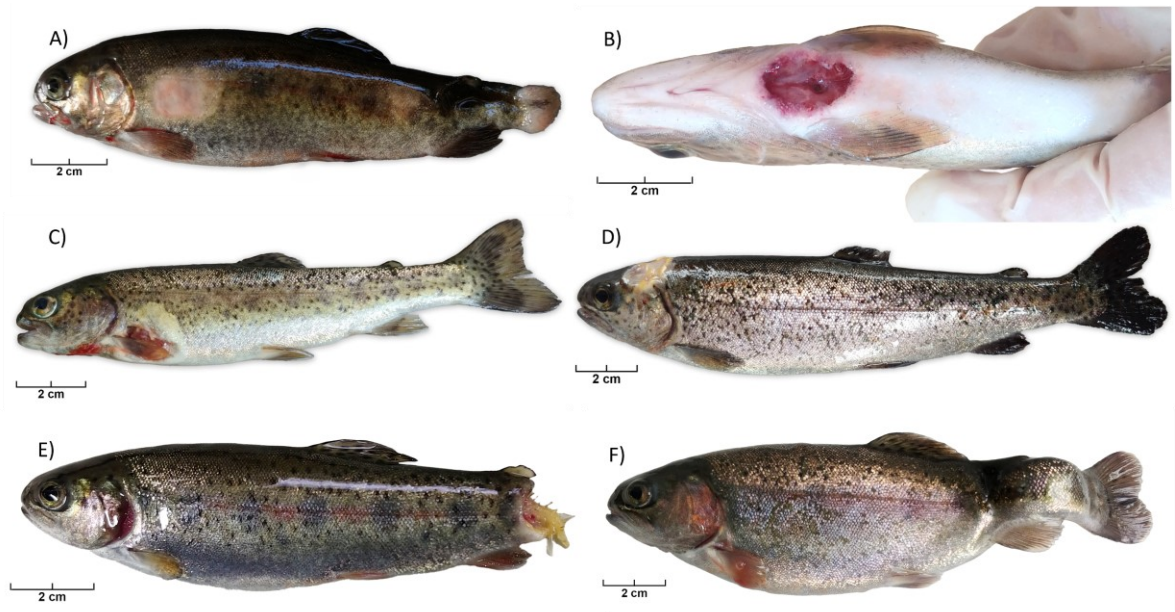


Figure 18: Gross lesions in fish testing positive for *Flavobacterium psychrophilum* and negative for other identified systemic fish pathogens: A) severe ulceration of caudal fin and shallow skin ulceration, as well as skin hyperpigmentation; B) deep skin ulceration with reddish margins; C) perforative skin ulceration with yellowish margins; D) skin ulceration with yellowish margins; E) complete loss of caudal fin and deep ulceration of caudal peduncle with exposure of vertebral column; moderate shortening of the operculum is also evident; F) S-shaped tail

Internally, the spleen (64/128, 50%) was mildly to severely enlarged (59/128, 46.1%), dark red (53/128, 41.4%) (Figure 19D, F), mottled brown and purple (2/128, 1.6%), or anaemic, light purple-red (10/128, 7.8%). Loss of definition of the splenic capsule and haemorrhage in the surrounding fatty tissue were also observed (4/128, 3.1%) (Figure 19C). The liver (62/128, 48.4%) was anaemic, light yellow, brown or pink (30/128, 23.4%), mottled light and dark brown (10/128, 7.8%), dark brown-red, consistent with hyperaemia (9/128, 7%), and/or had petechiae and ecchymoses (18/128, 14.1%) (Figure 19C). Moderate to severe hepatic atrophy (11/128, 8.6%) or moderate enlargement (7/128, 5.5%) were also observed, and in some cases, the gallbladder was severely distended (6/128, 4.7%) (Figure 19D, E). The kidneys (29/128, 22.7%), especially the posterior part, were multifocally or diffusely mildly to severely convex (23/128, 18%) and in some cases mottled brown and purple (4/128, 3.1%) (Figure 19F) or anaemic, light brown-purple (11/128, 8.6%). Intestinal lesions (5/128, 3.9%) included hyperaemia (3/128, 2.3%) and haemorrhage (2/128, 1.6%) of the intestine, as well as reddening of the vent in one fish. Petechiae and/or ecchymoses were observed in the heart (4/128, 3.1%) and the skeletal muscles (2/128, 1.6%), around the pyloric caeca (3/128, 2.3%), and in the mesenteric fat (2/128, 1.6%) (Figure 19D). In addition, the fish showed 1–2 mm black spots consistent with MMCs in the mesenteric fat and/or in the intestinal serosa (21/128, 16.4%). The fish were mildly to severely cachectic (21/128, 16.4%) (Figure 19E), and mild ascites was observed in a few individuals (5/128, 3.9%).

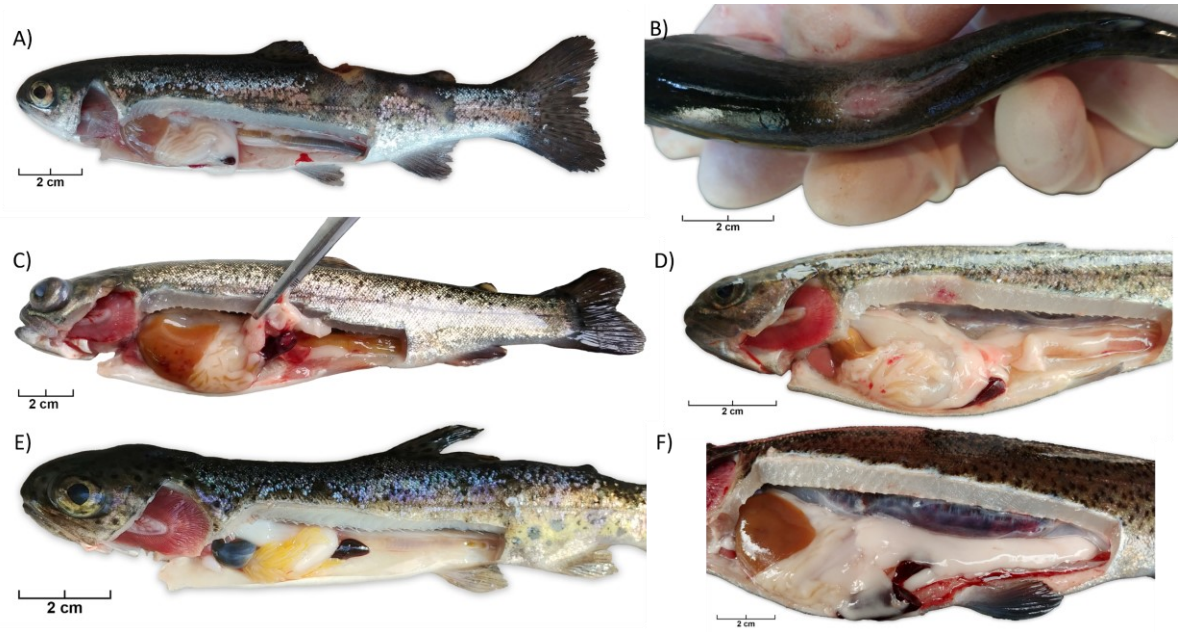


Figure 19: Gross lesions in fish testing positive for *Flavobacterium psychrophilum*: A) saddleback-like lesion due to skin ulceration behind the dorsal fin and shallow ulceration with scale loss extending laterally; anaemia of the gills and liver can also be observed; B) ulceration of the dorsal fin and skin hyperpigmentation; C) perisplenitis with loss of definition of the splenic capsule and haemorrhage in surrounding adipose tissue, as well as in the liver; exophthalmos is also evident; D) haemorrhage around the pyloric caeca and in the skeletal muscle tissue; moderate hepatic atrophy and mild splenic enlargement are also apparent; E) severe cachexia with severe hepatic atrophy and distended gall bladder; F) moderately enlarged kidney and spleen

4.3.1.2 *Flavobacterium branchiophilum*

The ranges of body weight, total length, and K for fish INF with or POS for *F. branchiophilum* were 24.5–48.5 g and 12–300.5 g, 12.5–19.5 cm and 11–29 cm, and 0.58–1.25 and 0.58–1.61, respectively. Analysis of necropsy variables for *F. branchiophilum* indicated that K of INF fish did not differ significantly from that of NH fish. On the other hand, INF fish had significantly higher necropsy fish indices (p = 0.016, mean 9.43 versus 1.87), as well as score values for gills (p = 0.006) and spleen (p = 0.009). The p-value, mean, median, and standard deviation for all necropsy variables analysed are shown in Table 11. It is important to note that the limited number of POS fish reduced the statistical power of the analysis.

F. branchiophilum-POS fish had anaemic, light pink gills (5/11, 45.5%), mild to severe clubbing of the gill filaments (4/11, 36.4%) with multifocal white nodules of up to 5 × 4 × 2 mm (2/11, 18.1%) (Figure 20A), and one fish had yellowish necrosis measuring 3 × 2 × 2 mm at the filament tips (Figure 20B). Additionally, two fish had mildly and severely shortened opercula (2/11, 18.1%), one fish had anaemic spleen and kidney, and some fish were moderately to severely cachectic (3/11, 27.3%).

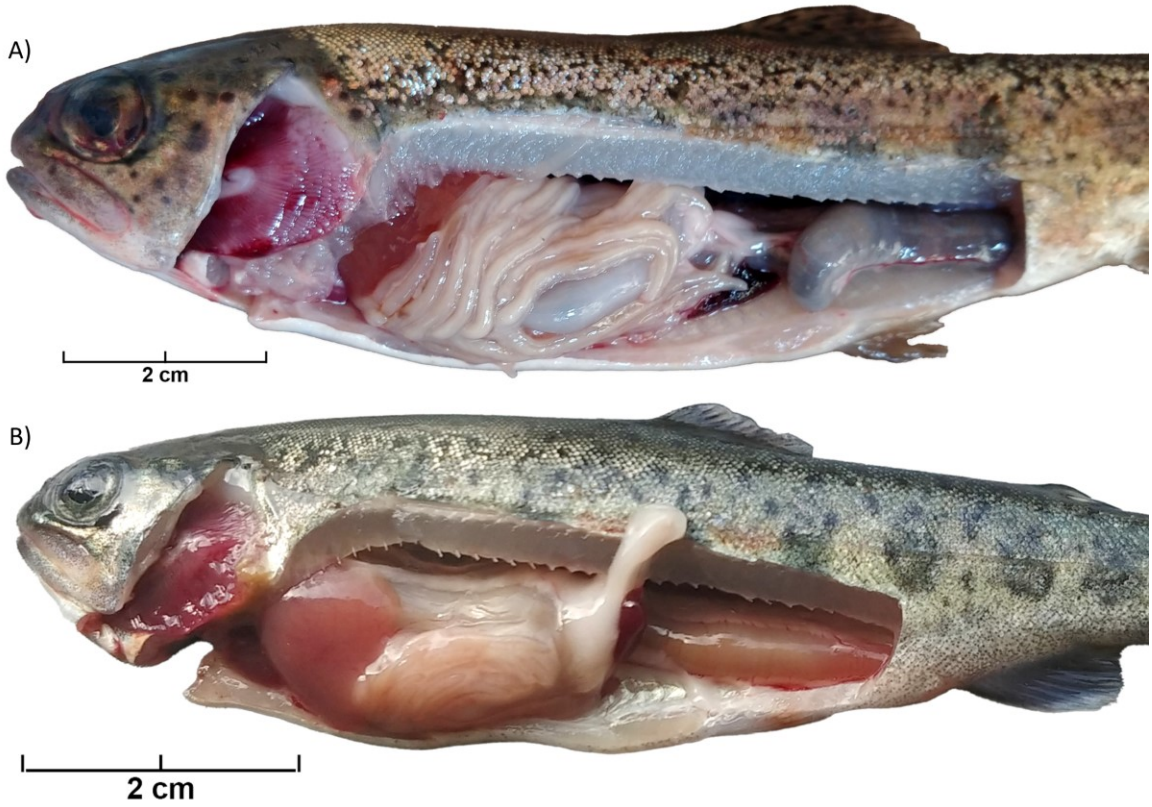


Figure 20: Gross lesions in fish testing positive for *Flavobacterium branchiophilum*: A) severe gill hyperplasia with clubbing of the filaments; B) yellowish necrosis of the gill filament tips

4.3.1.3 Lesser-known *Flavobacterium* species

Various gross lesions were also observed in some of the fish that tested POS for lesser-known *Flavobacterium* spp. and negative for other identified systemic fish pathogens, including *F. psychrophilum.* These lesions included erosions of skin and fins, shallow to deep skin ulcerations, and, in some fish, also internal lesions

(Figure 21). However, statistical power was limited or inadequate to support the statistical analysis of gross lesions.

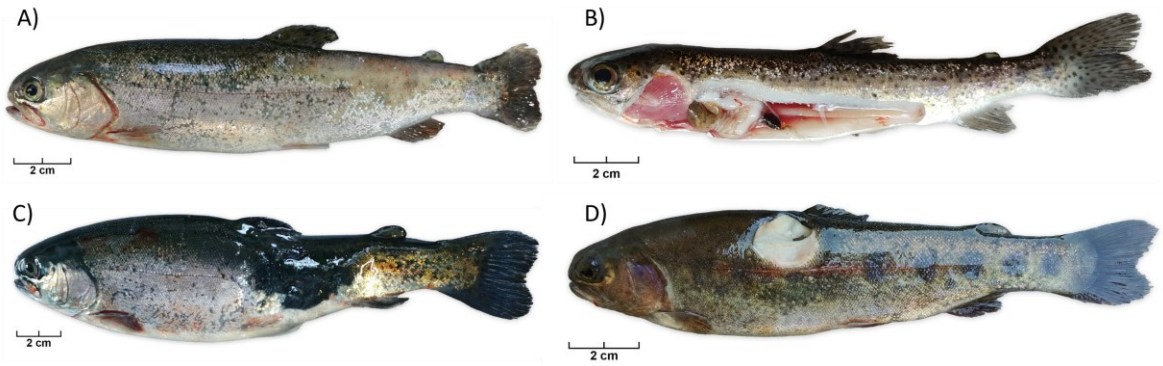


Figure 21: Gross lesions in some of the fish that tested positive for lesser-known *Flavobacterium* species and negative for other identified systemic fish pathogens: A) shallow skin ulceration on the dorsal part of the caudal peduncle and caudal fin from which numerous *Flavobacterium bernardetii* colonies were isolated; B) mild erosion of the dorsal and caudal fins, multifocal scale loss, mild gill anaemia, light to dark grey-brown mottled liver, mild ascites and severe cachexia in a fish with numerous isolated FX3 colonies from the skin and individual colonies from the liver; C) large concave and hyperpigmented chronic skin lesion from which numerous FX10 colonies were isolated; D) deep skin ulceration from which numerous FX26 colonies were isolated

4.3.2 Histopathological lesions

4.3.2.1 *Flavobacterium psychrophilum*

The indices of all organs analysed, except pancreas, were significantly higher in *F. psychrophilum*-INF fish compared to NH fish ($p < 0.05$), as shown by the Mann-Whitney U test per fish tank and a subsequent Fisher meta-test over individual *p*-values (Table 12, Figure 22).

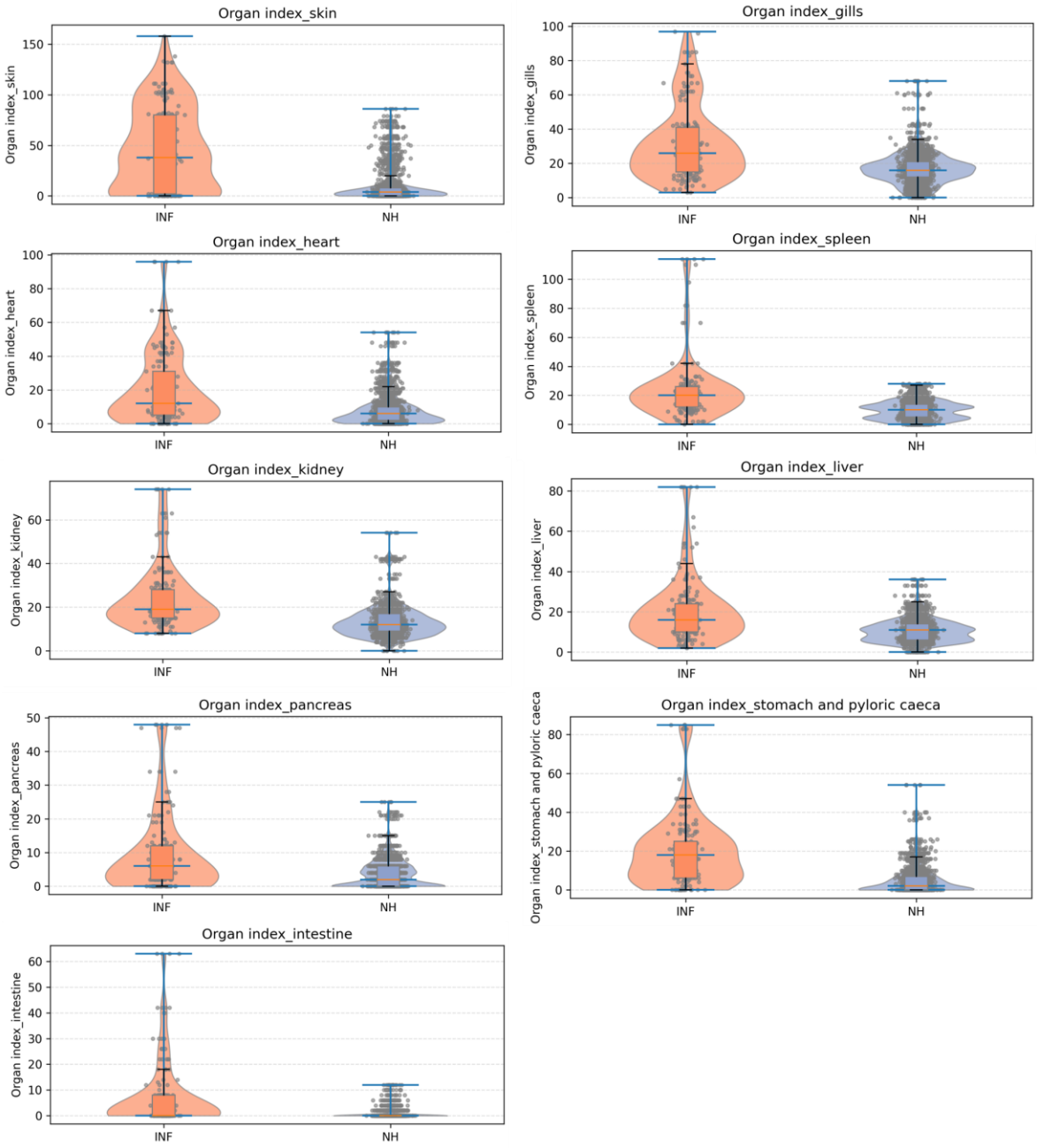
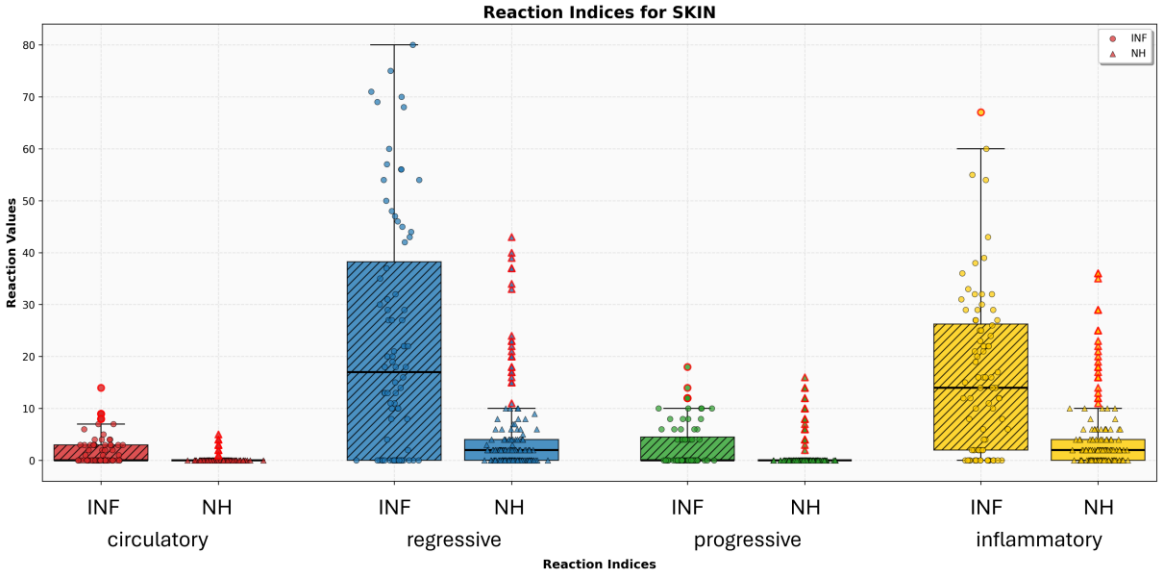


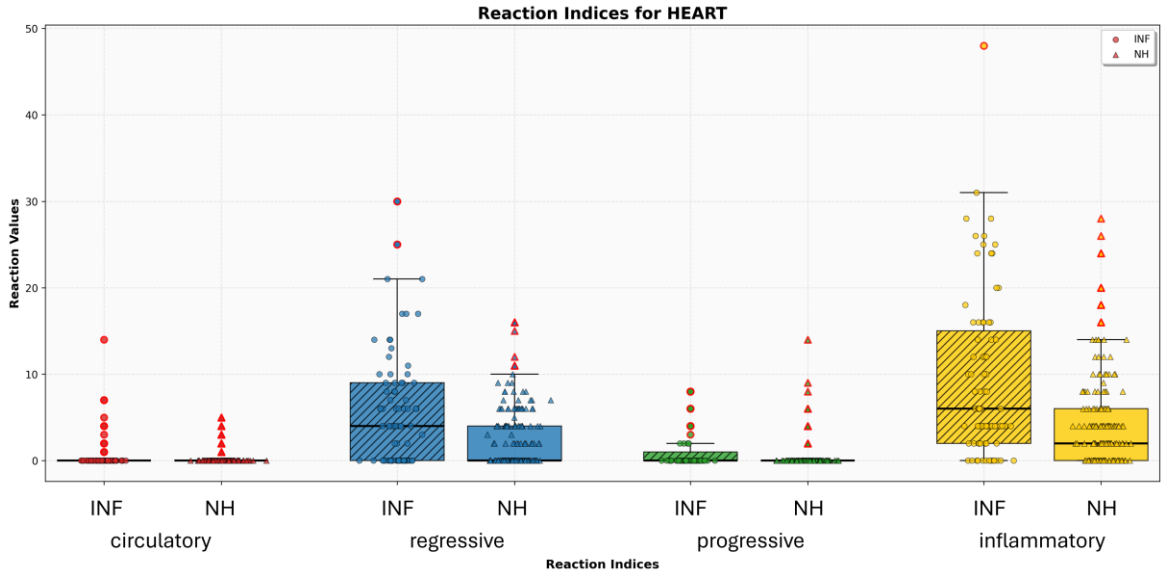
Figure 22: Violin boxplots showing the values of the organ indices for skin, gills, heart, spleen, kidney, liver, pancreas, stomach with pyloric caeca, and intestine in fish infected with *Flavobacterium psychrophilum* (INF) and negative healthy (NH) fish

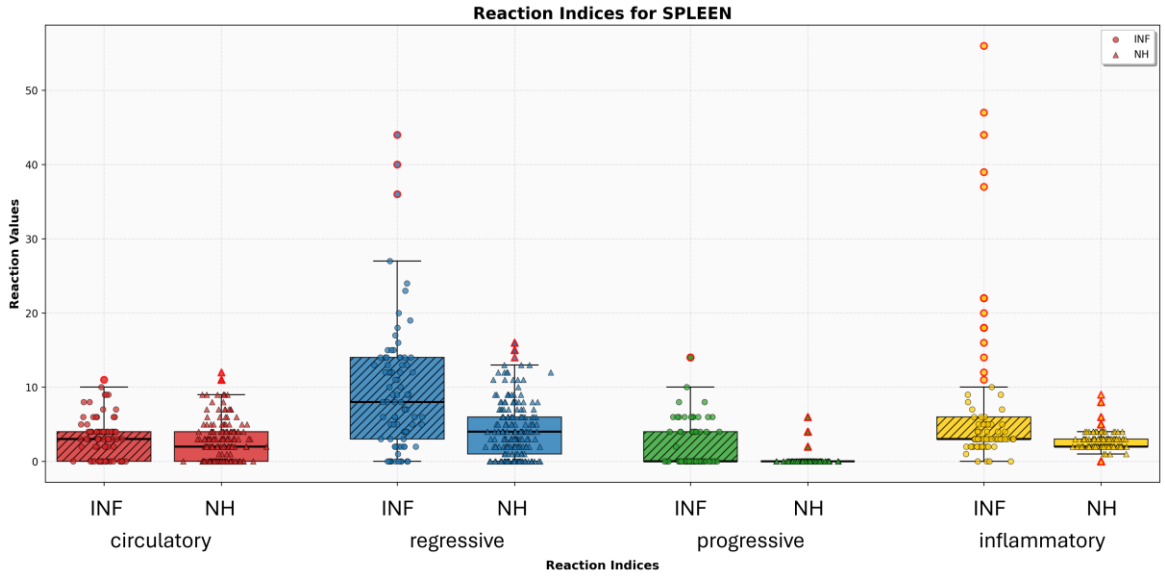
The analysis also revealed that the regressive and inflammatory reaction indices were significantly higher in most of the organs examined in the *F. psychrophilum*-INF fish (7/9 organs, except pancreas, and intestine), followed by the circulatory indices (6/9 organs, except heart, pancreas, and intestine), while the progressive

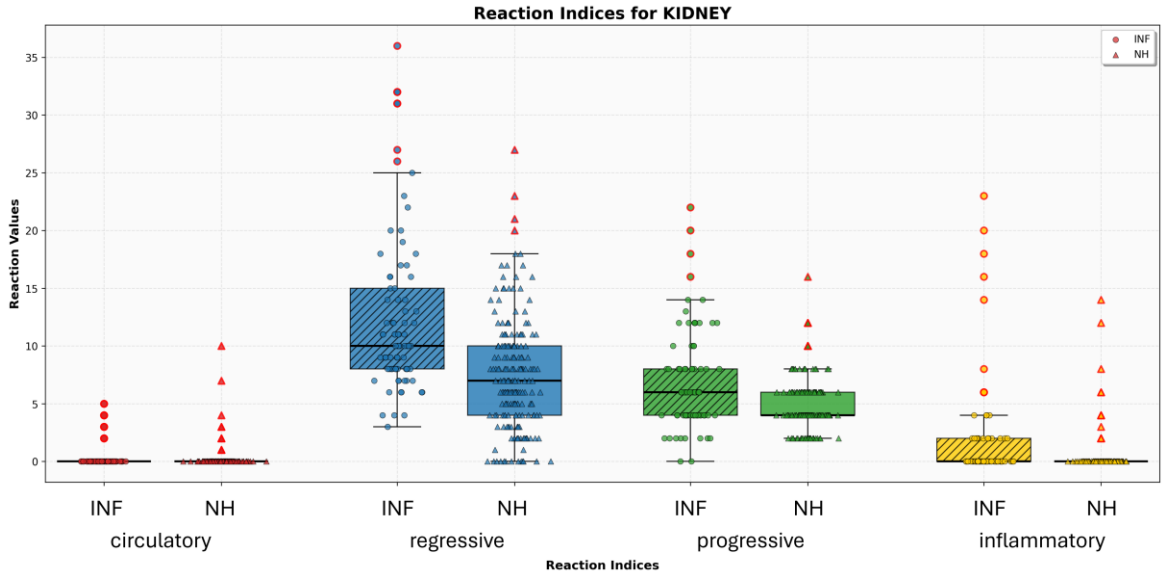
indices were significantly different only in the gills, spleen, and kidney (Table 12, Figure 23).

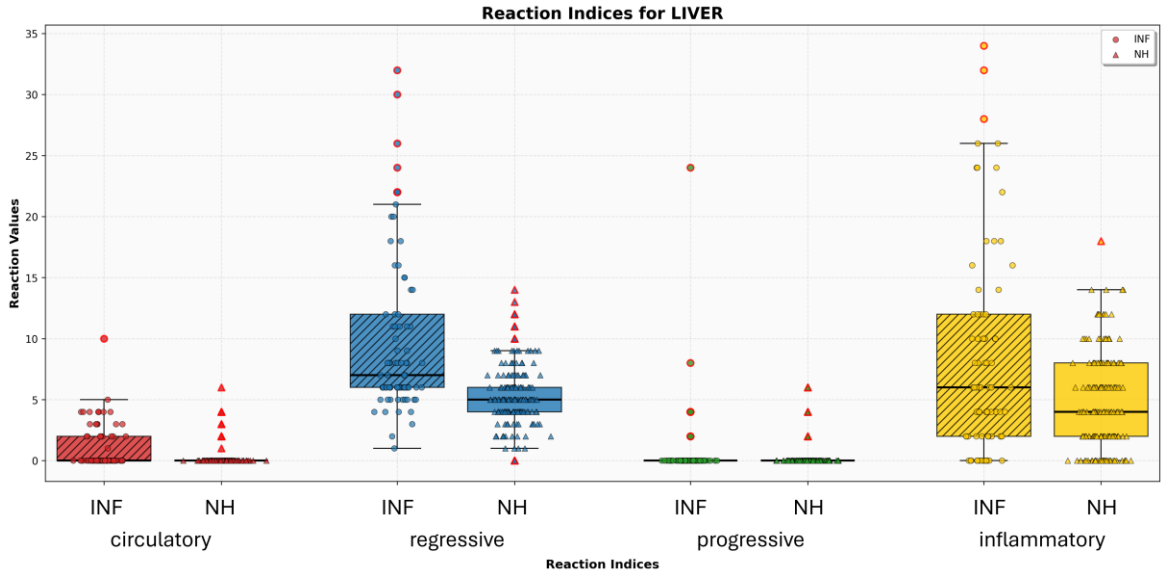


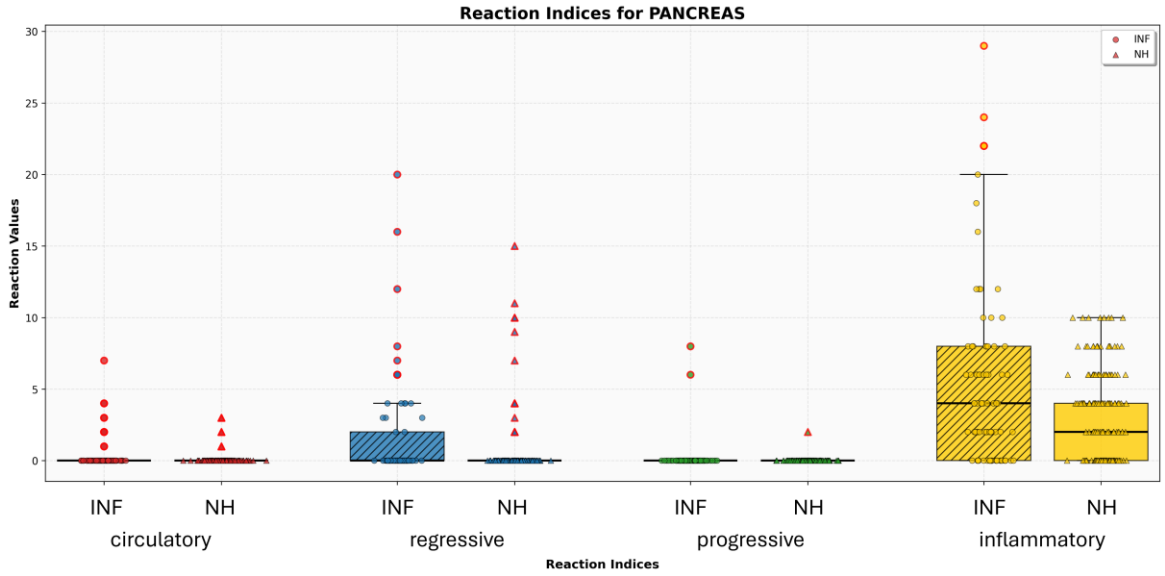


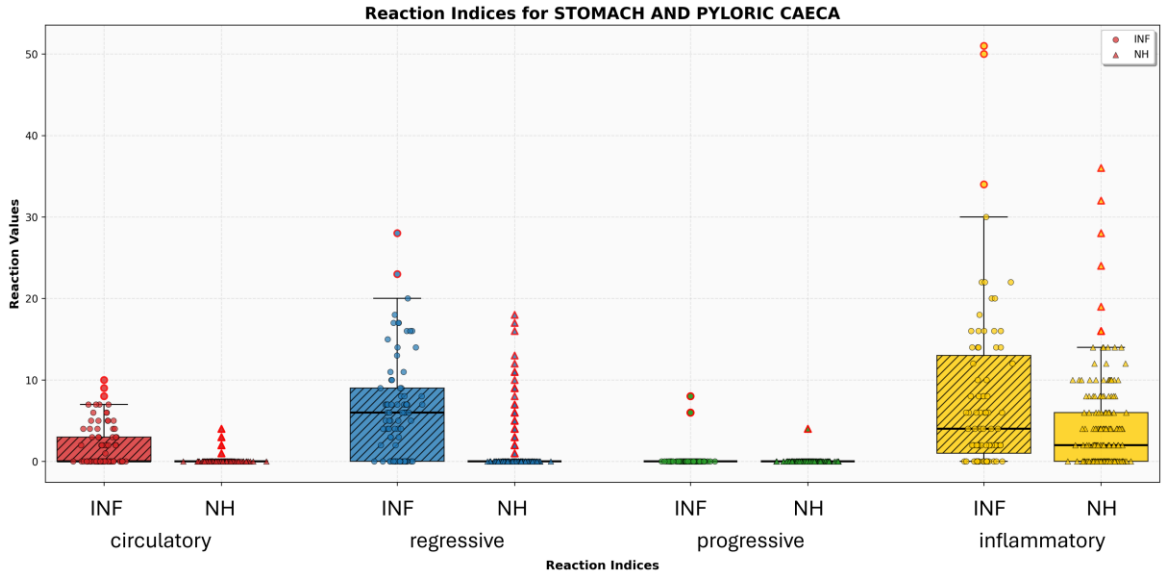

Reaction Indices for HEART
Reaction Values
INF
NH
INF NH INF NH INF NH INF NH
circulatory regressive progressive inflammatory
Reaction Indices


Reaction Indices for SPLEEN
Reaction Values
INF
NH
INF NH INF NH INF NH INF NH
circulatory regressive progressive inflammatory
Reaction Indices


Reaction Indices for KIDNEY
Reaction Values
INF
NH
INF NH INF NH INF NH INF NH
circulatory regressive progressive inflammatory
Reaction Indices


Reaction Indices for LIVER
Reaction Values
INF
NH
INF NH INF NH INF NH INF NH
circulatory regressive progressive inflammatory
Reaction Indices


Reaction Indices for PANCREAS
Reaction Values
INF
NH
INF NH INF NH INF NH INF NH
circulatory regressive progressive inflammatory
Reaction Indices


Reaction Indices for STOMACH AND PYLORIC CAECA
Reaction Values
INF
NH
INF NH INF NH INF NH INF NH
circulatory regressive progressive inflammatory
Reaction Indices

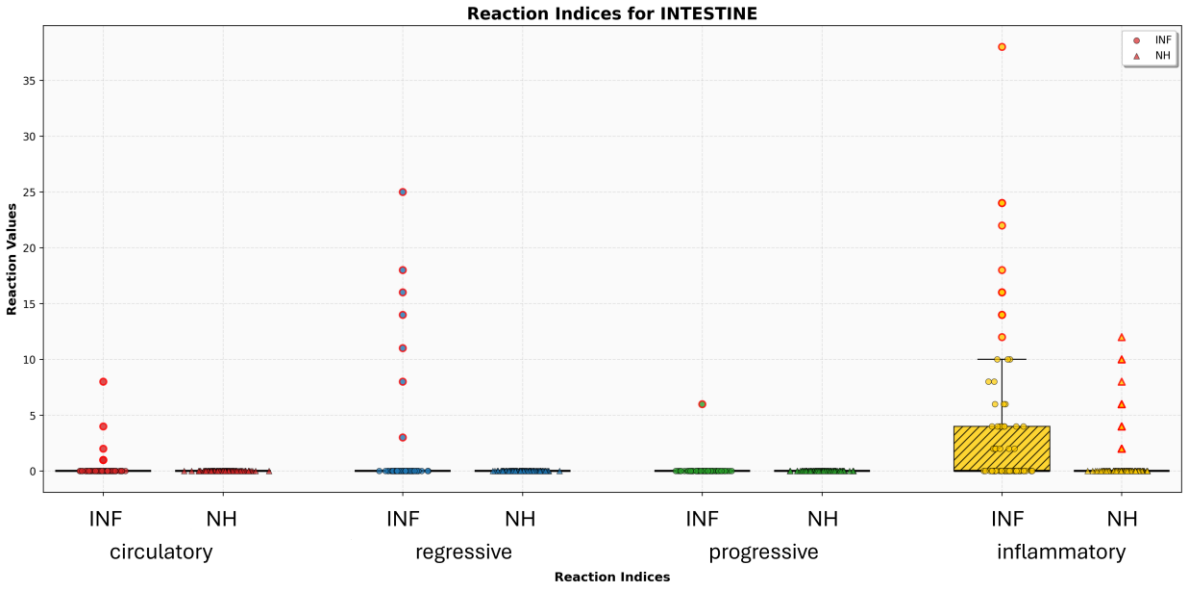


Figure 23: Boxplots showing the values of the reaction indices for skin, gills, heart, spleen, kidney, liver, pancreas, stomach with pyloric caeca, and intestine in fish infected with *Flavobacterium psychrophilum* (INF) and negative healthy (NH) fish

Several circulatory, regressive, progressive, and inflammatory lesions showed significantly higher values in the organs of *F. psychrophilum*-INF fish, and the results for each organ are described in detail below. The *p*-values, means, medians, and standard deviations for all histopathological variables analysed are shown in Table 12. This table also shows the percentage of INF fish in which the organ indices, reaction indices, and score values for individual lesions exceeded the median value of the NH fish.

Table 12: Histopathological (HP) variables associated with *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* infections analysed per fish tank using the Mann–Whitney U test and Fisher's combined probability test, including mean, median, and standard deviation (sd) of each variable, as well as percentage (%) of infected (INF) fish with a variable value above the median of negative healthy (NH) fish; significant *p*-values ($p < 0.05$) are shown in red

HP VARIABLES	*F. psychrophilum*		*F. branchiophilum*
	Per fish INF/NH fish	Per sampled organ INF/NH fish	INF/NH fish
Gill index	***p* = 0.001** **mean = 31.32/16.43** **median = 25.50/16.00** **sd = 23.16/9.70** **% INF: 66.67**	***p* = 0.100** **mean = 30.67/16.43** **median = 24.00/16.00** **sd = 22.39/9.70**	***p* = 0.724** **mean = 34.25/16.70** **median = 34.50/16.00** **sd = 18.52/9.95**

Table 12 continued

Table 12 continued

Gill RI circulatory	***p* = 0.025** mean = 2.16/1.44 median = 1.00/0.00 sd = 2.86/2.12 % INF: 55.56	*p* = 0.846 mean = 1.29/1.44 median = 0.50/0.00 sd = 2.07/2.12	*p* = 0.969 mean = 3.75/1.39 median = 3.00/1.00 sd = 4.11/2.05
Significant lesions	Lamellae/filaments_ haemorrhage *p* = 0.007 mean = 0.88/0.23 median = 0.00/0.00 sd = 1.33/0.62 % INF: 37.04	/	/
Gill RI regressive	***p* = 0.001** mean = 8.96/4.44 median = 5.50/4.00 sd = 8.81/3.79 % INF: 55.56	*p* = 0.114 mean = 9.29/4.44 median = 6.00/4.00 sd = 9.52/3.79	*p* = 0.602 mean = 8.75/4.60 median = 8.00/4.00 sd = 7.97/3.94
Significant lesions	Epithelium_degeneration *p* = 0.016 mean = 1.98/1.64 median = 2.00/2.00 sd = 1.45/1.15 % INF: 34.57 Epithelium_necrosis *p* = 0.010 mean = 0.85/0.32 median = 1.00/0.00 sd = 0.90/0.55 % INF: 55.56 Supporting tissue_necrosis *p* = 0.021 mean = 0.57/0.04 median = 0.00/0.00 sd = 1.17/0.30 % INF: 22.22	/	/
Gill RI progressive	***p* < 0.001** mean = 11.22/6.19 median = 9.00/6.00 sd = 6.35/3.07 % INF: 80.25	*p* = 0.068 mean = 10.50/6.19 median = 9.00/6.00 sd = 4.80/3.07	*p* = 0.645 mean = 16.25/6.31 median = 16.00/6.00 sd = 10.59/3.17
Significant lesions	Hypertrophy of epithelial cells *p* < 0.001 mean = 2.88/0.85 median = 3.00/1.00 sd = 1.55/0.92 % INF: 79.01 Hyperplasia of epithelial cells *p* = 0.002 mean = 2.54/1.79 median = 2.00/2.00 sd = 1.40/0.88 % INF: 45.68 Lamellar fusion *p* = 0.019 mean = 1.52/0.81 median = 1.00/1.00 sd = 1.41/0.83 % INF: 41.98	Hypertrophy of epithelial cells *p* < 0.001 mean = 2.67/0.85 median = 2.00/1.00 sd = 1.43/0.92	Filament fusion *p* = 0.010 mean = 1.50/0.01 median = 1.50/0.00 sd = 1.29/0.15
Gill RI inflammatory	***p* = 0.002** mean = 8.98/4.36 median = 6.00/4.00 sd = 8.43/3.54 % INF: 55.56	*p* = 0.098 mean = 9.58/4.36 median = 6.00/4.00 sd = 9.55/3.54	*p* = 0.853 mean = 5.50/4.40 median = 6.00/4.00 sd = 2.52/3.60

Table 12 continued

Table 12 continued

Significant lesions	Filaments/lamellae_ lymphocytes *p* = 0.012 mean = 1.61/1.42 median = 1.00/1.00 sd = 1.08/0.90 % INF: 45.68 Filaments/lamellae_ macrophages *p* = 0.007 mean = 0.80/0.35 median = 1.00/0.00 sd = 0.79/0.51 % INF: 64.20 Filaments/lamellae_ neutrophils *p* = 0.018 mean = 0.64/0.08 median = 0.00/0.00 sd = 1.13/0.42 % INF: 28.40	Filaments/lamellae_ macrophages *p* = 0.037 mean = 0.83/0.35 median = 1.00/0.00 sd = 0.82/0.51	/
Skin index	***p* < 0.001** **mean = 42.76/9.50** **median = 36.00/2.50** **sd = 41.29/17.34** **% INF: 70.00**	***p* < 0.001** **mean = 60.61/9.50** **median = 55.00/2.50** **sd = 39.90/17.34**	***p* = 0.798** **mean = 13.75/10.32** **median = 12.00/4.00** **sd = 12.97/18.34**
Skin RI circulatory	***p* < 0.001** mean = 1.78/0.17 median = 0.00/0.00 sd = 2.86/0.71 % INF: 41.25	***p* < 0.001** mean = 2.76/0.17 median = 2.00/0.00 sd = 3.32/0.71	*p* = 1.000 mean = 0.00/0.16 median = 0.00/0.00 sd = 0.00/0.70
Significant lesions	Dermis_haemorrhage *p* = 0.004 mean = 0.97/0.12 median = 0.00/0.00 sd = 1.47/0.55 % INF: 36.25	Dermis_haemorrhage *p* < 0.001 mean = 1.32/0.12 median = 1.00/0.00 sd = 1.52/0.55 Muscle_haemorrhage *p* < 0.001 mean = 1.17/0.00 median = 0.00/0.00 sd = 2.04/0.00	/
Skin RI regressive	***p* < 0.001** mean = 22.35/4.25 median = 16.00/2.00 sd = 22.93/8.22 % INF: 68.75	***p* < 0.001** mean = 33.07/4.25 median = 29.00/2.00 sd = 23.31/8.22	*p* = 0.779 mean = 6.75/4.59 median = 5.00/2.00 sd = 7.63/8.78

Table 12 continued

Table 12 continued

Significant lesions	Epidermis_erosion *p* = 0.001 mean = 1.37/0.26 median = 0.00/0.00 sd = 1.73/0.85 % INF: 42.50 Epidermis_degeneration *p* = 0.020 mean = 1.68/0.81 median = 1.00/1.00 sd = 1.67/1.00 % INF: 48.75 Basement membrane defect (ulceration) *p* < 0.001 mean = 1.62/0.14 median = 0.00/0.00 sd = 2.08/0.61 % INF: 42.50 Dermis_loss of scales/bone resorption *p* < 0.001 mean = 1.81/0.28 median = 0.00/0.00 sd = 2.18/0.95 % INF: 47.50 Dermis_necrosis *p* < 0.001 mean = 1.70/0.31 median = 1.00/0.00 sd = 1.89/0.96 % INF: 51.25	Epidermis_erosion *p* < 0.001 mean = 1.66/0.26 median = 0.00/0.00 sd = 1.84/0.85 Epidermis_degeneration *p* = 0.002 mean = 2.34/0.81 median = 2.00/1.00 sd = 1.62/1.00 Epidermis_necrosis *p* = 0.001 mean = 0.68/0.08 median = 0.00/0.00 sd = 0.91/0.31 Basement membrane defect (ulceration) *p* < 0.001 mean = 2.39/0.14 median = 3.00/0.00 sd = 2.30/0.61 Dermis_loss of scales/bone resorption *p* < 0.001 mean = 2.98/0.28 median = 3.00/0.00 sd = 2.22/0.95 Dermis_necrosis *p* < 0.001 mean = 2.37/0.31 median = 3.00/0.00 sd = 1.96/0.96 Muscle_degeneration *p* < 0.001 mean = 1.10/0.01 median = 0.00/0.00 sd = 1.92/0.14 Muscle_necrosis *p* < 0.001 mean = 1.20/0.00 median = 0.00/0.00 sd = 2.08/0.07	/
Skin RI progressive	*p* = 0.086 mean = 2.73/1.14 median = 0.00/0.00 sd = 4.39/3.05 % INF: 35.00	*p* = 0.103 mean = 3.56/1.14 median = 0.00/0.00 sd = 4.46/3.05	*p* = 0.997 mean = 1.50/1.27 median = 0.00/0.00 sd = 3.00/3.21
Significant lesions	/	/	/
Skin RI inflammatory	***p* < 0.001** mean = 15.89/3.95 median = 14.00/2.00 sd = 15.63/7.00 % INF: 67.50	***p* < 0.001** mean = 21.22/3.95 median = 21.00/2.00 sd = 14.98/7.00	*p* = 0.645 mean = 5.50/4.30 median = 4.00/2.00 sd = 4.73/7.31

Table 12 continued

Table 12 continued

Significant lesions	Epidermis_neutrophils *p* = 0.036 mean = 0.54/0.06 median = 0.00/0.00 sd = 0.94/0.32 % INF: 31.25 Dermis_lymphocytes *p* = 0.001 mean = 1.62/0.62 median = 1.00/0.00 sd = 1.55/1.30 % INF: 66.25 Dermis_macrophages *p* < 0.001 mean = 0.89/0.21 median = 1.00/0.00 sd = 1.11/0.55 % INF: 53.75 Dermis_neutrophils *p* < 0.001 mean = 1.27/0.20 median = 0.00/0.00 sd = 1.70/0.70 % INF: 46.25 Dermis_exudate *p* < 0.001 mean = 1.57/0.30 median = 0.00/0.00 sd = 1.80/0.92 % INF: 47.50	Epidermis_neutrophils *p* = 0.002 mean = 0.71/0.06 median = 0.00/0.00 sd = 1.03/0.32 Dermis_lymphocytes *p* < 0.001 mean = 2.00/0.62 median = 2.00/0.00 sd = 1.32/1.30 Dermis_macrophages *p* < 0.001 mean = 1.02/0.21 median = 1.00/0.00 sd = 1.04/0.55 Dermis_neutrophils *p* < 0.001 mean = 1.59/0.20 median = 1.00/0.00 sd = 1.73/0.70 Dermis_exudate *p* < 0.001 mean = 2.32/0.30 median = 3.00/0.00 sd = 1.68/0.92 Muscle_ lymphocytes *p* = 0.002 mean = 0.78/0.03 median = 0.00/0.00 sd = 1.42/0.26 Muscle_macrophages *p* = 0.021 mean = 0.46/0.00 median = 0.00/0.00 sd = 0.95/0.00 Muscle_neutrophils *p* < 0.001 mean = 0.85/0.00 median = 0.00/0.00 sd = 1.53/0.00 Muscle_exudate *p* = 0.008 mean = 0.90/0.00 median = 0.00/0.00 sd = 1.69/0.00	/
Heart index	***p* = 0.004** **mean = 17.86/7.78** **median = 10.00/4.00** **sd = 19.14/9.91** **% INF: 69.62**	/	***p* = 0.537** **mean = 4.00/8.00** **median = 4.00/4.00** **sd = 3.27/9.79**
Heart RI circulatory	*p* = 0.557 mean = 0.77/0.40 median = 0.00/0.00 sd = 2.14/1.06 % INF: 20.25	/	*p* = 0.996 mean = 0.00/0.42 median = 0.00/0.00 sd = 0.00/1.06
Significant lesions	/	/	/
Heart RI regressive	***p* = 0.011** mean = 6.37/2.42 median = 4.00/0.00 sd = 7.35/3.62 % INF: 64.56	/	*p* = 0.919 mean = 0.00/2.53 median = 0.00/0.00 sd = 0.00/3.62
Significant lesions	/	/	/
Heart RI progressive	*p* = 0.809 mean = 1.22/0.33 median = 0.00/0.00 sd = 2.40/1.53 % INF: 25.32	/	*p* = 1.000 mean = 0.00/0.31 median = 0.00/0.00 sd = 0.00/1.45

Table 12 continued

Table 12 continued

Significant lesions	/	/	/
Heart RI inflammatory	***p* = 0.019** mean = 9.50/4.64 median = 6.00/2.00 sd = 9.61/5.73 % INF: 73.42	/	*p* = 0.797 mean = 4.00/4.76 median = 4.00/2.00 sd = 3.27/5.71
Significant lesions	/	/	/
Spleen index	***p* < 0.001** **mean = 22.45/9.54** **median = 19.00/9.00** **sd = 20.94/6.19** **% INF: 82.72**	***p* < 0.001** **mean = 27.69/9.54** **median = 21.00/9.00** **sd = 24.82/6.19**	***p* = 0.498** **mean = 16.50/9.58** **median = 16.50/9.00** **sd = 6.14/6.24**
Spleen RI circulatory	*p* = 0.097 mean = 3.16/2.45 median = 3.00/2.00 sd = 2.77/2.68 % INF: 60.49	***p* = 0.034** mean = 3.62/2.45 median = 4.00/2.00 sd = 2.12/2.68	*p* = 0.274 mean = 3.25/2.38 median = 3.50/2.00 sd = 0.96/2.62
Significant lesions	Hyperaemia *p* = 0.022 mean = 2.31/1.80 median = 3.00/2.00 sd = 1.85/1.70 % INF: 54.32	Hyperaemia *p* = 0.014 mean = 2.65/1.80 median = 3.00/2.00 sd = 1.79/1.70	/
Spleen RI regressive	***p* < 0.001** mean = 9.66/4.40 median = 8.00/4.00 sd = 8.71/3.94 % INF: 69.14	***p* < 0.001** mean = 10.27/4.40 median = 8.50/4.00 sd = 10.53/3.94	*p* = 0.485 mean = 10.00/4.43 median = 10.50/4.00 sd = 5.35/3.98
Significant lesions	Parenchyma_atrophy/ lymphopaenia *p* = 0.001 mean = 1.89/0.43 median = 0.00/0.00 sd = 2.15/1.12 % INF: 45.68	Parenchyma_atrophy/ lymphopaenia *p* < 0.001 mean = 2.08/0.43 median = 0.00/0.00 sd = 2.37/1.12	/
Spleen RI progressive	*p* = 0.058 mean = 2.05/0.29 median = 0.00/0.00 sd = 3.26/1.07 % INF: 34.57	***p* = 0.026** mean = 2.38/0.29 median = 0.00/0.00 sd = 4.12/1.07	*p* = 0.992 mean = 0.00/0.30 median = 0.00/0.00 sd = 0.00/1.14
Significant lesions	/	Parenchyma_hyperplasia *p* = 0.026 mean = 0.92/0.14 median = 0.00/0.00 sd = 1.38/0.53	/
Spleen RI inflammatory	***p* < 0.001** mean = 7.58/2.39 median = 3.00/2.00 sd = 10.90/1.49 % INF: 77.78	***p* = 0.001** mean = 11.42/2.39 median = 4.50/2.00 sd = 12.70/1.49	*p* = 0.372 mean = 3.25/2.48 median = 2.50/2.00 sd = 1.89/1.64
Significant lesions	Parenchyma_activation of RES *p* < 0.001 mean = 2.81/2.13 median = 3.00/2.00 sd = 1.23/1.16 % INF: 65.43	Parenchyma_activation of RES *p* = 0.021 mean = 3.08/2.13 median = 3.00/2.00 sd = 1.13/1.16	/
Kidney index	***p* < 0.001** **mean = 21.95/13.04** **median = 18.00/12.00** **sd = 12.84/6.87** **% INF: 85.19**	***p* = 0.003** **mean = 25.09/13.04** **median = 18.00/12.00** **sd = 15.36/6.87**	***p* = 0.649** **mean = 18.75/13.29** **median = 19.00/12.00** **sd = 3.30/7.16**
Kidney RI circulatory	*p* = 0.353 mean = 0.60/0.17 median = 0.00/0.00 sd = 1.37/0.96 % INF: 18.52	***p* = 0.019** mean = 1.11/0.17 median = 0.00/0.00 sd = 1.76/0.96	*p* = 1.000 mean = 0.00/0.17 median = 0.00/0.00 sd = 0.00/0.94
Significant lesions	/	/	/

Table 12 continued

Table 12 continued

Kidney RI regressive	***p* < 0.001** mean = 12.65/7.35 median = 10.00/7.00 sd = 7.24/4.66 % INF: 81.48	***p* = 0.006** mean = 14.31/7.35 median = 11.00/7.00 sd = 8.26/4.66	*p* = 0.232 mean = 16.25/7.44 median = 16.50/7.00 sd = 2.75/4.68
Significant lesions	Tubule_degeneration *p* < 0.001 mean = 3.11/1.60 median = 3.00/2.00 sd = 1.31/1.25 % INF: 67.90	Tubule_degeneration *p* = 0.006 mean = 3.20/1.60 median = 4.00/2.00 sd = 1.26/1.25	/
Kidney RI progressive	***p* = 0.005** mean = 6.74/5.13 median = 6.00/4.00 sd = 4.07/2.34 % INF: 56.79	***p* = 0.025** mean = 7.23/5.13 median = 6.00/4.00 sd = 4.69/2.34	*p* = 0.340 mean = 2.50/5.22 median = 2.00/4.00 sd = 1.00/2.40
Significant lesions	Haematopoietic tissue_hyperplasia *p* < 0.001 mean = 1.20/0.19 median = 0.00/0.00 sd = 1.55/0.66 % INF: 44.44 Tubule_regeneration *p* = 0.021 mean = 2.06/2.35 median = 2.00/2.00 sd = 0.80/0.87	Haematopoietic tissue_hyperplasia *p* = 0.005 mean = 1.46/0.19 median = 0.00/0.00 sd = 1.74/0.66	/
Kidney RI inflammatory	*p* = 0.080 mean = 1.96/0.39 median = 0.00/0.00 sd = 4.61/1.63 % INF: 30.86	*p* = 0.167 mean = 2.43/0.39 median = 0.00/0.00 sd = 5.31/1.63	*p* = 0.891 mean = 0.00/0.45 median = 0.00/0.00 sd = 0.00/1.80
Significant lesions	/	/	/
Liver index	***p* = 0.001** **mean = 19.50/10.48** **median = 15.00/10.00** **sd = 15.85/5.71** **% INF: 67.90**	***p* = 0.004** **mean = 19.61/10.48** **median = 16.00/10.00** **sd = 16.02/5.71**	***p* = 0.613** **mean = 14.00/10.54** **median = 11.00/10.00** **sd = 9.76/5.90**
Liver RI circulatory	*p* = 0.217 mean = 1.00/0.34 median = 0.00/0.00 sd = 1.77/0.99 % INF: 32.10	***p* = 0.025** mean = 1.29/0.34 median = 0.00/0.00 sd = 2.21/0.99	*p* = 0.940 mean = 0.50/0.33 median = 0.00/0.00 sd = 1.00/1.01
Significant lesions	/	Liver_haemorrhage *p* = 0.047 mean = 0.94/0.03 median = 0.00/0.00 sd = 1.62/0.25	/
Liver RI regressive	***p* < 0.001** mean = 9.70/5.22 median = 7.00/5.00 sd = 6.52/2.57 % INF: 80.25	***p* = 0.015** mean = 9.48/5.22 median = 6.00/5.00 sd = 6.84/2.57	*p* = 0.415 mean = 10.00/5.20 median = 8.00/5.00 sd = 5.48/2.67
Significant lesions	Liver_decreased vacuolation *p* = 0.001 mean = 5.40/4.22 median = 6.00/4.00 sd = 0.96/1.61 % INF: 86.42	Liver_degeneration *p* = 0.022 mean = 0.68/0.09 median = 0.00/0.00 sd = 1.51/0.48 Liver_necrosis *p* = 0.047 mean = 0.71/0.07 median = 0.00/0.00 sd = 1.30/0.30	/

Table 12 continued

Table 12 continued

Liver RI progressive	*p* = 0.999 mean = 0.78/0.14 median = 0.00/0.00 sd = 2.98/0.78 % INF: 14.82	*p* = 0.730 mean = 1.23/0.14 median = 0.00/0.00 sd = 4.40/0.78	*p* = 1.000 mean = 0.00/0.13 median = 0.00/0.00 sd = 0.00/0.74
Significant lesions	/	/	/
Liver RI inflammatory	***p* = 0.021** mean = 8.02/4.75 median = 6.00/4.00 sd = 8.32/3.82 % INF: 51.85	*p* = 0.058 mean = 7.61/4.75 median = 4.00/4.00 sd = 7.13/3.82	*p* = 0.191 mean = 3.50/4.86 median = 2.00/4.00 sd = 4.43/3.93
Significant lesions	/	Liver_neutrophils *p* = 0.005 mean = 0.71/0.02 median = 0.00/0.00 sd = 1.42/0.21	/
Pancreas index	***p* = 0.078** **mean = 7.68/3.40** **median = 4.00/2.00** **sd = 9.95/4.28** **% INF: 55.56**	/	***p* = 0.866** **mean = 5.00/3.67** **median = 4.00/2.00** **sd = 3.83/4.63**
Pancreas RI circulatory	*p* = 0.968 mean = 0.39/0.06 median = 0.00/0.00 sd = 1.15/0.36 % INF: 13.58	/	*p* = 1.000 mean = 0.00/0.06 median = 0.00/0.00 sd = 0.00/0.37
Significant lesions	/	/	/
Pancreas RI regressive	*p* = 0.590 mean = 1.72/0.52 median = 0.00/0.00 sd = 3.63/1.93 % INF: 27.16	/	*p* = 0.991 mean = 0.00/0.65 median = 0.00/0.00 sd = 0.00/2.20
Significant lesions	/	/	/
Pancreas RI progressive	*p* = 1.000 mean = 0.18/0.01 median = 0.00/0.00 sd = 1.11/0.14 % INF: 2.47	/	*p* = 1.000 mean = 0.00/0.01 median = 0.00/0.00 sd = 0.00/0.13
Significant lesions	/	/	/
Pancreas RI inflammatory	*p* = 0.089 mean = 5.39/2.81 median = 4.00/2.00 sd = 6.21/3.00 % INF: 53.09	/	*p* = 0.852 mean = 5.00/2.95 median = 4.00/2.00 sd = 3.83/3.10
Significant lesions	/	/	/
Stomach and pyloric caeca index	***p* < 0.001** **mean = 16.83/5.22** **median = 12.50/2.00** **sd = 16.55/8.23** **% INF: 83.54**	/	***p* = 0.637** **mean = 16.25/5.14** **median = 10.50/2.00** **sd = 12.53/8.01**
Stomach and pyloric caeca RI circulatory	***p* < 0.001** mean = 1.94/0.18 median = 0.00/0.00 sd = 2.63/0.68 % INF: 44.44	/	*p* = 0.732 mean = 2.00/0.19 median = 1.50/0.00 sd = 2.45/0.70

Table 12 continued

Table 12 continued

Significant lesions	Mucosa_intercellular oedema *p* = 0.007 mean = 0.76/0.08 median = 0.00/0.00 sd = 1.30/0.45 % INF: 28.40 Other layers_intercellular oedema *p* = 0.002 mean = 1.00/0.10 median = 0.00/0.00 sd = 1.41/0.47 % INF: 35.80	/	/
Stomach and pyloric caeca RI regressive	***p* < 0.001** mean = 6.47/1.32 median = 6.00/0.00 sd = 6.27/3.14 % INF: 72.15	/	*p* = 0.090 mean = 5.75/1.34 median = 4.50/0.00 sd = 6.95/3.08
Significant lesions	Glands_degeneration *p* < 0.001 mean = 1.42/0.17 median = 1.00/0.00 sd = 1.53/0.47 % INF: 60.76 Glands_necrosis *p* < 0.001 mean = 0.58/0.02 median = 0.00/0.00 sd = 0.76/0.14 % INF: 44.30	/	/
Stomach and pyloric caeca RI progressive	*p* = 1.000 mean = 0.51/0.04 median = 0.00/0.00 sd = 1.81/0.39 % INF: 7.59	/	*p* = 1.000 mean = 0.00/0.03 median = 0.00/0.00 sd = 0.00/0.37
Significant lesions	/	/	/
Stomach and pyloric caeca RI inflammatory	***p* = 0.026** mean = 7.86/3.68 median = 4.00/2.00 sd = 10.34/5.58 % INF: 54.43	/	*p* = 0.825 mean = 8.50/3.57 median = 7.00/2.00 sd = 7.19/5.46
Significant lesions	Mucosa_lymphocytes *p* = 0.011 mean = 1.26/0.69 median = 1.00/0.00 sd = 1.51/1.05 % INF: 54.32	/	/
Intestine index	***p* = 0.013** **mean = 5.46/0.61** **median = 0.00/0.00** **sd = 11.14/1.78** **% INF: 44.16**	/	***p* = 0.912** **mean = 3.00/0.61** **median = 0.00/0.00** **sd = 6.00/1.78**
Intestine RI circulatory	*p* = 1.000 mean = 0.21/0.00 median = 0.00/0.00 sd = 1.05/0.00 % INF: 6.49	/	*p* = 1.000 mean = 0.00/0.00 median = 0.00/0.00 sd = 0.00/0.00
Significant lesions	/	/	/
Intestine RI regressive	*p* = 0.860 mean = 1.25/0.00 median = 0.00/0.00 sd = 4.44/0.00 % INF: 9.09	/	*p* = 1.000 mean = 0.00/0.00 median = 0.00/0.00 sd = 0.00/0.00
Significant lesions	/	/	/

Table 12 continued

Table 12 continued

Intestine RI progressive	*p* = 1.000 mean = 0.08/0.00 median = 0.00/0.00 sd = 0.69/0.00 % INF: 1.30	/	*p* = 1.000 mean = 0.00/0.00 median = 0.00/0.00 sd = 0.00/0.00
Significant lesions	/	/	/
Intestine RI inflammatory	***p* = 0.015** mean = 3.92/0.61 median = 0.00/0.00 sd = 7.19/1.78 % INF: 42.86	/	*p* = 0.912 mean = 3.00/0.61 median = 0.00/0.00 sd = 6.00/1.78
Significant lesions	Mucosa_lymphocytes *p* = 0.050 mean = 0.83/0.23 median = 0.00/0.00 sd = 1.36/0.63 % INF: 35.06	/	/

RI, reaction index

The skin index exceeded the median of NH fish in 70% of *F. psychrophilum*-INF fish. The circulatory, regressive, and inflammatory reaction indices were significantly higher in INF fish and exceeded the healthy median in 41.25%, 68.75%, and 67.50% of INF fish, respectively. The difference for the progressive index was not significant. Circulatory lesions included haemorrhage in the dermis and muscle tissue. Regressive lesions included erosion, hydropic or vacuolar degeneration, and necrosis of the epidermis, ulceration, necrosis of the dermis, loss or resorption of scales or bone by osteoclasts, as well as hydropic degeneration and Zenker's degeneration (necrosis) of muscle tissue. Inflammatory lesions included infiltration of the epidermis with neutrophils and infiltration with lymphocytes, macrophages, and neutrophils in the dermis and muscle tissue, accompanied by serous or fibrinous exudate in these layers (Table 12, Figure 24A). Few to numerous filamentous bacteria, identified as *F. psychrophilum* by bacteriological investigation, were occasionally observed in the superficial necrotic tissue of skin and fin ulcerations or adhering to and surrounding exposed bone (Figure 24B). Some other lesions were observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. These lesions included hyperplasia of epithelial cells (28.75% of INF fish), as well as regeneration (2.5%) and fibrosis (1.25%) of muscle tissue.

The gill index exceeded the median of NH fish in 66.67% of *F. psychrophilum*-INF fish. The circulatory, regressive, progressive, and inflammatory reaction indices

were significantly higher in INF fish and exceeded the healthy median in 55.56%, 55.56%, 80.25%, and 55.56% of INF fish, respectively. Circulatory lesions included haemorrhage in the lamellae and filaments, while regressive lesions included hydropic or vacuolar degeneration and necrosis of the epithelium and necrosis of the supporting tissue of lamellae and filaments. Progressive lesions were hypertrophy and hyperplasia of epithelial cells and lamellar fusion. Inflammatory lesions were characterised by infiltration of the lamellae and filaments with lymphocytes, macrophages, and neutrophils (Figure 24C–E) (Table 12). Low to moderate numbers of filamentous bacteria, identified as *F. psychrophilum* by bacteriological investigation, were occasionally observed along the gill epithelium and in the necrotic epithelial and supporting tissue, leading to exposure of the gill filament cartilage in severe cases (Figure 24C). Several other lesions were observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. These lesions included intercellular oedema in the lamellae and filaments (24.69% of INF fish), lamellar aneurysms (18.52%), thrombosis in the lamellae or central venous sinus of the filaments (3.7%) (Figure 24E), hyperplasia of goblet cells (33.33%), fusion of filaments (8.64%), as well as necrosis (9.87%) and infiltration with lymphocytes (16.05%), macrophages (16.05%), and neutrophils (11.11%) in the gill arches. Perivasculitis of the efferent branchial arterioles was also observed (Figure 24F).

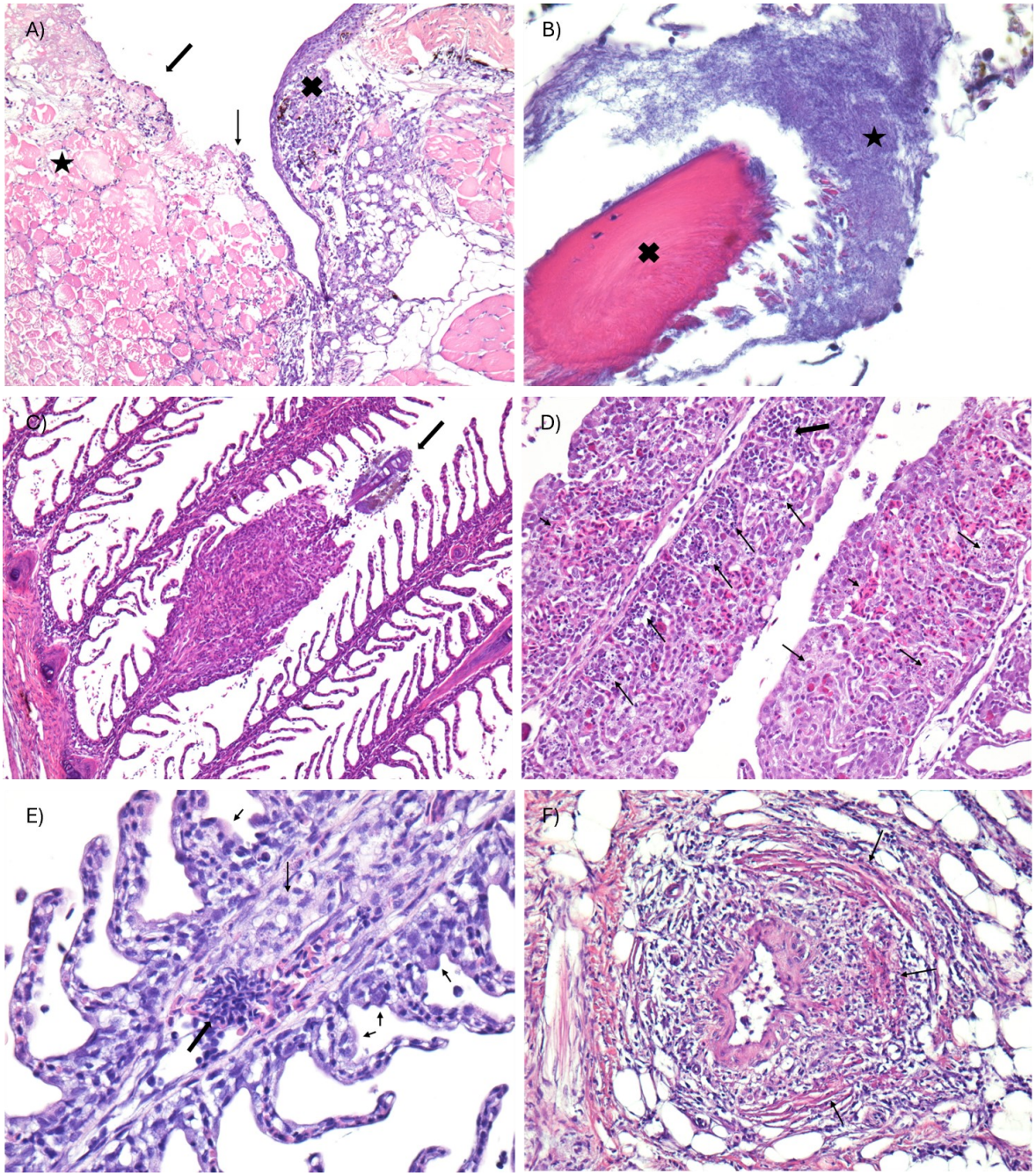


Figure 24: Histopathological lesions in skin and gills of fish infected with *Flavobacterium psychrophilum*: A) shallow skin ulceration (thick arrow shows the ulceration and thin arrow its margin) next to the dorsal fin with Zenker's degeneration of the underlying skeletal muscle tissue (star) as well as mild to moderate macrophage and neutrophil infiltration in the affected area and surrounding tissues (X) (100× magnification); B) numerous filamentous *F. psychrophilum* bacteria (star) adhering to and surrounding the caudal vertebra (X), which is exposed due to severe peduncle ulceration and necrosis (400× magnification); C) severe necrosis of the gill filament tip with exposed cartilage surrounded by filamentous *F. psychrophilum* bacteria (arrow), along with severe hyperplasia and lamellar fusion in the proximal part of the affected filament (100× magnification); D) severe gill epithelial hyperplasia with lamellar fusion and multifocal small areas

of necrosis (long thin arrow), multifocal haemorrhage (short thin arrow) and mild lymphocyte infiltration (thick arrow) (200× magnification); E) thrombus (thick arrow) adherent to the endothelium of the central venous sinus of the gill filament with a mild macrophage infiltrate (long thin arrow) in the surrounding connective tissue as well as mild hypertrophy, hyperplasia (short thin arrows) and vacuolar degeneration of the epithelium (400× magnification); F) lymphocytic and neutrophilic perivasculitis of the gill arch efferent branchial arteriole with multifocal small areas of necrosis (arrows) (200× magnification); haematoxylin and eosin staining

In 69.62% of *F. psychrophilum*-INF fish, the heart index was above the median of NH fish. The indices for regressive and inflammatory reactions were significantly higher in INF fish and exceeded the healthy median in 64.56% and 73.42% of INF fish, respectively. The difference in the circulatory and progressive indices was not significant. Additionally, no lesion showed significant differences between INF and NH fish (Table 12). However, several lesions were observed in INF fish, including haemorrhage in the epicardium and myocardium (13.92% and 8.86% of INF fish, respectively), thrombosis of myocardial blood vessels (1.27%), hyaline, hydropic or vacuolar degeneration of cardiomyocytes (62.03%) (Figure 25A), necrosis in the epicardium, myocardium and endocardium (6.33%, 30.38% and 2.53%, respectively), myocardial regeneration (22.78%), as well as infiltration with lymphocytes, macrophages and neutrophils in the epicardium (41.77%, 63.29% and 8.86%, respectively), myocardium (43.04%, 13.92% and 13.92%, respectively), and endocardium (16.46%, 6.33% and 2.53%, respectively) (Figure 25B, C). Thrombosis of the endocardium was observed in three fish (Figure 25A). In two fish, inflammation and necrosis of the endocardium involved the bulboventricular and atrioventricular valves (Figure 25B). Necrosis in the myocardium was observed, particularly at the compact/spongy interface of the ventricular myocardium (Figure 25C).

The spleen index exceeded the median of NH fish in 82.72% of *F. psychrophilum*-INF fish. The circulatory, regressive, progressive, and inflammatory reaction indices were significantly higher in INF fish and exceeded the healthy median in 60.49%, 69.14%, 34.57%, and 77.78% of INF fish, respectively. Significant differences were observed in splenic parenchyma for hyperaemia in circulatory lesions, lymphopaenia in regressive lesions, hyperplasia of the white pulp in

progressive lesions, and activation of RES in inflammatory lesions (Table 12). Several other lesions were observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. The splenic parenchyma and capsule exhibited haemorrhage (27.16% of INF fish), necrosis (11.11% and 4.94%, respectively), and infiltration with macrophages (14.81% and 11.11%, respectively) and neutrophils (13.58% and 7.41%, respectively) along with serous or fibrinous exudate (3.70%). Additionally, activation of MMCs and infiltration with EGCs (18.52%) were observed in the splenic parenchyma (48.15%), and the capsule showed infiltration with lymphocytes (9.88%) (Figure 25D).

In 85.19% of *F. psychrophilum*-INF fish, the kidney index was above the median of NH fish. The circulatory, regressive, and progressive reaction indices were significantly higher in INF fish and exceeded the healthy median in 18.52%, 81.48%, and 56.79% of INF fish, respectively. The difference in the inflammatory index was not significant. Significant differences were observed for hydropic, vacuolar, or hyaline droplet degeneration of renal tubules in regressive lesions and hyperplasia of haematopoietic tissue in progressive lesions (Figure 25E, Table 12). A significant difference was also observed in tubule regeneration. This was the only case in which the mean value was higher in NH fish than in INF fish. Several other lesions were observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. These lesions included haemorrhage (14.81% of INF fish), thrombosis (1.23%), necrosis (12.35%), nephrocalcinosis (2.47%), fibrosis (1.23%), activation of MMCs (34.57%), and infiltration with macrophages, neutrophils and EGCs (11.11%, 8.64% and 18.52%, respectively) along with serous or fibrinous exudate (1.23%) in the haematopoietic tissue, as well as necrosis of the surrounding renal tubules (8.64%) (Figure 25E).

The liver index exceeded the median of NH fish in 67.90% of *F. psychrophilum*-INF fish. The circulatory, regressive, and inflammatory reaction indices were significantly higher in INF fish and exceeded the healthy median in 32.10%, 80.25%, and 51.85% of INF fish, respectively. The difference in progressive index was not significant. Significant differences were observed for haemorrhage in circulatory lesions, decreased hepatocellular vacuolation, micro- or macrovesicular

fatty and/or vacuolar degeneration, and necrosis of hepatocytes in regressive lesions and infiltration with neutrophils in inflammatory lesions (Figure 25F, Table 12). Several other lesions were observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. These lesions included hyperaemia (11.11% of INF fish), thrombosis (1.23%), activation of MMCs (38.27%), liver regeneration (9.88%), fibrosis (3.70%), and infiltration with lymphocytes, macrophages, and EGCs (38.27%, 16.05%, and 4.94%, respectively) (Figure 25F).

None of the histopathological variables analysed for the pancreas showed significant differences between *F. psychrophilum*-INF and NH fish (Table 12). However, several lesions were observed in INF fish, including haemorrhage (13.58% of INF fish), necrosis (13.58%), fibrosis (1.23%), and infiltration with lymphocytes, macrophages, and neutrophils (35.80%, 48.15%, and 16.05%, respectively).

The stomach and pyloric caeca index exceeded the median of NH fish in 83.54% of *F. psychrophilum*-INF fish. The circulatory, regressive, and inflammatory reaction indices were significantly higher in INF fish and exceeded the healthy median in 44.44%, 72.15%, and 54.43% of INF fish, respectively. The difference in progressive index was not significant. Significant differences were observed for intercellular oedema in the mucosa and other tissue layers in circulatory lesions, vacuolar degeneration and necrosis of the gastric glands in regressive lesions, and infiltration of the mucosa with lymphocytes in inflammatory lesions (Table 12). Other lesions were also observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. Lesions observed in the mucosa and other layers included haemorrhage (4.94% and 2.47% of INF fish, respectively), necrosis (7.41% and 4.94%, respectively) and infiltration with macrophages (45.68% and 13.58%, respectively), and neutrophils (4.94% and 6.17%, respectively), as well as atrophy of the gastric glands (5.06%).

The intestine index exceeded the median of NH fish in 44.16% of *F. psychrophilum*-INF fish. The inflammatory reaction index was significantly

higher in INF fish and exceeded the healthy median in 35.06% of INF fish, respectively. The difference in other reaction indices was not significant. Only infiltration of the mucosa with lymphocytes showed a significant difference in inflammatory lesions (Table 12). Several other lesions were observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. Lesions observed in the mucosa and other layers included haemorrhage (2.60% of INF fish for each layer), necrosis (2.60% and 3.90%, respectively), and infiltration with macrophages (18.18% and 3.90%, respectively) and neutrophils (1.30% and 2.60%, respectively).

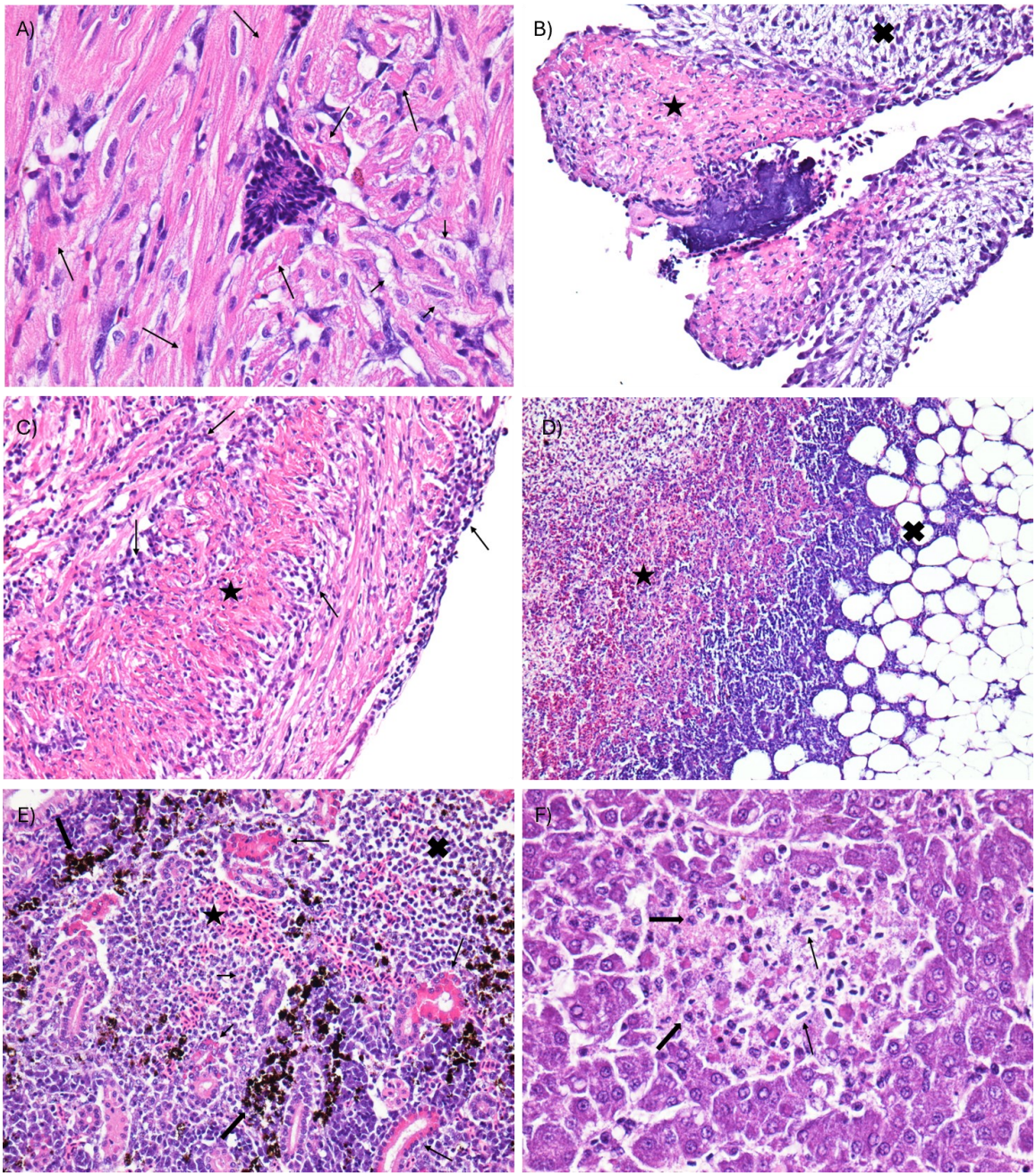


Figure 25: Histopathological lesions in internal organs of fish infected with *Flavobacterium psychrophilum*: A) thrombus adherent to the endocardium overlying the spongy myocardium with mild hyaline (long arrows) and hydropic (short arrows) degeneration (400× magnification); B) necrotising endocarditis of the atrioventricular valves (X) with vegetations (star) infiltrated by neutrophils and lymphocytes (200× magnification); C) necrosis (star) at the compact/spongy interface of the ventricular myocardium and moderate infiltration of epicardium, myocardium and endocardium with lymphocytes and neutrophils (arrows) (HE, 200× magnification); D) necrosis and haemorrhage (star) in the splenic parenchyma with severe lymphocytic and neutrophilic perisplenitis (100× magnification); E) infiltration of kidney haematopoietic tissue with neutrophils (X), haemorrhage (star) and necrosis of tubules (short thin arrow) in the affected area, hyaline droplet degeneration of the surrounding kidney tubules (long thin arrow) and increased number and

size of melanomacrophage centres (thick arrow) (200× magnification); F) small liver necrosis with mild neutrophil (thick arrow) infiltration and erythocytes (thin arrow) indicating haemorrhage; note the lack of hepatocellular vacuolation (400× magnification); haematoxylin and eosin staining

4.3.2.2 *Flavobacterium branchiophilum*

In *F. branchiophilum*, analysis of the histopathological variables showed that none of the histopathological variables analysed showed significant differences between INF and NH fish, except for fusion of the gill filaments (p = 0.010). The *p*-values, means, medians, and standard deviations for all histopathological variables analysed are shown in Table 12. It is important to note that the limited number of POS fish reduced the statistical power of the analysis. Other lesions were also observed in INF fish. However, analysis per fish tank revealed no statistically significant differences. These lesions included haemorrhage and intercellular oedema in the lamellae and filaments, lamellar aneurysms, hydropic or vacuolar degeneration and necrosis of epithelium, hypertrophy and hyperplasia of epithelial cells, hyperplasia of goblet cells, infiltration with lymphocytes, macrophages, and neutrophils in the lamellae and filaments, as well as neutrophils in the gill arch. Few to numerous filamentous bacteria, identified as *F. branchiophilum* with bacteriological investigation, were occasionally observed along the gill epithelium and in the necrotic epithelial tissue (Figure 26).

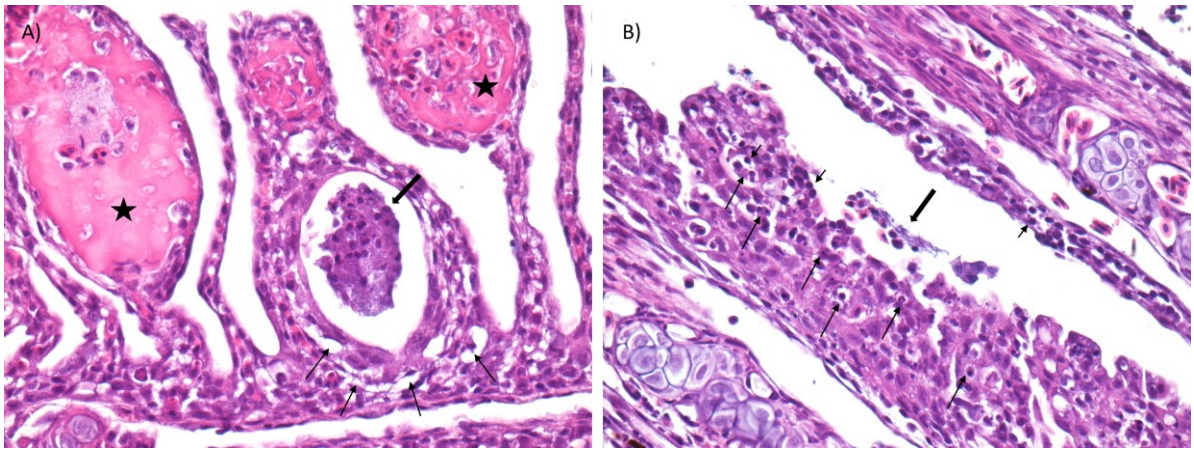


Figure 26: Histopathological lesions in gills of fish infected with *Flavobacterium branchiophilum*: A) a group of *F. branchiophilum* bacteria and sloughed epithelial cells (thick arrow) in a pseudocyst, lamellar aneurysms (star) and hydropic or vacuolar degeneration (thin arrows) of gill epithelium (400× magnification); B) filamentous *F. branchiophilum* bacteria (thick arrow) along the gill

epithelium with hyperplasia and fusion of gill lamellae; the epithelium shows mild lymphocytic infiltration (short thin arrows) and multifocal single-cell necrosis (long thin arrows) (400× magnification); haematoxylin and eosin staining

4.3.2.3 Lesser-known *Flavobacterium* species

Analysis of histopathological variables was only possible for *F. hercynium*, *F. hydatis,* and *F. frigidimaris*. The analysis per sampled organ showed that *F. hercynium*-INF fish had significantly higher score values for skin ulceration (p = 0.035, mean = 0.43/0.17, median = 0.00/0.00, sd = 0.79/0.68) than NH fish, *F. hydatis*-INF fish had significantly higher score values for hypertrophy (p = 0.022, mean = 3.75/0.88, median = 4.00/1.00, sd = 2.06/0.94), and hyperplasia (p = 0.018, mean = 2.00/1.80, median = 1.50/2.00, sd = 1.41/0.90) of gill epithelial cells, while *F. frigidimaris*-INF fish did not show any significantly different lesions. For other lesser-known *Flavobacterium* spp. the statistical power was limited or inadequate to support the statistical analysis of the histopathological lesions. However, in the internal organs that tested POS for *Flavobacterium* spp. some histopathological lesions were observed in fish that were otherwise negative for other identified systemic fish pathogens, including *F. psychrophilum*. In the spleen of a diseased fish from F3 that tested POS for *F. bernardetii,* we observed moderate atrophy of the white pulp, moderate hyperaemia, mildly increased number of MMCs, and infiltration with individual EGCs. Moderate atrophy of the white pulp was also observed in the spleen of an apparently healthy fish from F3 that tested POS for FX18, as well as moderate activation of the RES. In the liver of a diseased fish from the summer sampling in F2 that tested POS for FX3, we observed diffusely severely decreased hepatocellular vacuolation, multifocal moderate microvesicular fatty degeneration, moderately increased number of MMCs, and multifocal moderate lymphocytic hepatitis. Similar lesions were observed in the liver of a diseased fish from the spring sampling in F2 that tested POS for FX24, except for infiltration with neutrophils. In the liver of an apparently healthy fish from the autumn sampling in F2 that tested POS for FX12, we observed diffusely severely decreased hepatocellular vacuolation, individual small areas of necrosis, and multifocal small lymphocyte infiltrates.

4.3.3 Histopathological and cytological investigation of filamentous bacteria

Histopathological examination revealed filamentous bacteria only in the skin and/or gills of 22 fish, but not in the internal organs. In 12 fish (54.6%), few to numerous filamentous bacteria were observed in skin ulcerations, including on the fins and jaws. Two of these fish tested negative in the bacteriological investigation, but the presence of gram-negative filamentous bacteria was confirmed by Gram staining. Other fish tested POS for *F. psychrophilum*. Low to moderate numbers of filamentous bacteria were detected in the gills of 9 fish (41%). Two fish tested POS for *F. branchiophilum* in the bacteriological investigation, while another two tested POS for *F. psychrophilum*. Five fish tested negative, but Gram staining confirmed the presence of Gram-negative filamentous bacteria in two fish, while additional, further tissue sections from three fish used for Gram staining did not contain bacteria. Additionally, filamentous bacteria were observed in both the gills and skin of one fish (4.6%), and bacteriological investigation identified *F. branchiophilum* in the gills and *F. psychrophilum* in the skin.

We did not observe any filamentous or other bacteria in the smears of gill and skin scrapes stained with Giemsa.

4.4 ANTIMICROBIAL SUSCEPTIBILITY

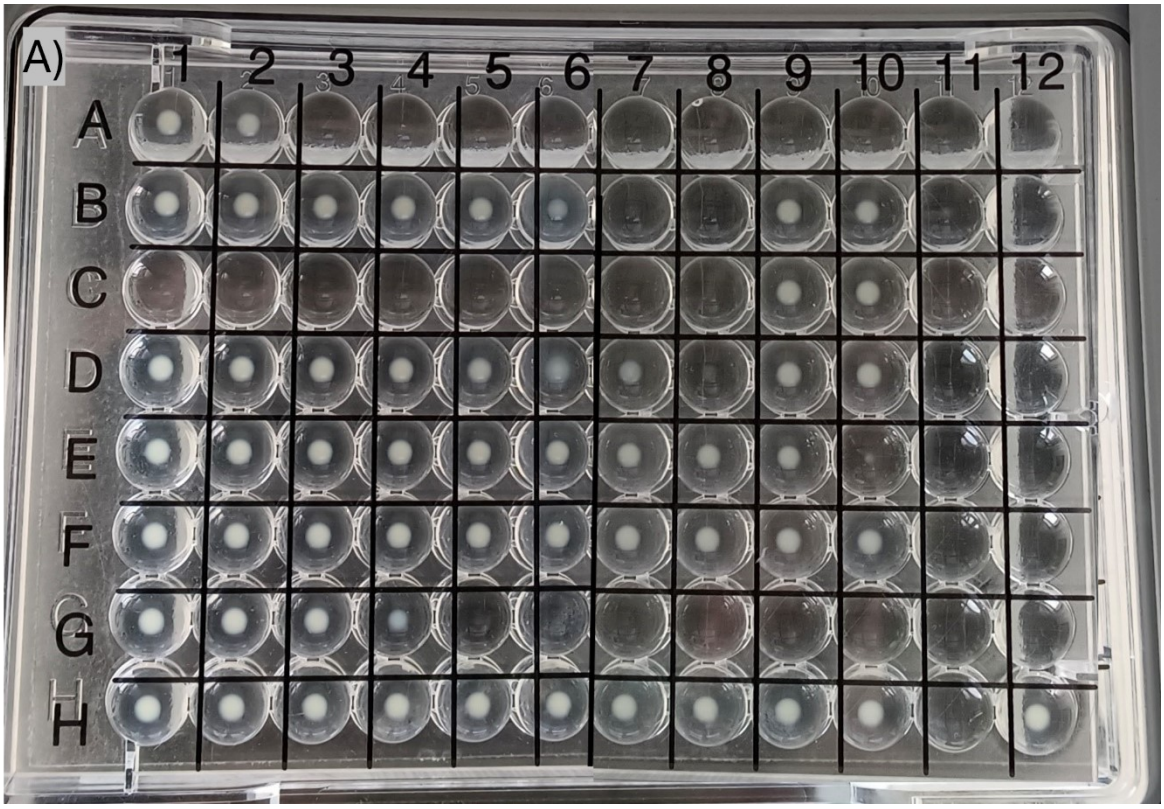
4.4.1 Quality control

The MICs determined for the quality control strain *E. coli* ATCC 25922 were within the acceptable ranges, and the MICs for the reference strain *F. psychrophilum* ATCC 49418^T were below the CO_{WT} value calculated for AMP and the ECVs established for *F. psychrophilum* for ENR, ERY, FLO, OXO, and OXY. In addition, the reference strain grew at the lower end of the concentration range tested for GEN (MIC 0.25 µg/ml) and MER (MIC ≤ 0.008 µg/ml) and at the upper end of the concentration range tested for CTZ (MIC 2 µg/ml) and TRS (MIC > 1/19 µg/ml).

The MICs for the 10 antimicrobials tested against the reference strain *F. psychrophilum* ATCC 49418^T are shown in Appendix 5.

4.4.2 Phenotypic AMR profiles of *Flavobacterium psychrophilum*

The phenotypic AST profiles were determined for 54 *F. psychrophilum* isolates. *F. psychrophilum* isolates grew well in DCAMHB and formed buttons with smooth or rhizoid margins (Figure 27). The distribution of MICs of selected *F. psychrophilum* isolates is shown in Figure 28. The MICs for the individual isolates are shown in Appendix 5.



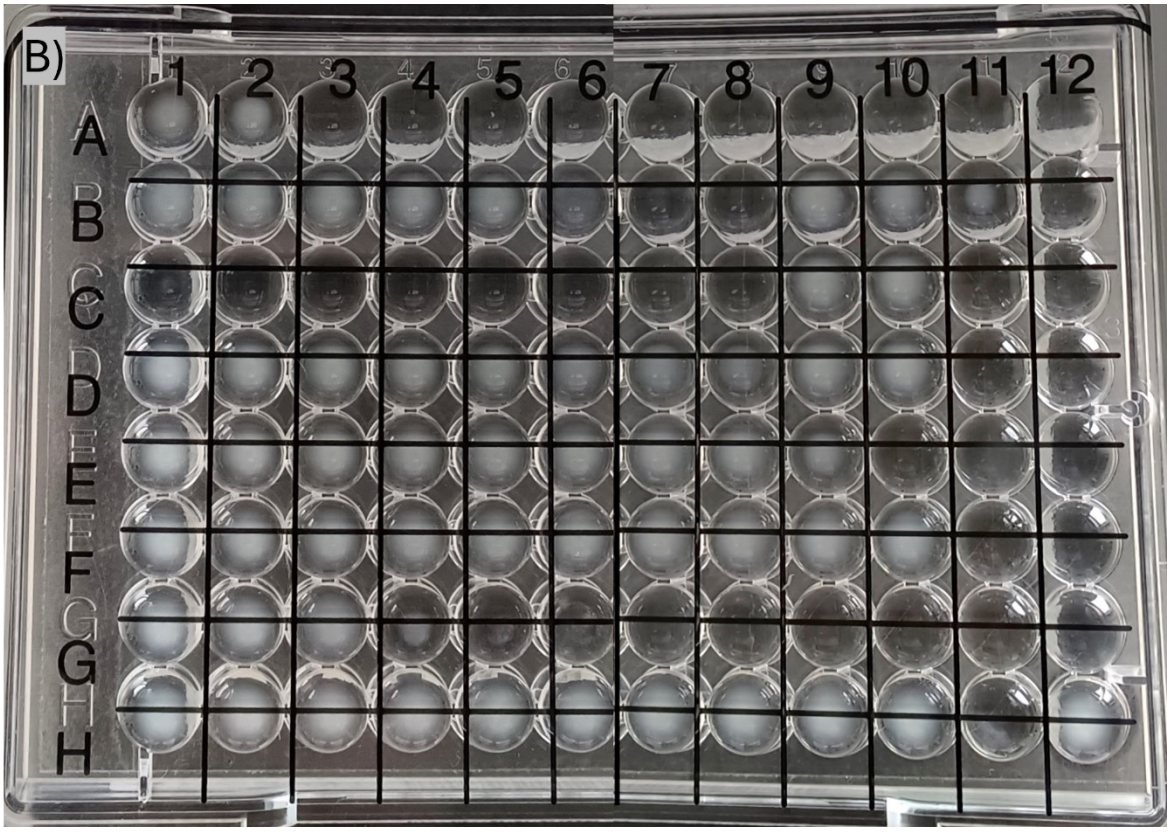


Figure 27: Microtitre plates for two selected *Flavobacterium psychrophilum* isolates. A) Isolate F1824 with smooth margins; B) Isolate F341 with rhizoid margins. For each microtitre plate, two images were merged between columns 6 and 7 so that all 96 wells are clearly visible

For AMP, most isolates exhibited MICs ≤ 0.12 µg/ml, while only two isolates were inhibited by higher concentrations, namely 0.5 and 4 µg/ml. Using the provisional CO_{WT} value of ≤ 0.125 µg/ml, 52 isolates (96.3%) were classified as WT and two isolates (3.7%) as NWT (Figure 28A).

The MICs for CTZ were unimodally distributed. All isolates grew at the upper end of the CTZ concentration range tested, with MICs between 1 and > 4 µg/ml. The calculated provisional CO_{WT} value was ≤ 8 µg/ml, which is the first concentration above the tested range for this antimicrobial (Figure 28B).

The MICs for the two quinolones, ENR and OXO, were bimodally distributed. Using the ECV of ≤ 0.03 µg/ml for ENR, seven isolates (13.0%) were classified as WT and 47 isolates (87.0%) as NWT (Figure 28C). Based on the ECV of ≤ 0.25 µg/ml for OXO, five isolates (9.3%) were classified as WT and 49 isolates (90.7%)

as NWT (Figure 28H). In both cases, the most frequently observed MICs exceeded the highest concentration tested for ENR (0.25 µg/ml) and OXO (1 µg/ml). It was not possible to calculate a valid CO_{WT} value for ENR and OXO due to a high percentage of the MICs being “above-scale”.

The MICs for ERY were unimodally distributed. Using the ECV of ≤ 8 µg/ml and the calculated CO_{WT} value of ≤ 4 µg/ml for ERY, all tested isolates were classified as WT (Figure 28D).

The MICs for FLO showed unimodal distribution. Based on the ECV of ≤ 2 µg/ml and the calculated CO_{WT} value of ≤ 1 µg/ml for FLO, all tested isolates were classified as WT (Figure 28E).

The MICs for GEN were unimodally distributed. All isolates were inhibited by the lower end of the GEN concentration range tested, with MICs between ≤ 0.06 and 0.25 µg/ml. It was not possible to calculate a valid CO_{WT} value due to a high percentage of the MICs being “below-scale” (Figure 28F).

All isolates were inhibited by the lowest tested concentration of MER (MIC ≤ 0.008 µg/ml). It was not possible to calculate a valid CO_{WT} value due to a high percentage of the MICs being “below-scale” (Figure 28G).

The distribution of MICs for OXY was bimodal. Using the ECV of ≤ 0.12 µg/ml for OXY, 10 isolates (18.5%) were classified as WT and 44 isolates (81.5%) as NWT. It was not possible to calculate a valid CO_{WT} value because the calculated standard deviation for the normalised distribution exceeded < 1.2 $\log_2$ µg/ml (Figure 28I).

The distribution of MICs for TRS was variable but appeared to be bimodal. Most isolates (88.9%) were inhibited by the upper end of the TRS concentration range tested (MICs ≥ 0.25/4.75 µg/ml), while only six isolates (11.1%) were inhibited by the lower end of the TRS concentration range tested, with MICs between 0.015 and 0.12 µg/ml. The most frequently observed MIC exceeded the highest

concentration of TRS (1/19 µg/ml) tested. It was not possible to calculate a valid CO_{WT} value because a high percentage of the MICs were “above-scale”, and the calculated standard deviation for the normalised distribution exceeded < 1.2 log_2 µg/ml (Figure 28J).

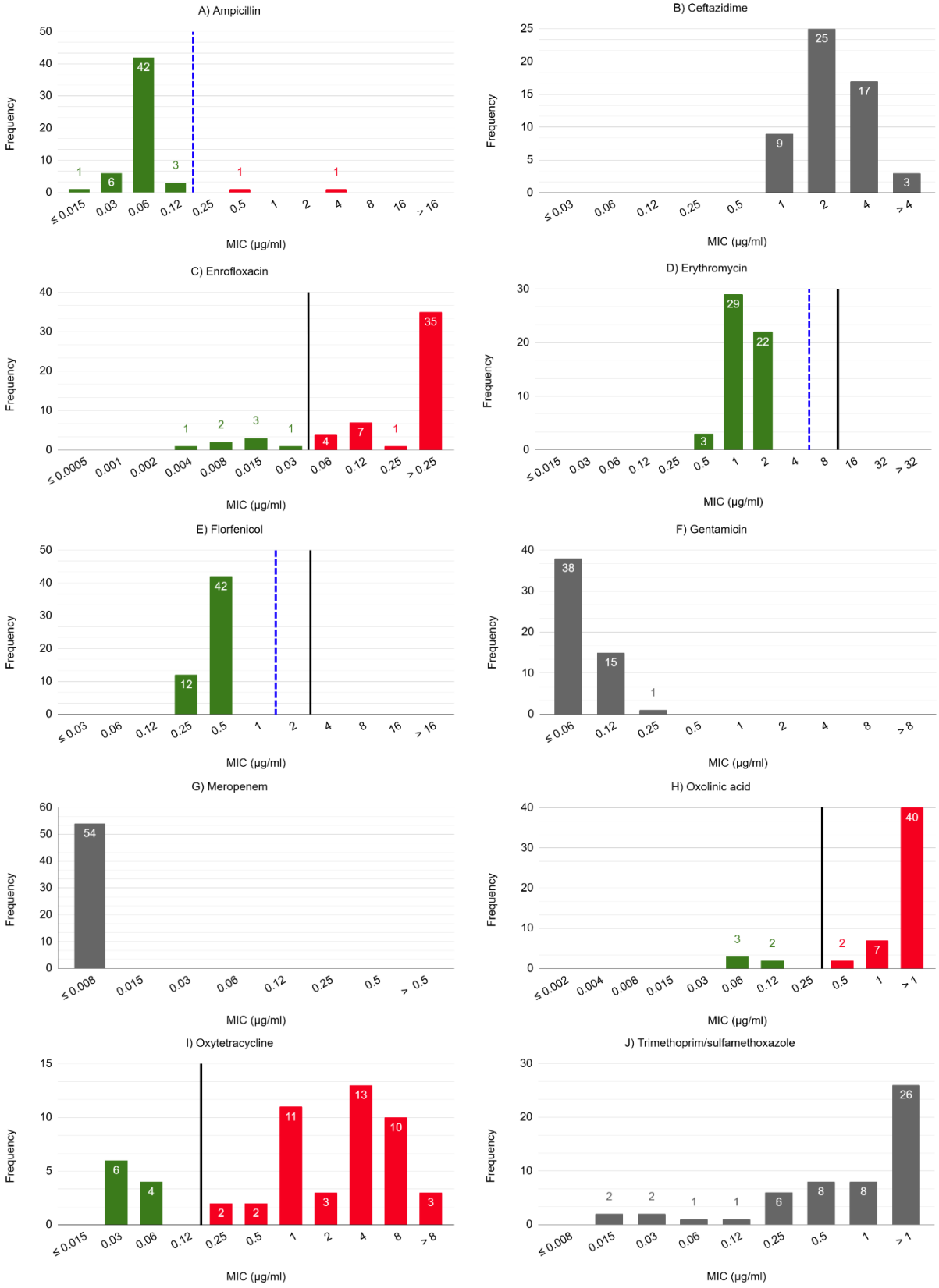
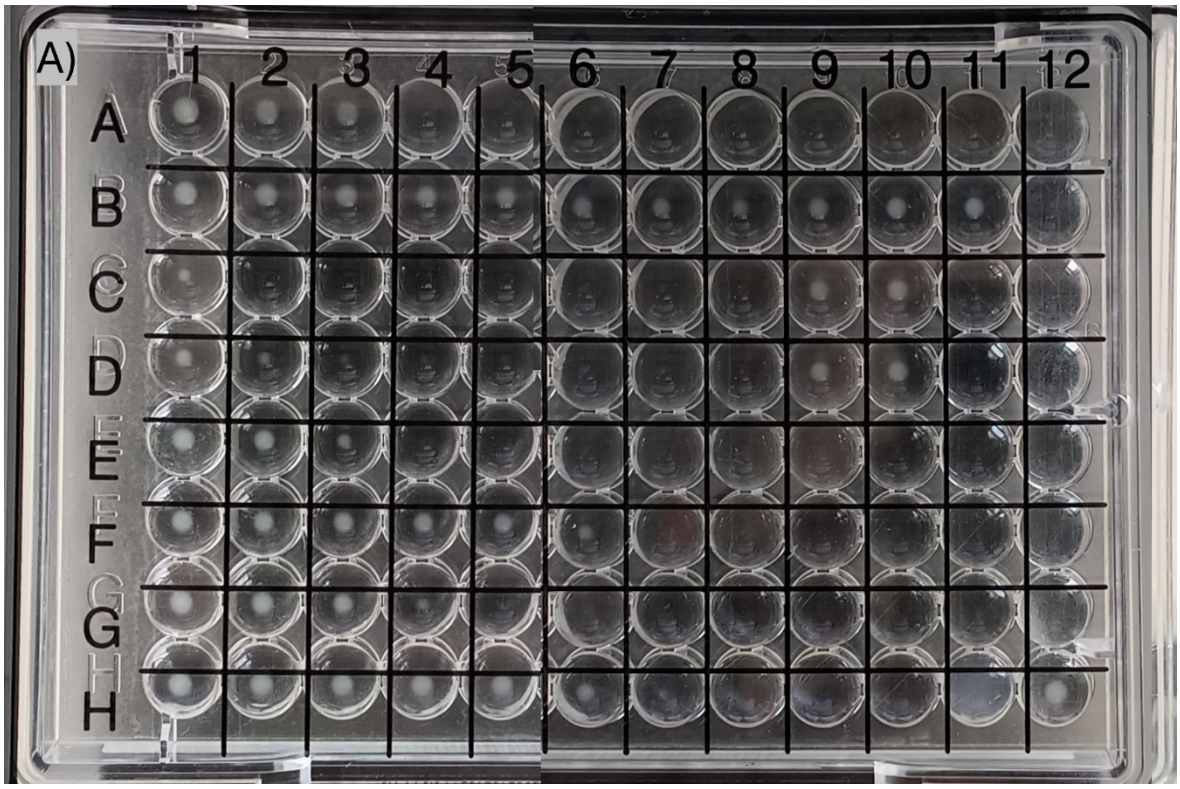


Figure 28: The distribution of minimum inhibitory concentrations (MICs) of 54 *Flavobacterium psychrophilum* isolates for 10 antimicrobials (A-J) determined using the broth microdilution method. The tested concentration range (µg/ml) is presented on the x-axis and the number of isolates on

the y-axis. The solid black and dashed blue vertical lines represent the epidemiological cut-off values (ECVs) and the provisional epidemiological cut-off values for wild type (CO_{WT}) for *F. psychrophilum*, respectively. Where possible, the green bars represent the wild type (WT) isolates, and the red bars represent the non-wild type (NWT) isolates

4.4.3 Phenotypic AMR profiles of *Flavobacterium branchiophilum*

The phenotypic AST profiles were determined for eight *F. branchiophilum* isolates. In general, the *F. branchiophilum* isolates grew better in the AO broth than in the DCAMHB+C, as shown by the size of the growth buttons (Figure 29). Nonetheless, the *F. branchiophilum* isolates showed sufficient growth in DCAMHB+C to allow determination of MICs for the tested antimicrobials. Considering the extent of possible technical variation (±1 dilution) in standardised AST, only minor differences were observed between the two media (Figure 30, Appendix 6). The addition of 5% foetal bovine serum or yeast extract did not improve the growth of *F. branchiophilum*.



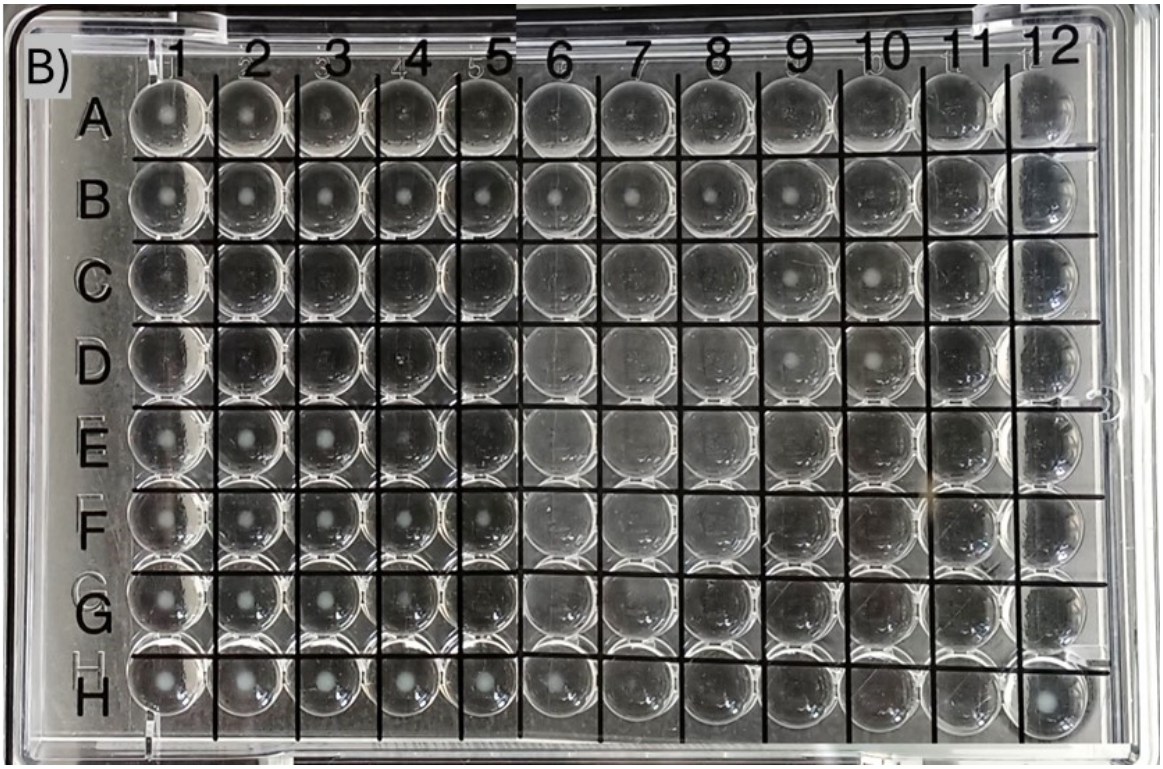


Figure 29: Microtitre plates for two selected *Flavobacterium branchiophilum* isolates. A) Isolate F1517 on Anacker and Ordal (AO) broth; B) Isolate F1628 on diluted cation-adjusted Mueller-Hinton broth supplemented with additional cations (DCAMHB+C). For each microtitre plate, two images were merged between columns 5 and 6 so that all 96 wells are clearly visible

The MICs for all 10 antimicrobials tested showed a unimodal distribution. The MICs determined by AST using the DCAMHB+C were 0.06–0.2 µg/ml for AMP, > 4 µg/ml for CTZ, 0.015–0.06 µg/ml for ENR, 1–2 µg/ml for ERY, 0.5–1 µg/ml for FLO, ≤ 0.06–0.25 µg/ml for GEN, ≤ 0.008 µg/ml for MER, 0.12–0.25 µg/ml for OXO, 0.25 µg/ml for OXY and 0.015–0.03 µg/ml for TRS. Notably, when using DCAMHB+C, the MICs for OXY for all isolates and ENR for one isolate were the first concentration above the ECV_FP. The remaining isolates had MICs just below the ECV_FP for ENR (0.015–0.03 µg/ml), while all isolates had MICs just below the ECV_FP for OXO (0.12–0.25 µg/ml), and the CO_{WT} calculated for *F. psychrophilum* for AMP (0.06–0.12 µg/ml). In addition, all MICs exceeded the highest tested concentration of CTZ and the lowest tested concentration of MER. The MICs for ERY (1–2 µg/ml) and FLO (0.5–1 µg/ml) were in the middle of the tested concentration range and below the ECV_FP. All isolates grew at the lower end of the tested concentration range for GEN (MICs ≤ 0.06–0.25 µg/ml) and TRS

(MICs 0.015–0.03 µg/ml). The distribution of MICs of eight *F. branchiophilum* isolates tested using both growth media is shown in Figure 30. The MICs for the individual isolates are shown in Appendix 6.

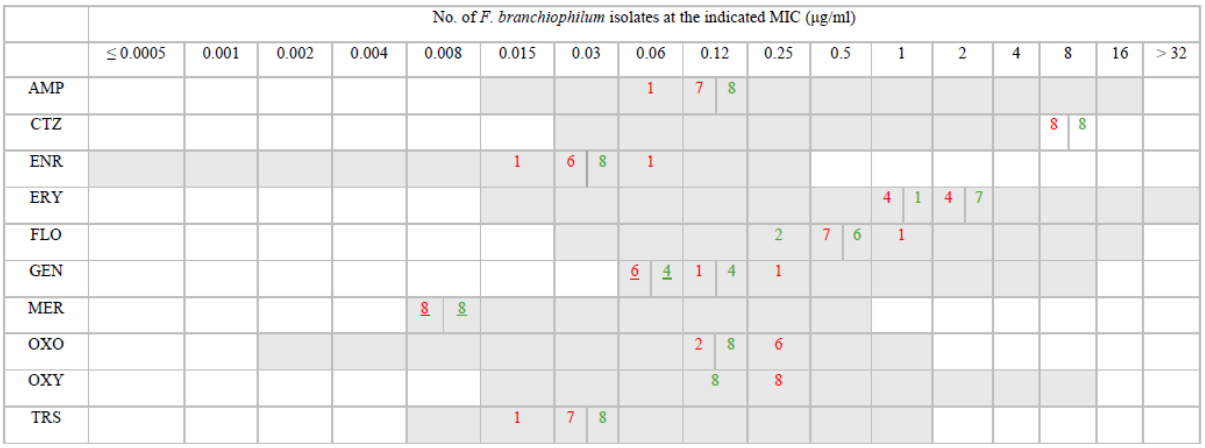


Figure 30: The distribution of minimum inhibitory concentrations (MICs) of eight *Flavobacterium branchiophilum* isolates determined using the broth microdilution method for AMP, ampicillin; CTZ, ceftazidime; ENR, enrofloxacin; ERY, erythromycin; FLO, florfenicol; GEN, gentamicin; MER, meropenem; OXO, oxolinic acid; OXY, oxytetracycline; TRS, trimethoprim/sulfamethoxazole. The range of dilutions tested (µg/ml) for each antimicrobial is shaded gray. MICs higher than the highest concentration tested are given as the first concentration above the tested range, and MICs equal to or lower than the lowest concentration tested are underlined. The red and green numbers represent the MICs determined using diluted cation-adjusted Mueller-Hinton broth with additional cations (DCAMHB+C) and Anacker and Ordal (AO) broth, respectively

4.4.4 Phenotypic AMR profiles of lesser-known *Flavobacterium* species

The MICs for both *F. bernardetii* isolates exceeded the highest concentration of CTZ tested. For TRS, the MIC for one isolate exceeded the tested concentration range, while the MIC for the other isolate was at the highest concentration tested (1/19 µg/ml). Both *F. bernardetii* isolates grew in the middle of the tested AMP concentration range (MICs 0.25 and 0.5 µg/ml) and exceeded the CO_{WT} value calculated for *F. psychrophilum*. Additionally, isolates had MICs below the ECV_FP for ENR (0.008 and 0.015 µg/ml), ERY (0.25 µg/ml), FLO (1 µg/ml), OXO (0.12 µg/ml), and OXY (0.12 µg/ml). Isolates also grew at the lower end of the tested concentration range for GEN (MICs 0.12 µg/ml) and MER (MICs ≤ 0.008 and 0.015 µg/ml). The MICs for individual isolates are shown in Appendix 6.

The MICs for both *F. branchiarum* isolates exceeded the highest tested concentrations of AMP, CTZ, ENR, and TRS. Isolates also exhibited MICs at the upper end or exceeding the concentration ranges tested for ERY (16 µg/ml), FLO (16 and > 16 µg/ml), GEN (8 µg/ml), OXO (1 and > 1 µg/ml), and OXY (2 and 4 µg/ml). MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, FLO, OXO, and OXY. Both isolates grew in the middle of the tested MER concentration range (MICs 0.06 µg/ml). The MICs for individual isolates are shown in Appendix 5.

Both *F. celericrescens* isolates exhibited MICs at the upper end or exceeding the concentration ranges tested for CTZ (> 4 µg/ml) and TRS (0.5/9.5 µg/ml). They also grew in the middle of the tested AMP concentration range, with MICs (0.25 µg/ml) at the first concentration above the CO_{WT} value calculated for *F. psychrophilum*. Isolates also exhibited MICs at the first concentration above the ECV_FP for ENR (0.06 µg/ml) and OXO (0.5 µg/ml) and the first concentration below the ECV_FP for OXY (0.12 µg/ml). Additionally, the MICs for ERY (0.12–4 µg/ml) and FLO (2 µg/ml) were below the ECV_FP for these antimicrobials. Isolates grew at the lower end of the tested GEN concentration range (MICs 0.12 µg/ml), and their growth was inhibited by the lowest tested concentration of MER. The MICs for individual isolates are shown in Appendix 6.

The MICs for both *F. collinsii* isolates exceeded the highest tested concentrations of AMP, CTZ, ERY, MER, and TRS. Isolates also exhibited MICs at the upper end or exceeding the concentration ranges tested for ENR (0.25 and > 0.25 µg/ml), FLO (8 and > 16 µg/ml), GEN (4 and > 8 µg/ml), OXO (0.5 and 1 µg/ml), and OXY (4 and > 8 µg/ml). The MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, FLO, OXO, and OXY. The MICs for individual isolates are shown in Appendix 5.

The *F. difficile* isolate exhibited MICs exceeding the highest tested concentrations of CTZ and TRS. Additionally, the MIC for ENR (0.06 µg/ml) was the first concentration above the ECV_FP. The isolate had MICs below the CO_{WT} value

calculated for *F. psychrophilum* for AMP (0.12 µg/ml) and below the ECV_FP for ERY (0.12 µg/ml), FLO (0.5 µg/ml), OXO (0.25 µg/ml), and OXY (0.12 µg/ml). Growth of the isolate was inhibited by the lowest tested concentrations of GEN (≤ 0.06 µg/ml) and MER (≤ 0.008 µg/ml). The MICs are shown in Appendix 6.

The MIC for *F. erciyesense* exceeded the highest tested concentration of CTZ, and the isolate also grew at the upper end of the tested concentration ranges for GEN (MIC 2 µg/ml) and TRS (MIC 0.5/9.5 µg/ml). Additionally, the MICs for AMP (0.25 µg/ml) and ENR (0.06 µg/ml) were the first concentrations above the CO_{WT} value calculated for *F. psychrophilum* and ECV_FP, respectively. The isolate had MICs below the ECV_FP for ERY (1 µg/ml), FLO (1 µg/ml), OXO (0.25 µg/ml), and OXY (0.06 µg/ml). Growth of the isolate was inhibited by the lowest tested concentration of MER (≤ 0.008 µg/ml). The MICs are shown in Appendix 5.

The MICs for the *F. hercynium* isolate exceeded the highest tested concentrations of CTZ, ENR, ERY, OXO, and TRS. The isolate also grew at the upper end of the tested concentration ranges for AMP (MIC 16 µg/ml), FLO (MIC 4 µg/ml), GEN (MIC 4 µg/ml), MER (MIC 0.25 µg/ml), and OXY (MIC 2 µg/ml). The MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, FLO, OXO, and OXY. The MICs are shown in Appendix 5.

F. oncorhynchi isolate exhibited MICs exceeding the highest tested concentrations of CTZ, ERY, and TRS. Additionally, the isolate grew at the upper end of the tested concentration ranges for AMP (MIC 16 µg/ml), ENR (MIC 0.25 µg/ml), FLO (MIC 8 µg/ml), GEN (MIC 8 µg/ml), MER (MIC 0.5 µg/ml), OXO (MIC 0.5 µg/ml), and OXY (MIC 2 µg/ml). The MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, FLO, OXO, and OXY. The MICs are shown in Appendix 5.

The MICs for the *F. piscis* isolate exceeded the highest tested concentrations of CTZ, ERY, and TRS. The isolate also exhibited MICs at the upper end of the tested concentration ranges for AMP (16 µg/ml), ENR (0.25 µg/ml), FLO (8 µg/ml),

GEN (4 µg/ml), MER (0.5 µg/ml), OXO (1 µg/ml), and OXY (4 µg/ml). The MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, FLO, OXO, and OXY. The MICs are shown in Appendix 5.

The MIC for *F. tiangeerense* exceeded the highest tested concentration of TRS, and the isolate also grew at the upper end of the tested concentration ranges for AMP (MIC 2 µg/ml), CTZ (MIC 4 µg/ml), and GEN (MIC 2 µg/ml). The MIC for AMP was above the CO_{WT} value calculated for *F. psychrophilum*. The isolate had MICs below the ECV_FP for ENR (0.03 µg/ml), ERY (0.5 µg/ml), FLO (1 µg/ml), OXO (0.25 µg/ml), and OXY (0.12 µg/ml). Growth of the isolate was inhibited by the lowest tested concentration of MER (≤ 0.008 µg/ml). The MICs are shown in Appendix 6.

F. tructae isolate exhibited MICs exceeding the highest tested concentrations of AMP, CTZ, FLO, and TRS. Additionally, the isolate exhibited MICs at the upper end of the tested concentration ranges for ENR (0.12 µg/ml), ERY (32 µg/ml), GEN (4 µg/ml), MER (0.5 µg/ml), and OXY (8 µg/ml). The MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, FLO, and OXY. The MIC for OXO (0.25 µg/ml) was the first dilution below the ECV_FP. The MICs are shown in Appendix 5.

The MICs for the three FX2 isolates exceeded the highest tested concentration of CTZ. Isolates also exhibited MICs at the upper end and exceeding the concentration range tested for OXY (4 and > 8 µg/ml) and TRS (0.5/9.5 and > 1/19 µg/ml). Isolates grew in the middle of the tested AMP concentration range, with MICs (0.5 and 1 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*. Additionally, the isolates had MICs below the ECV_FP for ENR (0.015 and 0.03 µg/ml), ERY (0.12–0.5 µg/ml), FLO (0.5 and 2 µg/ml), and OXO (0.12 and 0.25 µg/ml). Isolates also grew at the lower end of the tested concentration ranges for GEN (MICs 0.25 µg/ml) and MER (MICs ≤ 0.008 and 0.03 µg/ml), except for one isolate that had an MIC (1 µg/ml) in the middle of the tested GEN concentration range. The MICs for individual isolates are shown in Appendix 6.

The three FX3 isolates exhibited MICs exceeding the highest tested concentration of CTZ and at the upper end of the concentration range tested for TRS (0.5/9.5 and 1/19 µg/ml). Isolates grew in the middle of the tested AMP concentration range, with MICs (0.5 and 1 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*. Additionally, the isolates had MICs below the ECV_FP for ENR (0.008 and 0.015 µg/ml), ERY (0.25 µg/ml), FLO (1 µg/ml), OXO (0.12 and 0.25 µg/ml), and OXY (0.06 and 0.12 µg/ml). Isolates also grew at the lower end of the tested concentration ranges for GEN (MICs ≤ 0.06 and 0.12 µg/ml) and MER (MICs 0.015 and 0.03 µg/ml). The MICs for individual isolates are shown in Appendix 6.

The MICs for the three FX5 isolates exceeded the highest tested concentrations of CTZ and TRS. Two isolates also grew in the middle of the tested AMP concentration range, with MICs (0.25 and 1 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*, while the third isolate had the MIC (0.12 µg/ml) at the first concentration below the CO_{WT} value. Additionally, the isolates had MICs below the ECV_FP for ENR (0.015 and 0.03 µg/ml), ERY (0.25–1 µg/ml), FLO (1 and 2 µg/ml), OXO (0.25 µg/ml), and OXY (0.12 µg/ml), except for one isolate with an MIC at the first concentration (0.25 µg/ml) above the ECV_FP for OXY. Isolates also grew at the lower end of the tested concentration ranges for GEN (MICs 0.12 and 0.25 µg/ml) and MER (MICs ≤ 0.008 and 0.015 µg/ml). The MICs for individual isolates are shown in Appendix 6.

Both FX9 isolates exhibited MICs at the upper end or exceeding the concentration ranges tested for CTZ (> 4 µg/ml) and TRS (1/19 and > 1/19 µg/ml). One isolate also grew in the middle of the tested AMP concentration range, with an MIC (0.25 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*, while the other isolate had an MIC at the first concentration (0.12 µg/ml) below the CO_{WT} value. Both isolates grew at the upper end of the tested concentration ranges for ENR (MICs 0.06 and 0.12 µg/ml) and OXO (MICs 0.5 µg/ml), exceeding the ECV_FP for these antimicrobials. Additionally, the isolates had MICs below the ECV_FP for ERY (0.5 µg/ml), FLO (0.5 and 1 µg/ml), and OXY (0.3 µg/ml). Both

isolates grew in the middle of the tested GEN concentration range (MICs 0.5 and 1 µg/ml), and their growth was inhibited by the lowest tested concentration of MER. The MICs for individual isolates are shown in Appendix 5.

Both FX10 isolates exhibited MICs exceeding the concentration range for CTZ and at the upper end of the concentration range tested for TRS (1/19 µg/ml). Isolates also grew in the middle of the tested AMP concentration range, with MICs (0.25 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*. Both isolates grew at the upper end of the tested concentration range for ENR (MICs 0.06 µg/ml), exceeding the ECV_FP. One isolate grew at the highest tested concentration of OXY (MIC 8 µg/ml), while the other exhibited an MIC (0.06 µg/ml) below the ECV_FP. Additionally, the isolates had MICs below the ECV_FP for ERY (1 µg/ml), FLO (1 µg/ml), and OXO (0.12 µg/ml). Both isolates exhibited MICs in the middle of the tested GEN concentration range (0.5 µg/ml), and their growth was inhibited by the lowest tested concentration of MER. The MICs for individual isolates are shown in Appendix 5.

All six FX15 isolates exhibited MICs at the upper end or exceeding the concentration ranges tested for CTZ (> 4 µg/ml) and TRS (from 0.25/4.75 to > 1/19 µg/ml). They also grew in the middle of the tested AMP concentration range, with MICs (0.5–1 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*, except for one isolate with an MIC at the first concentration (0.12 µg/ml) below the CO_{WT} value. All isolates grew at the upper end of the tested ENR concentration range (MICs 0.06–0.25 µg/ml), exceeding the ECV_FP. Two isolates had MICs at the first concentration (0.5 µg/ml) above the ECV_FP for OXO, while the other isolates exhibited MICs just below (0.12–0.25 µg/ml) this value. Additionally, the MICs for ERY (4–8 µg/ml), FLO (1–2 µg/ml), and OXY (0.12 µg/ml) were just below the ECV_FP for these antimicrobials. All isolates grew in the middle of the tested GEN concentration range (MICs 0.5–2 µg/ml) and at the lower end of the tested MER concentration range (MICs 0.015–0.03 µg/ml). The MICs for individual isolates are shown in Appendix 5.

Both FX18 isolates exhibited MICs exceeding the concentration range for CTZ and at the upper end of the concentration range tested for TRS (0.5/9.5 µg/ml). One isolate also grew in the middle of the tested AMP concentration range, with an MIC (0.5 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*, while the other isolate had an MIC (0.06 µg/ml) below this value. Additionally, the isolates had MICs below the ECV_FP for ENR (0.004 and 0.008 µg/ml), ERY (0.12 and 0.25 µg/ml), FLO (1 µg/ml), OXO (0.06 and 0.12 µg/ml), and OXY (0.12 µg/ml). Both isolates grew at the lower end of the tested concentration ranges for GEN (MICs 0.12 and 0.25 µg/ml) and MER (MICs ≤ 0.008 and 0.015 µg/ml). The MICs for individual isolates are shown in Appendix 6.

The two FX23 isolates had MICs exceeding the highest tested concentrations of CTZ, ERY, and TRS. Additionally, the isolates exhibited MICs at the upper end of the tested concentration ranges for AMP (8 and 16 µg/ml), ENR (0.25 µg/ml), FLO (4 and 16 µg/ml), GEN (8 µg/ml), MER (0.25 and 0.5 µg/ml), OXO (1 µg/ml), and OXY (4 µg/ml). The MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, FLO, OXO, and OXY. The MICs for individual isolates are shown in Appendix 5.

Both FX27 isolates exhibited MICs exceeding the tested concentration ranges for CTZ and TRS. They also grew in the middle of the tested AMP concentration range, with MICs (1 and 2 µg/ml) exceeding the CO_{WT} value calculated for *F. psychrophilum*, as well as in the middle of the tested concentration ranges for GEN (MICs 0.5 and 1 µg/ml) and MER (MICs 0.06 and 0.12 µg/ml). Additionally, the isolates had MICs below the ECV_FP for ENR (0.03 µg/ml), ERY (0.5 and 4 µg/ml), FLO (1 µg/ml), OXO (0.12 µg/ml), and OXY (0.06 µg/ml). The MICs for individual isolates are shown in Appendix 5.

The two FX32 isolates exhibited MICs exceeding the concentration range for CTZ and at the upper end of the concentration range tested for TRS (0.25/4.75 and 0.5/9.5 µg/ml). Isolates also grew in the middle of the tested concentration ranges for AMP (MICs 0.25 µg/ml) and GEN (MICs 0.5 µg/ml). The MICs for AMP were the first concentration above the CO_{WT} value calculated for *F. psychrophilum*. One

isolate exhibited an MIC (0.06 µg/ml) at the first concentration above, and the other an MIC (0.03 µg/ml) at the first concentration below the ECV_FP for ENR. Additionally, the isolates had MICs below the ECV_FP for ERY (0.25 and 0.5 µg/ml), FLO (0.5 µg/ml), OXO (0.12 µg/ml), and OXY (0.03 µg/ml). Both isolates were inhibited by the lowest concentration of MER tested. The MICs for individual isolates are shown in Appendix 5.

Both FX36 isolates had MICs exceeding the highest tested concentrations of CTZ and TRS. Additionally, the isolates exhibited MICs at the upper end or exceeding the tested concentration ranges for AMP (4 µg/ml), ENR (0.12 µg/ml), ERY (16 and 32 µg/ml), GEN (2 and 4 µg/ml), MER (0.12 µg/ml), OXO (1 and > 1 µg/ml), and OXY (0.5 and 1 µg/ml). The MICs exceeded the CO_{WT} value calculated for *F. psychrophilum* for AMP and the ECV_FP for ENR, ERY, OXO, and OXY. The MICs for FLO (2 µg/ml) were the first dilution below the ECV_FP for this antimicrobial. The MICs for individual isolates are shown in Appendix 5.

4.4.5 Genotypic AMR profiles of *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* isolates

Seven *F. psychrophilum* isolates selected for WGS represent six different phenotypic resistotypes based on AST for AMP, ENR, ERY, FLO, OXO, and OXY. Resistotype 4, which includes isolates that were NWT for ENR, OXO, and OXY, was the most prevalent (66.7%). WGS confirmed the presence of a T83A or T83V substitution in GyrA in five of seven sequenced *F. psychrophilum* isolates. All five isolates with this substitution were classified as NWT, and the two isolates without substitution were classified as WT for OXO. The presence of this substitution did not influence the susceptibility of *F. psychrophilum* to ENR (Table 13). No AMR genes or other AMR-associated mutations were detected in the analyzed genomes of *F. psychrophilum* and *F. branchiophilum* isolates. All *F. psychrophilum* isolates harboured 1–3 small plasmids, but none encoded AMR genes. No plasmids were found in the *F. branchiophilum* isolates.

Table 13: Seven *Flavobacterium psychrophilum* isolates selected for whole-genome sequencing, representing six different resistotypes based on phenotypic antimicrobial susceptibility testing for ampicillin (AMP), enrofloxacin (ENR), erythromycin (ERY), florfenicol (FLO), oxolinic acid (OXO), and oxytetracycline (OXY); GyrA substitution associated with resistance to quinolones is also shown

R	No.	%	Isolate	AMP	ENR	ERY	FLO	OXO	OXY	GyrA substitution
1	1	1.8	F88	0.06	0.015	1	0.25	0.12	0.06	Absent
2	2	3.7	F1186	4	> 0.25	1	0.5	**> 1**	1	T83V
3	9	16.7	F1187	0.06	0.12	1	0.5	**> 1**	0.06	T83A
4	36	66.7	F1519	0.06	> 0.25	1	0.5	**> 1**	8	T83V
5	4	7.4	F1719	0.03	0.008	1	0.5	0.06	0.5	Absent
6	2	3.7	F1516	0.03	0.03	0.5	0.25	**0.5**	1	T83A
6	2	3.7	F621	≤ 0.015	0.015	1	0.25	**0.5**	0.25	T83A

R, resistotype; No., number of isolates (N = 54) exhibiting this resistotype; %, percentage of isolates exhibiting this resistotype. The minimum inhibitory concentrations (MICs; in µg/ml) above the epidemiological cut-off value (ECV) or the epidemiological cut-off value for wild type (CO_{WT}) are marked in red, and the MICs below are marked in green. Five of the seven *F. psychrophilum* isolates had MICs for OXO above the ECV and also had a corresponding T83A or T83V substitution in GyrA; these MICs are highlighted in bold and underlined. The remaining two isolates had no such substitution and had MICs for OXO below the ECV.

4.5 WATER QUALITY PARAMETERS

Analysis of the water quality parameters showed that the water temperature in all sampled fish tanks ranged between 6.6 and 15.8 °C, dissolved oxygen between 7.28 and 12.11 mg/l, and pH between 7.45 and 8.42. Wilcoxon signed-rank tests showed that water temperature consistently increased by an average of 0.10 °C (p = 0.0014) across 26 paired inlet-outlet measurements, while dissolved oxygen and pH consistently decreased by an average of 0.74 mg/l (p < 0.00001) and 0.061 units (p < 0.00001), respectively, as the water passed through the fish tanks. Detailed information on the water quality parameters for each sampled fish tank can be found in Table 14.

Table 14: Measurements of water temperature (T), pH, and dissolved oxygen (O) at the inlet and outlet of the sampled fish tanks for each longitudinal (LONG) and individual (IND) sampling in fish farms F1 to F4

SAMPLING	FISH TANK	T inlet (°C)	T outlet (°C)	pH inlet	pH outlet	O inlet (mg/l)	O outlet (mg/l)
LONG1 – F1 AUTUMN	1	9.4	9.4	7.625	7.595	10.53	9.46
	2	9.5	9.5	7.740	7.615	10.70	9.93
	3	9.4	9.4	7.623	7.636	9.70	9.25
LONG2 – F2 AUTUMN	1	12.6	12.6	8.386	8.358	9.45	9.29
	2	12.8	12.9	8.351	8.283	9.69	9.02
	3	13.0	13.1	8.336	8.310	10.24	9.95
LONG3 – F1 WINTER	1	9.3	9.3	7.564	7.445	10.97	8.59
	2	9.3	9.3	7.758	7.737	10.95	10.68
	3	9.3	9.3	7.545	7.508	7.55	7.51
LONG4 – F2 WINTER	1	8.4	8.4	8.273	8.255	11.59	11.39
	2	8.2	8.2	8.276	8.265	11.94	11.60
	3	8.0	8.0	8.283	8.265	12.11	11.77
LONG5 – F1 SPRING	1	9.6	9.7	7.738	7.633	10.17	8.82
	2	9.4	9.6	7.803	7.721	11.17	9.53
	3	9.4	9.5	7.599	7.589	10.25	10.04
LONG6 – F2 SPRING	1	11.9	11.9	8.138	8.112	10.55	10.07
	2	11.8	12.2	8.232	8.094	11.18	10.10
	3	11.4	11.5	8.059	8.011	11.14	10.45
LONG7 – F1 SUMMER	1	9.8	10.4	7.625	7.485	10.45	7.89
	2	10.4	10.7	7.519	7.503	8.48	8.41
	3	9.7	9.7	7.544	7.485	8.57	7.28
LONG8 – F2 SUMMER	1	14.7	14.8	8.137	8.090	10.43	10.22
	2	15.1	15.1	8.045	7.883	9.79	9.43
	3	15.6	15.8	8.176	8.118	10.08	9.58
IND1 – F3	1	6.6	6.6	8.316	8.162	10.36	9.23
IND2 – F4	1	14.0	14.2	8.420	8.372	9.98	9.32

The Kruskal–Wallis tests showed that the water quality parameters (temperature, pH, and dissolved oxygen at both the inlet and outlet) showed significant differences between the seasons. Water temperature ($p < 0.001$) was highest in summer (mean 13.0 °C) and lowest in winter (mean 8.8 °C), with autumn and spring intermediate. Dissolved oxygen ($p < 0.001$) showed the opposite pattern to temperature and was lowest in summer (mean 9.75 mg/l) and highest in winter (mean 10.85 mg/l). The pH varied only modestly and was slightly alkaline throughout the year, but still showed significant differences between the seasons ($p = 0.004$), being highest in spring (mean 8.06) and lowest in winter (mean 7.95). Kruskal–Wallis tests also showed that all measured water quality parameters

showed significant differences between the four fish farms (p < 0.001). F1 had consistently cold water (approximately 9.5 °C) with the lowest pH (approximately 7.61), F4 the warmest (approximately 14 °C), and most alkaline water (approximately 8.4), F3 the coldest (approximately 6.6 °C) but similarly alkaline water (approximately 8.24), and F2 with an intermediate water temperature (about 12 °C) and the highest dissolved oxygen (approximately 10.45 mg/l). Almost all pairwise Dunn comparisons remained after Holm adjustment (p ≤ 0.05), underscoring that each fish farm has a distinct physico-chemical profile. Additionally, most water quality parameters showed significant differences between samplings, including temperature at the inlet (Kruskal–Wallis, p < 0.01) and outlet (ANOVA, p < 0.01), pH at the inlet (ANOVA, p < 0.01) and outlet (Kruskal–Wallis, p < 0.01), and dissolved oxygen at the outlet (ANOVA, p < 0.01), except for dissolved oxygen at the inlet (Kruskal–Wallis, p = 0.05). The mean water quality parameters per sampling are shown in Figure 31.

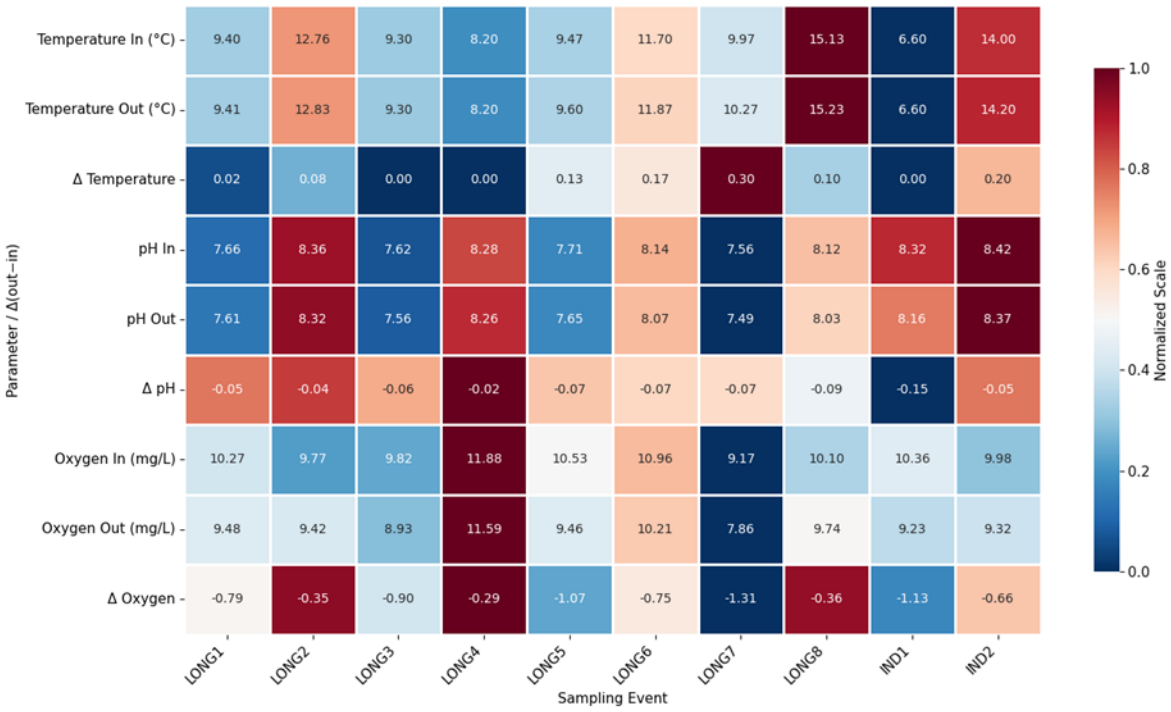


Figure 31: Mean water quality parameters per sampling; heatmap with normalised scale indicates low (0) or high (1) values, allowing comparison across water parameters and sampling points (LONG, longitudinal sampling; IND, individual sampling)

Across the LONG samplings, prevalence of *F. psychrophilum* infections was broadly similar in both farms and showed only modest seasonal variation. In both F1 and F2, the lowest infection rates were observed in autumn (F2 7.4%; F1 13.0%), although the seasonal patterns showed significant differences between the farms. In F2, infection rates increased through winter (16.7%) and spring (13.0%), reaching a peak in summer (22.2%). In contrast, F1 showed a pronounced increase in winter (20.4%), followed by lower rates in spring (9.3%) and summer (11.1%). When the data from both farms were aggregated, crude seasonal infection rates were approximately 10.2% in autumn, 11.1% in spring, 16.7% in summer, and 18.5% in winter. A chi-squared test indicated no statistically significant overall effect of season ($\chi^2 = 4.49$, df = 3, $p = 0.2136$). However, a GEE model with farm-level clustering revealed that only winter differed significantly from the autumn baseline, with fish sampled in winter having approximately twice the odds of being infected (coefficient = - 0.696, $p = 0.007$). No statistically significant differences were found for spring, summer, or between farms (F1 and F2).

The Pearson and Spearman correlation analyses revealed no significant correlation between *F. psychrophilum* infection and water quality parameters or seasonal changes (Table 15). However, the GEE model, accounting for clustering by fish tank, showed a significant association between pH at the inlet and *F. psychrophilum* infection (coefficient = +0.5531, $p = 0.011$), while temperature and dissolved oxygen showed a negative but non-significant association. For each unit increase in inlet pH, the odds of infection increased by approximately 74%, assuming that temperature and dissolved oxygen remained the same.

In contrast, *F. branchiophilum* infection correlated significantly with increased water temperature at the inlet and outlet (Pearson r = 0.410 and 0.420; Spearman $\rho = 0.471$ and 0.465, respectively) and strongly correlated with the summer season (Pearson r = 0.837; Spearman $\rho = 0.896$; $p < 0.001$). Additionally, there was a weak but significant negative correlation with dissolved oxygen at the outlet (Pearson r = - 0.395, $p = 0.046$) (Table 15).

Table 15: Pearson and Spearman correlation analyses to investigate the correlation between *Flavobacterium psychrophilum* and *Flavobacterium branchiophilum* infections and water quality parameters, including water temperature (T), pH, and dissolved oxygen (O) at the inlet and outlet of sampled fish tanks, as well as sampled seasons; significant *p*-values ($p < 0.05$) are shown in red

Water parameters and seasons	*F. psychrophilum*	*F. branchiophilum*
T inlet	Pearson r = - 0.008 ($p = 0.968$); Spearman ρ = - 0.185 ($p = 0.366$)	**Pearson r = 0.410 ($p = 0.037$); Spearman ρ = 0.471 ($p = 0.015$)**
T outlet	Pearson r = - 0.027 ($p = 0.897$); Spearman ρ = - 0.185 ($p = 0.367$)	**Pearson r = 0.420 ($p = 0.032$); Spearman ρ = 0.465 ($p = 0.017$)**
T inlet-outlet	Pearson r = 0.302 ($p = 0.134$); Spearman ρ = 0.321 ($p = 0.110$)	Pearson r = - 0.266 ($p = 0.189$); Spearman ρ = - 0.307 ($p = 0.126$)
pH inlet	Pearson r = 0.057 ($p = 0.782$); Spearman ρ = - 0.006 ($p = 0.977$)	Pearson r = - 0.191 ($p = 0.350$); Spearman ρ = - 0.307 ($p = 0.128$)
pH outlet	Pearson r = 0.027 ($p = 0.896$); Spearman ρ = - 0.033 ($p = 0.873$)	Pearson r = - 0.211 ($p = 0.301$); Spearman ρ = - 0.308 ($p = 0.126$)
pH inlet-outlet	Pearson r = 0.191 ($p = 0.350$); Spearman ρ = 0.182 ($p = 0.373$)	Pearson r = 0.148 ($p = 0.469$); Spearman ρ = 0.257 ($p = 0.206$)
O inlet	Pearson r = - 0.028 ($p = 0.894$); Spearman ρ = - 0.017 ($p = 0.934$)	Pearson r = - 0.372 ($p = 0.061$); Spearman ρ = - 0.387 ($p = 0.051$)
O outlet	Pearson r = - 0.045 ($p = 0.829$); Spearman ρ = - 0.085 ($p = 0.679$)	**Pearson r = - 0.395 ($p = 0.046$);** Spearman ρ = - 0.309 ($p = 0.124$)
O inlet-outlet	Pearson r = 0.032 ($p = 0.875$); Spearman ρ = 0.097 ($p = 0.636$)	Pearson r = 0.086 ($p = 0.675$); Spearman ρ = 0.014 ($p = 0.946$)
Autumn	Pearson r = - 0.275 ($p = 0.174$); Spearman ρ = - 0.272 ($p = 0.180$)	Pearson r = - 0.278 ($p = 0.169$); Spearman ρ = - 0.298 ($p = 0.140$)
Winter	Pearson r = 0.198 ($p = 0.332$); Spearman ρ = 0.222 ($p = 0.275$)	Pearson r = - 0.278 ($p = 0.169$); Spearman ρ = - 0.298 ($p = 0.140$)
Spring	Pearson r = - 0.097 ($p = 0.639$); Spearman ρ = - 0.064 ($p = 0.754$)	Pearson r = - 0.308 ($p = 0.125$); Spearman ρ = - 0.330 ($p = 0.100$)
Summer	Pearson r = 0.170 ($p = 0.408$); Spearman ρ = 0.111 ($p = 0.588$)	**Pearson r = 0.837 ($p < 0.001$); Spearman ρ = 0.896 ($p < 0.001$)**

4.6 OTHER FISH PATHOGENS

Several other fish pathogens were identified using the bacteriological, histopathological, and parasitological investigations, often in combination within the same individual, indicating a high frequency of concurrent infections. Information on the fish pathogens identified with bacteriological and histopathological investigations and concurrent isolation of *F. psychrophilum* and/or *F. branchiophilum* is given in Table 16.

Bacteriological investigation with MALDI-TOF MS identification revealed the presence of several bacterial species in the internal organs of the sampled fish, namely *Aeromonas salmonicida* subsp. *salmonicida* (n = 2), *Aeromonas* spp.

(n = 9), *Carnobacterium maltaromaticum* (n = 3), *Citrobacter gillenii* (n = 2), *Lactococcus garvieae* (n = 7), and *Pseudomonas* spp. (n = 2) (Table 16). Bacteria of the genus *Aeromonas*, except *A. salmonicida* subsp. *salmonicida*, and *Pseudomonas* could only be identified to genus level using MALDI-TOF MS.

Histopathological investigation confirmed the bacteriological findings of a systemic infection with *A. salmonicida* subsp. *salmonicida* in one fish, with numerous groups of rod bacteria observed in several internal organs on HE stained slides. Additionally, Gram staining revealed gram-positive cocci in the macrophages infiltrating several internal organs of one fish, indicating a systemic infection with *Renibacterium salmoninarum*. Single or multifocal cyst-like structures filled with intracellular bacteria were observed in the gill epithelium, indicating epitheliocystis disease (n = 6). Moderate to severe fungal infection by *Saprolegnia* spp. was detected in the gills, skin, and mouth (n = 9). Additionally, various parasites were identified, namely few to numerous *Acanthocephalus* spp. in the digestive system (n = 19), few to numerous amoebae exhibiting different morphologies (lined on the surface of the gill epithelium or freely between gill filaments or in pseudocysts) in the gills (n = 22), *Dermocystidium* spp. in a fin of one fish and individual to numerous *Tetracapsuloides bryosalmonae* in the kidneys and less frequently in other organs (spleen, liver gills and skin) (n = 11) (Table 16).

Table 16: Information on the other pathogens identified with bacteriological and histopathological investigations of fish organs, including number (No.) of diseased (D) and apparently healthy (H) fish per fish farm (F) and fish tank (T), organ of detection and connection with the isolation of *Flavobacterium psychrophilum* and/or *Flavobacterium branchiophilum*

OTHER PATHOGENS	**No.**	**ORGAN OF DETECTION**	***F. psychrophilum* isolation source**	***F. branchiophilum* isolation source**
BACTERIOLOGICAL INVESTIGATION				
BACTERIA				
Aeromonas salmonicida* subsp. *salmonicida (2 fish)	F2 summer T2 D1	spleen	gills, skin, spleen, kidney, liver	-
	F2 summer T3 D1	spleen, kidney, liver	-	-

Table 16 continued

Table 16 continued

***Aeromonas* spp.** (9 fish)	F1 autumn T3 D1	spleen, kidney, liver	-	-
	F2 autumn T1 D1, H1	D: kidney; H: spleen	-	-
	F2 autumn T2 D1	kidney	kidney	-
	F2 winter T3 H1	spleen, kidney, liver	-	-
	F2 summer T1 D1	spleen, kidney, liver	skin, liver	-
	F4 D2	spleen, kidney, liver	D1: liver	-
Carnobacterium maltaromaticum (3 fish)	F2 summer T1 D1	liver, spleen	-	-
	F4 D2	D1st: spleen, kidney, liver; D2nd: spleen	-	-
Citrobacter gillenii (2 fish)	F2 summer T1 H1	spleen	gills	-
	F2 summer T3 H1	spleen, kidney, liver	-	-
Lactococcus garvieae (7 fish)	F2 summer T1 D2	spleen, kidney, liver	skin	D1: gills
	F2 summer T2 D1	spleen, kidney, liver	skin	-
	F2 summer T3 D4	spleen, kidney, liver	-	-
***Pseudomonas* spp.** (2 fish)	F2 autumn T2 D1	liver, spleen	-	-
	F4 D1	liver, spleen	-	-
HISTOPATHOLOGICAL INVESTIGATION				
BACTERIA				
Renibacterium salmoninarum (1 fish)	F2 summer T3 D1	macrophages infiltrating the gills, skin, heart, spleen, kidney, stomach, pyloric caeca, and pancreas	-	-
Epitheliocystis (6 fish)	F2 winter T1 D1	gills	-	-
	F1 spring T3 D1	gills	-	-
	F2 spring T2 D1, H1	gills	-	-
	F1 summer T1 D1, H1	gills	H: skin	-

Table 16 continued

Table 16 continued

FUNGAE				
***Saprolegnia* spp.** (9 fish)	F1 autumn T3 D1	mouth	-	-
	F1 winter T1 D1	skin	gills, skin, spleen	-
	F2 spring T2 D1	skin, fins	skin, spleen, kidney, liver	-
	F2 spring T3 D2	D1st: mouth; D2nd: gills	D1st: skin, spleen, kidney, liver; D2nd: spleen, kidney, liver	-
	F2 summer T1 D2	D1st: gills; D2nd: fins	skin	D1st: gills
	F2 summer T2 D1	fins	-	-
	F2 summer T3 D1	gills	-	gills
PARASITES				
***Acanthocephalus* spp.** (19 fish)	F1 autumn T1 D1	pyloric caeca, intestine	-	-
	F2 autumn T2 H1	intestine	-	-
	F2 winter T1 H2	H1st: pyloric caeca; H2nd: intestine	-	-
	F2 winter T3 D1, H1	D: pyloric caeca, intestine; H: intestine	D: skin, spleen	-
	F2 spring T1 H1	intestine	-	-
	F2 spring T3 H1	intestine	-	-
	F2 summer T1 2D, H4	D1st: pyloric caeca; D2nd: intestine; H1–-3: intestine; H4th: abdominal cavity, intestine	D1st: gills, skin; D2nd: skin; H1st: gills	-
	F2 summer T2 2D, H3	D: intestine; H1st, 2nd: intestine; H3rd: pyloric caeca	H1st: gills	-

Table 16 continued

Table 16 continued

Amoebae (22 fish)	F2 autumn T1 D1	gills	-	-
	F1 winter T1 D3	gills	D1: gills, spleen, kidney, liver	-
	F1 spring T1 D2	gills	D1: skin	-
	F1 spring T3 D2	gills	D1: gills, skin, kidney	-
	F2 spring T2 D1	gills	-	-
	F2 spring T3 D5	gills	D1st: skin, spleen, kidney, liver; D2nd: spleen, kidney, liver; D3rd: skin	-
	F1 summer T1 D1	gills	-	-
	F2 summer T1 D3	gills	D1st: skin, liver; D2nd: gills, skin; D3rd: skin	-
	F2 summer T2 D2	gills	D1st: gills, skin	D2nd: gills
	F2 summer T3 D1, H1	gills	-	-
***Dermocystidium* spp.** (1 fish)	F2 autumn T2 D1	fin	-	-
Tetracapsuloides bryosalmonae (11 fish)	F2 autumn T2 D4, H1	D1st, 2nd: kidney; D3rd: gill, kidney; D4th: gills, skin, spleen, kidney; H: gill, kidney	-	-
	F2 autumn T3 D1	kidney	-	-
	F2 winter T1 D1	kidney	-	-
	F2 summer T2 D2, H2	D1st: gills, kidney; D2nd: gills, spleen, kidney, liver; H1st: spleen, kidney, liver, stomach; H2nd: spleen	D1st: gills, skin, spleen, kidney, liver; D2nd: gills, skin, spleen	-

F, fish farm; T, fish tank; D, diseased fish; H, apparently healthy fish

Parasitological investigation of the gill and skin smears stained with Giemsa revealed the presence of several different parasites, namely mild to severe infestation of the gills and skin with *Apiosoma* spp. (gills 4/540, 0.7%; skin 75/540, 13.9%), *Chilodonella* spp. (gills 31/540, 5.7%; skin 25/540, 4.6%), *I. necator* (gills

20/540, 3.7%; skin 47/540, 8.7%), Monogenea (*Gyrodactylus* spp. or *Dactylogyrus* spp.) (gills 106/540, 19.6%; skin 268/540, 49.6%), *Trichodina* spp. (gills 31/540, 5.7%; skin 64/540, 11.9%), and mild infestation with *Ichthyophthirius multifiliis* (gills 20/540, 3.7%; skin 10/540, 1.9%). A mild to severe infestation with *Epistylis* spp. (21/540, 3.9%) and a mild infestation with *Riboscyphidia* spp. (2/540, 0.4%) was only found in the skin samples.

The Pearson chi-squared test showed that infection with *F. psychrophilum* was significantly associated with infestation of the gills ($p = 0.002$) and skin ($p < 0.0001$) with Monogenea. No other pathogens identified with parasitological or histopathological investigation were significantly associated with *F. psychrophilum* infections.

Hierarchical clustering of fish based on infection with other fish pathogens, identified with parasitological or histopathological investigation, revealed distinct clusters of both fish and fish pathogens, reflecting patterns of co-occurrence and common pathogen profiles, with the heatmap showing varying levels of pathogen presence across individuals. Notably, the skin-associated parasites *Gyrodactylus* spp. and/or *Dactylogyrus* spp. were represented in most fish clusters. The co-occurrence of gill-associated pathogens, such as *Apiosoma* spp., *Gyrodactylus* spp., and/or *Dactylogyrus* spp., *I. necator,* and *Trichodina* spp., was also evident in certain fish groups. Some parasites, including *Epistylis* spp. and *T. bryosalmonae*, occurred more sporadically and were not strongly clustered. Clustering also shows a group of fish with minimal parasite occurrence in all fish pathogens assessed. The clustering of fish pathogens on the y-axis highlights common coinfections (Figure 32).

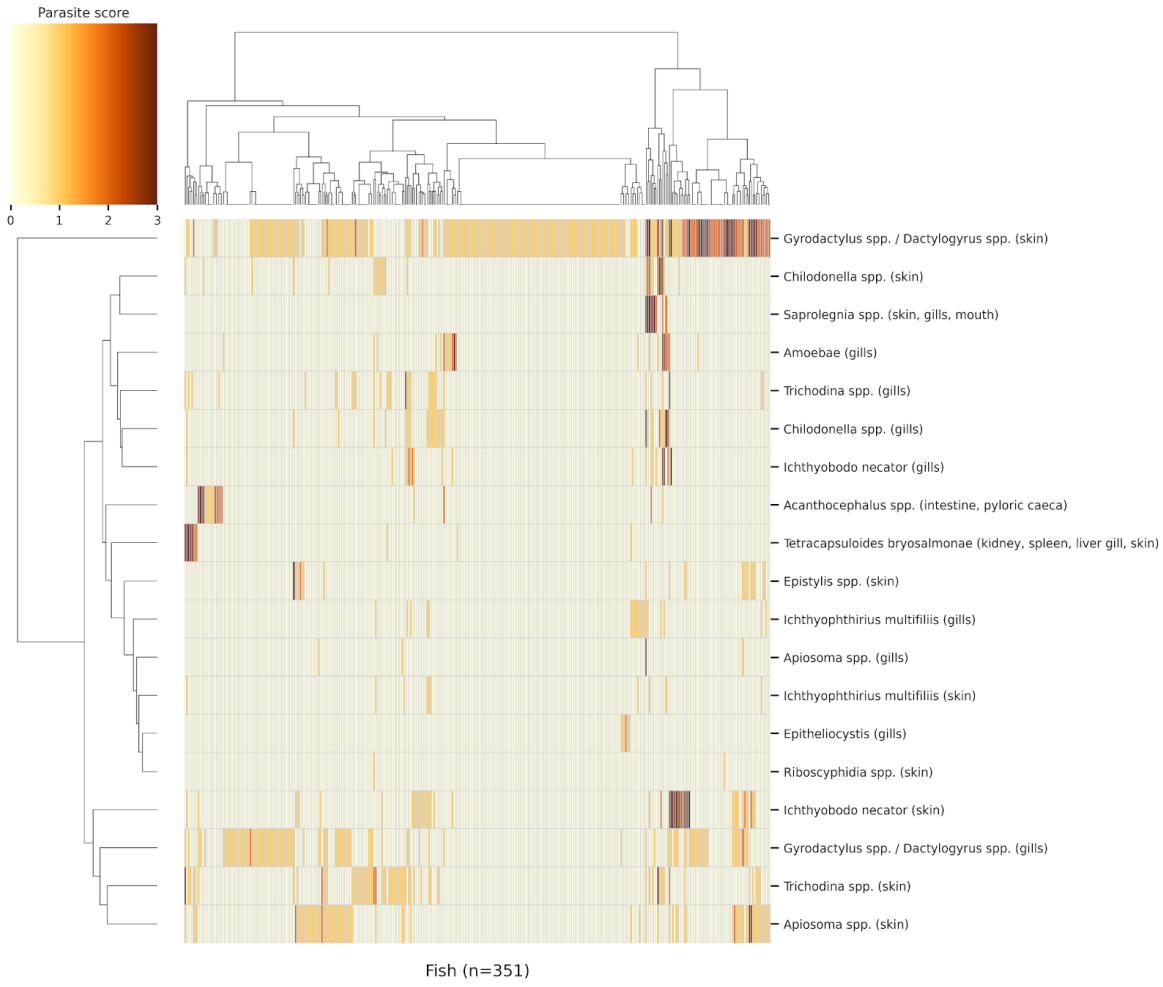


Figure 32: Hierarchical clustering of fish (n = 351; at least one other fish pathogen) based on semiquantitatively assessed infection with other fish pathogens. The heatmap shows infection severity from 0 (none) to 3 (severe). In the top dendrogram, fish are clustered based on similar infection profiles, and in the left dendrogram, fish pathogens are clustered based on their co-occurrence in the same fish

5 DISCUSSION

5.1 FLAVOBACTERIA IN SLOVENE FARMED RAINBOW TROUT AND THEIR ASSOCIATION WITH PATHOLOGICAL LESIONS

In recent decades, aquaculture has expanded significantly worldwide, surpassing capture fisheries in the production of aquatic animals for human consumption (FAO, 2024). However, the farming environment is known to favour the occurrence and spread of infectious diseases, including those caused by opportunistic and facultative pathogens, such as flavobacteria (Pulkkinen et al., 2010; Lee et al., 2023). To our knowledge, flavobacteria in Slovenia have so far only been detected in freshwater (Mulec et al., 2021a; Eržen, 2024), seawater (Turk and Tinta, 2021), and ice (Jarčič, 2021; Mulec et al., 2021b), as well as in rumen contents (Ramšak and Avguštin, 1998), and in certain animals, such as the olm (*Proteus anguinus*) (Bokan, 2013) and jellyfish (Tinta et al., 2012). To mitigate the negative impact of flavobacteria on fish aquaculture and implement effective management practices, it is important to understand the occurrence and aetiology of flavobacterioses and the characteristics of flavobacteria present in the aquaculture environment. In this research, at least one *Flavobacterium* spp. was isolated from one or more organs in 89.3% of all sampled rainbow trout, most frequently from the gills and skin, which was not surprising given their ubiquitous distribution in nature (Bernardet and Bowman, 2006) but in some cases also from the internal organs (16.9% of POS fish). We isolated several different flavobacteria from the gills and skin of most fish. As observed in other studies reviewed by Bernardet and Bowman (Bernardet and Bowman, 2006), the morphology of flavobacteria was highly diverse, even within a single species, while some different species showed a very similar morphology, which is why accurate identification of the isolates is always recommended. This research is the first to confirm the presence of flavobacteria in Slovene fish, including the well-known fish pathogens *F. psychrophilum* and *F. branchiophilum*. Notably, no species belonging to the *F. columnare* group were detected, which could be due to the low water temperatures recorded in this research (up to 15.8 °C) because the disease occurs most frequently when water

temperatures rise above 15 °C to around 20 °C (Suomalainen et al., 2005; Declercq et al., 2013a). The lesser-known species identified in this study include *F. araucananum, F. bernardetii, F. collinsii, F. frigidimaris, F. hercynium, F. hydatis, F. oncorhynchi, F. piscis, F. plurextorum, F. tiangeerense* and *F. tructae,* which have previously been associated with disease in fish as reviewed by Heckman et al. (2023)*,* presumably causing external lesions (Anderson and Ordal, 1961; Strohl and Tait, 1978; Carson et al., 1993) and/or systemic infections (Flemming et al., 2007; Kämpfer et al., 2012; Loch, 2012; Suebsing and Kim, 2012; Zamora et al., 2012). Among lesser-known species, the pathogenicity of *F. tructae* has been experimentally demonstrated (Loch, 2012; Loch and Faisal, 2016). It should be noted that *F. araucananum* and *F. plurextorum* were only identified by MALDI-TOF MS in this study. However, the Bruker MALDI Biotyper matching hints table indicates that these species share highly similar MSPs with certain other *Flavobacterium* species, making reliable identification at the species level difficult. Filamentous bacteria were observed in the histological preparations of gills and skin of some fish that tested negative for *F. psychrophilum* and *F. branchiophilum*, indicating that isolation was not always successful or that the observed bacteria may belong to the lesser-known *Flavobacterium* species. Other studies investigating the diversity of fish-associated flavobacteria are scarce, but they also reported the isolation of numerous lesser-known flavobacteria and putative new species from gills, skin, and internal organs. Some of the isolates were obtained from diseased fish exhibiting pathologies similar to those commonly observed in BCWD or BGD. These studies underscore the complexity of the aetiologies of flavobacterioses and the growing number of different flavobacteria suspected of causing disease in fish (Loch, 2012; Loch and Faisal, 2014, 2015; Verma and Rathore, 2015; Mwega et al., 2019; Heckman et al., 2023). Notably, experimental infections with some uncharacterised *Flavobacterium* spp. resulted in mortality, development of gross lesions, and reisolation of the bacteria from internal organs (Loch, 2012; Loch and Faisal, 2014). Lee et al. (2023) concluded that some recently described *Flavobacterium* spp. are likely saprophytic or commensal bacteria that may behave as opportunistic pathogens.

Studies of flavobacterial communities in healthy and diseased fish are needed to enable differentiation between commensal or mutualistic species and pathogenic species (Loch, 2012). However, there are few diagnostic methods available for the lesser-known flavobacteria, making their identification challenging (Loch and Faisal, 2015). In this research, MALDI-TOF MS enabled rapid and reliable identification of *F. psychrophilum*, *F. branchiophilum,* and several lesser-known flavobacteria, as has been observed in other studies (Strepparava et al., 2012b; Pérez-Sancho et al., 2017; Saticioglu et al., 2021a; Saticioglu et al., 2021b; Zurbuchen et al., 2023). Most isolates obtained in this research could not be classified to species level using MALDI-TOF MS. WGS and subsequent ANI analysis of selected isolates identified only to the genus level, particularly those isolated from internal organs or in greater numbers from skin or gills, as well as those with frequently observed colony morphologies and/or MALDI-TOF MS results, revealed that most WGS-typed isolates (74.1%, 60/81) represented 41 putative new flavobacterial species. Further studies are required before these can be recognised as validly described new species. The latter should follow the IJSEM minimal standards for the description of new bacterial species and include a comprehensive polyphasic typing approach (Bernardet et al., 2002; Riesco and Trujillo, 2024). The Bruker MALDI-TOF MS reference database used in this research contained MSPs for 48 *Flavobacterium* species. The spectra for some species identified with WGS, namely *F. bernardetii*, *F. branchiarum*, *F. celericrescens*, *F. collinsii*, *F. cupriresistens*, *F. difficile*, *F. erciyesense,* and *F. tructae*, were not yet included. Following the expansion of the in-house *Flavobacterium* MALDI-TOF MS database, some of the selected isolates with previously unreliable results were identified as *F. branchiarum* and *F. tructae,* as well as several putative new species (FX3, FX6, FX9, FX10, FX15, FX18, and FX32). These results underscore the usefulness of MALDI-TOF MS for the identification of flavobacteria, provided that the species-specific MSP is available in the database. Additionally, in the MSP dendrogram by MALDI-TOF MS, isolates of the same species typically clustered together. In contrast, the two FX36 isolates formed separate clusters, although WGS and ANI analyses confirmed that they belong to the same species, indicating possible contamination in the MSP of one isolate. It should be noted that some closely related flavobacterial species have

very similar MSPs, making it difficult to differentiate between these species using MALDI-TOF MS. The WGS revealed that five isolates identified as *Flavobacterium* spp. by MALDI-TOF MS belong to the genera *Kaistella* and *Pedobacter*. Therefore, further comparative studies between MALDI-TOF MS and WGS are needed to assess the reliability of MALDI-TOF MS for the identification of lesser-known or closely related flavobacterial species. Of the 12 lesser-known species identified by WGS, all corresponded to the best matches based on the 16S rRNA gene sequence similarity, except for *F. cupriresistens*, which was more accurately identified using the *gyrB* gene. This indicates that the 16S rRNA gene provided higher taxonomic resolution than *gyrB* in this dataset. This observation is somewhat unexpected because although the 16S rRNA gene remains the most commonly used phylogenetic marker for bacterial identification, some studies suggest that the *gyrB* gene may provide better species-level discriminatory power (Peeters and Willems, 2011; Galloway-Peña et al., 2014; Reichley et al., 2017; Kumru et al., 2020; Heckman et al., 2023; Gélinas et al., 2024). Notably, most isolates (34/60, 56.7%) representing putative new species showed 16S rRNA gene sequence identities of 99–100% with their closest matches, exceeding the 99% threshold used for species-level identification of flavobacteria (Dong et al., 2016; Edgar, 2018). These results confirm that ANI analysis is the most accurate approach for the identification of *Flavobacterium* species (Gélinas et al., 2024).

F. psychrophilum was the most common pathogenic flavobacterium in Slovene farmed fish sampled in this research. It was isolated in 23.7% of all sampled fish, both in clinically diseased fish (77.3%) and in apparently healthy fish (22.7%) from all sampled organs, from all fish farms, and in all seasons. MLST analysis showed that the *F. psychrophilum* isolates (n = 7) belonged to different clonal complexes, namely CC-ST1 and CC-ST10, which are widely distributed in Europe and associated with high virulence in rainbow trout (Nilsen et al., 2014; Knupp et al., 2019; Sundell et al., 2019), as well as several newly identified sequence types. However, given the limited number of isolates analysed, further studies are needed to characterise the genetic diversity of *F. psychrophilum* in Slovene farmed rainbow trout better. F3 had a significantly higher percentage (37%) of *F. psychrophilum*-INF fish compared to the other samplings. When analysed by

fish farm, the prevalence of infection was lowest in F1 and F2 (about 14%), while F3 and F4 had significantly higher infection rates (about 24–28%). Fish from F3 and F4 had significantly higher odds for infection compared to those from F1 and F2, while F1 was not significantly different from F2. Therefore, infection with *F. psychrophilum* was probably the cause or contributed to the increased mortality observed in F3 and F4. Notably, isolation from the skin (n = 2) and gills (n = 0) of fish from F4 was significantly lower compared to the other samplings, although an equal or greater number of fish tested POS in the internal organs (n = 13), which may be due to the formalin treatment performed a few days before sampling. High concentrations of formalin can inactivate flavobacteria (Grabowski et al., 2004), while a study using bath treatment with low concentrations of formalin (0.017%) observed fewer but similar numbers of *F. branchiophilum* cells after treatment (Ostland et al., 1995b).

F. psychrophilum-INF fish had a significantly lower body weight and total length than NH fish, while the difference in K was not significant when analysed per sampling. However, no significant difference was found when these variables were analysed per fish tank. The association with lower body weight and length per sampling supports well-established understanding that younger fish are more susceptible to infection (Starliper, 2011). Body weight, total length and K of *F. psychrophilum*-INF, and POS fish ranged from 4–300.5 g and 4–650 g, 7–30.5 cm and 7–40 cm and 0.59–2.07 and 0.5–2.07, respectively, indicating that different life stages were affected, from juveniles to yearlings, which is also consistent with the literature (Lorenzen et al., 1997). Notably, the three fish with the highest K had a shortened body resulting from severe caudal fin and peduncle ulceration due to infection with *F. psychrophilum*, which led to a misleadingly high K value. The results per fish tank and the fact that no significant difference in K was found indicate that *F. psychrophilum* infection did not influence the body condition of fish in this research. In contrast, we found one study in which a correlation was found between *F. psychrophilum* and lower K (Boutin et al., 2014). Infection with *F. psychrophilum* was associated with a significantly higher necropsy fish index, as well as score values of all organs except the opercula, stomach with pyloric caeca, and intestine. The gross lesions observed in POS fish were

consistent with those described in the literature (Kent et al., 1989; Lorenzen et al., 1991; Bruno, 1992; Ekman and Norrgren, 2003; Bruno et al., 2013; Loch and Faisal, 2017), including erosions and ulcerations of the skin and fins, in severe cases resulting in saddleback-like lesions, complete loss of the caudal fin and severe ulceration of the caudal peduncle, S-shaped tails, as well as skin hyperpigmentation, ocular lesions, thickened gills, enlarged spleen and perisplenitis, anaemia of the gills, liver, kidney and spleen, intestinal hyperaemia, haemorrhage in different organs, and mild ascites. The liver atrophy observed in this study had not previously been described in the literature on *F. psychrophilum* infections and was more likely associated with cachexia rather than a direct effect of the *F. psychrophilum* infection, as all but one of the affected fish had a low K. Gross enlargement of the liver and kidney were also not yet reported in rainbow trout infected with *F. psychrophilum*. In the enlarged livers and kidneys, we histologically observed haemorrhage, degeneration, necrosis, and inflammation, which have also been described in other studies (Lorenzen et al., 1991; Bruno, 1992; Aly et al., 2013).

The analysis of the histopathological variables revealed that the indices of all organs, except the pancreas, were significantly higher in *F. psychrophilum*-INF fish compared to NH fish. The regressive, inflammatory, and circulatory reaction indices were significantly higher in most of the analysed organs of *F. psychrophilum*-INF fish, while the progressive indices were significantly different only in the gills, spleen, and kidney. Histopathological investigation revealed haemorrhage, necrosis, degeneration, and infiltration with lymphocytes, macrophages, and neutrophils in most of the examined organs of *F. psychrophilum*-INF fish. However, there were only a few lesions that showed significant differences between *F. psychrophilum*-INF and NH fish. In the skin, these lesions included hydropic or vacuolar degeneration, necrosis, erosion, ulceration and infiltration of the epidermis with neutrophils, haemorrhage, and necrosis of the dermis, loss or resorption of scales or bone, haemorrhage, hydropic degeneration, and Zenker's degeneration of the muscle tissue, as well as infiltration with lymphocytes, macrophages, and neutrophils in the dermis and muscle tissue, accompanied by serous or fibrinous exudate. Gill lesions that

showed significant differences included haemorrhage and necrosis in the lamellae and filaments, hydropic or vacuolar degeneration, necrosis, hypertrophy, and hyperplasia of the epithelial cells, along with lamellar fusion and infiltration of the lamellae and filaments with lymphocytes, macrophages, and neutrophils. In the splenic parenchyma, significant differences were found for hyperaemia, atrophy, and hyperplasia of the white pulp and activation of RES. In the kidney, significantly different lesions included hydropic, vacuolar, or hyaline droplet degeneration of the renal tubules and hyperplasia of the haematopoietic tissue. A significant difference was also observed in tubule regeneration. This was the only case in which the mean value was higher in NH fish than in INF fish, indicating a reduced capacity of tubule regeneration in INF fish, which has also not been previously described. In the liver, significant differences were observed for haemorrhage, decreased hepatocellular vacuolation, micro- or macrovesicular fatty, and/or vacuolar degeneration, necrosis, and infiltration with neutrophils. Significantly different lesions in the stomach and pyloric caeca included intercellular oedema in the mucosa and other tissue layers, vacuolar degeneration and necrosis of the gastric glands, and infiltration of the mucosa with lymphocytes. Only the infiltration of the mucosa with lymphocytes showed a significant difference in the intestine. There were no significant differences between INF and NH fish in the heart and pancreas, except for the heart index and the regressive and inflammatory reaction indices in the heart. The lesions described are generally in good agreement with the existing literature (Lorenzen et al., 1991; Bruno, 1992; Ekman and Norrgren, 2003; Ferguson, 2006; Nilsen et al., 2011; Aly et al., 2013; Soares et al., 2019; Park et al., 2023). However, this is one of the few studies in which the histopathological lesions in flavobacterial infections are assessed semiquantitatively and an NH control group is used (Ostland et al., 1990; Ekman and Norrgren, 2003; Marancik et al., 2014). The spleen is usually the most affected internal organ, and perisplenitis is a very common and possibly pathognomonic lesion in *F. psychrophilum* infections (Nilsen et al., 2011). In contrast, in this research, we found no lesions in the splenic capsule that showed significant differences between INF and NH fish. Haemorrhage, necrosis, and inflammation in the splenic parenchyma and capsule, as well as in the kidney haematopoietic tissue, have been described previously (Ekman and Norrgren,

2003; Bruno et al., 2013; Marancik et al., 2014) and were also observed in *F. psychrophilum*-INF fish in this research, but showed no significant differences. The significant difference in hyperplasia of the kidney haematopoietic tissue and splenic white pulp in *F. psychrophilum*-INF fish in this study reflects increased white blood cell production during infection, while the atrophy of splenic white pulp indicates mobilisation of lymphocytes from the spleen to peripheral tissues. We found only one study describing white pulp atrophy in Siberian sturgeons (*Acipenser baerii*) infected with *F. psychrophilum* (Chinchilla et al., 2023). Hyperplasia of kidney haematopoietic tissue and splenic white pulp has not yet been described in *F. psychrophilum* infections. Fish confirmed to be infected with *T. bryosalmonae*, which is known to cause such lesions, were excluded from the analyses. Some infested individuals may have remained undetected, albeit it is unlikely that mild infestations would significantly affect the results. Additionally, Gram staining of kidneys with granulomatous inflammation revealed the presence of gram-positive cocci in only one fish. However, in practice, Gram staining of *R. salmoninarum* may not always be successful. Therefore, it is possible that more fish were infected than were detected. Lesions in the heart have also been described in some studies (Rangdale, 1995; Cipriano and Holt, 2005; Nilsen et al., 2011), and several lesions were observed in this study, but did not show a significant difference. Studies investigating the histopathological lesions in the stomach, pyloric caeca, intestine, and pancreas of fish infected with *F. psychrophilum* are lacking, although gross lesions in the intestine are often described (Ekman and Norrgren, 2003; Bruno et al., 2013; Knupp and Loch, 2023). The bacterium has been detected in these organs of naturally infected ayu, although no histopathological lesions were found (Liu et al., 2001; Miwa and Nakayasu, 2005), as well as in the intestines of salmonids showing hyperaemia (Ekman and Norrgren, 2003). The gastric glands may show vacuolation (Park et al., 2023), which was also described in this research.

F. branchiophilum was only isolated from the gills of 11 fish (2.04% of all fish sampled) from F1 and F2 during summer sampling. Like *F. psychrophilum*, it was mostly isolated from diseased fish (72.73%) and also from apparently healthy fish (27.27%). Only individual *F. branchiophilum* colonies were observed during

primary isolation. Given its fastidious nature, it is possible that the bacterium was present in other fish, but the isolation was not successful. The ranges of body weight, total length, and K for *F. branchiophilum*-INF and POS fish were 24.5–48.5 g and 12–300.5 g, 12.5–19.5 cm and 11–29 cm, and 0.58–1.25 and 0.58–1.61, respectively. Therefore, fish of various life stages were affected, which is consistent with the available literature (Ferguson et al., 1991; Ferguson, 2006). Analysis of necropsy variables for *F. branchiophilum* revealed that K of INF fish did not differ significantly from that of NH fish. No other studies were found that analysed the association between K and *F. branchiophilum* infection. On the other hand, INF fish had significantly higher necropsy fish indices, as well as score values for gills and spleen. *F. branchiophilum*-POS fish showed anaemic gills, mild to severe clubbing of the gill filaments, one fish had focal gill necrosis, two fish had shortened opercula, and one fish also exhibited anaemia of the spleen and kidney. Analysis of histopathological variables showed that only the fusion of the gill filaments showed significant differences between *F. branchiophilum*-INF and NH fish, indicating severe hyperplasia of the gill epithelium in INF fish. Other lesions were also observed in *F. branchiophilum*-INF fish, including haemorrhage, necrosis, and inflammation in the gill lamellae and filaments, but these did not show any significant differences. The gross and histopathological lesions observed in the gills of INF fish were consistent with lesions reported in the literature (Ostland et al., 1990; Speare et al., 1991; Ferguson, 2006; Frasca et al., 2018).

It is noteworthy that 75.98% of the isolates could not be classified to the species level during this research. A total of 2652 isolates belonging to the genus *Flavobacterium* were identified by MALDI-TOF MS, of which 2105 were preserved in storage. The high biodiversity within the genus, combined with the limited MSPs for *Flavobacterium* spp., available in the current Bruker MALDI-TOF MS reference database, prevented identification to species level for most isolates. This was the main limitation of the study because the low number of isolates that could be reliably identified to species level prevented the analysis of associations between infections with most of the lesser-known *Flavobacterium* spp. and the presence of gross and histopathological lesions. The large number of stored isolates enables future studies to determine their species identity using additional methods and to

further investigate their pathogenic potential. Several lesser-known and putative new species were isolated in greater numbers from the gills and/or skin, and also from internal organs of fish exhibiting gross and/or histopathological lesions, indicating a pathogenic potential. Analysis of histopathological lesions was only possible for *F. hercynium, F. hydatis,* and *F. frigidimaris*, which were isolated from gills and skin of diseased and apparently healthy fish in this research. The results showed that *F. hercynium*-INF fish had significantly higher score values for skin ulceration than NH fish, and *F. hydatis*-INF fish had significantly higher score values for hypertrophy and hyperplasia of gill epithelial cells. Analysis for *F. frigidimaris* did not show any significantly different lesions. Some studies reported the isolation of these species from diseased fishes, including salmonids, namely *F. hercynium* from fins, gills, kidney, and swim bladder (Loch et al., 2013; Loch and Faisal, 2014; Verma and Rathore, 2015), *F. hydatis* from gills (Strohl and Tait, 1978; Loch and Faisal, 2014; Mantareva et al., 2022), and *F. frigidimaris* from gills (Loch and Faisal, 2014). However, these studies did not report the pathological lesions observed, except for *F. hydatis*, which has been described to cause pathological lesions similar to BGD. Additionally, only for *F. hydatis* we found a study describing histopathological lesions in the gills of *F. hydatis*-INF Russian sturgeon (*Acipencer gueldenstaedtii*), including epithelial hyperplasia (Timur et al., 2010). Our results show that *F. hercynium* and *F. hydatis* may indeed cause pathological lesions in fish, while the pathological significance of *F. frigidimaris* was not evident. Further research is required to determine whether these flavobacteria meet Koch's postulates for pathogenicity. Hierarchical clustering between sampled fish and *Flavobacterium* spp. isolated from more than 1% of individuals showed no significant association between clustering patterns and metadata variables, such as health status, season, or sampling. The role of many flavobacteria in fish health remains unclear, including whether they are transient inhabitants or part of the commensal microflora, and which newly described species are opportunistic pathogens.

It is important to note that the pathological lesions observed in this research may not be solely attributed to the recovered flavobacteria because these fish were naturally infected, and other pathogens could have contributed to the lesions.

5.2 ANTIMICROBIAL SUSCEPTIBILITY OF SLOVENE *FLAVOBACTERIUM* SPECIES ISOLATES

This research investigated the antimicrobial susceptibility of *F. psychrophilum*, *F. branchiophilum,* and several lesser-known *Flavobacterium* spp. Antimicrobials approved for use in aquaculture in different countries were tested (Cabello et al., 2013), including FLO and OXY used in Slovenia (JAZMP, 2025), as well as CTZ and MER, which are primarily used in human medicine. Information on antimicrobial susceptibility of flavobacterial isolates provides the basis for appropriate treatment selection and prudent use of antimicrobials in Slovene aquaculture. Considering the limited number of published standardised AST studies for *F. psychrophilum*, our results make a valuable contribution to the global and especially European understanding of susceptibility of this important aquaculture pathogen to antimicrobials.

The *F. psychrophilum* isolates were mostly WT for AMP, ERY, and FLO, but showed high NWT frequencies for OXY (81.5%) and quinolones (87.0% for ENR and 90.7% for OXO). Additionally, all isolates grew at the lower end of the concentration ranges tested for GEN and MER and probably represent WT strains, while most isolates grew at the upper end of the ranges tested for CTZ and TRS. Other studies using the standardised method reported similar findings (Smith et al., 2016; Van Vliet et al., 2017; Ngo et al., 2018; Söderlund et al., 2018; Jarau et al., 2019; Saticioglu et al., 2019; Li et al., 2021; Vaibarová et al., 2024). Studies that used non-standardised broth microdilution methods were also included for comparison (Hesami et al., 2010; Henríquez-Núñez et al., 2012). However, it is important to consider that valid comparisons between studies are only possible when standardised and harmonised methods and growth media are used. The calculated provisional CO_{WT} value of ≤ 0.125 µg/ml for AMP aligns with previous research using the NRI method (Miranda et al., 2016; Smith et al., 2016; Ngo et al., 2018). Few studies reported a high frequency (71.0–82.3%) of reduced susceptibility to AMP, possibly due to its extensive use, including in *F. psychrophilum* infections (Hesami et al., 2010; Li et al., 2021). Few studies

reported low to moderate frequencies (up to 20%) of NWT isolates for ERY (Hesami et al., 2010; Saticioglu et al., 2019; Vaibarová et al., 2024). ERY is primarily used to treat infections caused by gram-positive bacteria. However, in a previous study, it was found to be more effective than FLO in reducing fish mortality and splenic bacterial load in *F. psychrophilum* infections (Jarau et al., 2019). Hesami et al. (2010) also stated that ERY could be used to treat *F. psychrophilum* infections and, if used prudently, could delay the emergence of widespread resistance to FLO. Surprisingly, despite the use of FLO in aquaculture for over 30 years, there are few published reports of *F. psychrophilum* strains with reduced susceptibility. Two NWT isolates (2/31) for FLO were found in China (Li et al., 2021), and only two studies reported high frequencies of reduced susceptibility to FLO, namely in 52.8% (38/72) of isolates from Canada (Hesami et al., 2010) and in 92.5% (37/40) of isolates from Chile (Henríquez-Núñez et al., 2012). Additionally, a recent study from the Czech Republic reported a low frequency of NWT isolates for FLO (11/70; 15.7%) while most remaining isolates had MICs at the first (44.3%) or second (37.2%) concentration below ECV (Vaibarová et al., 2024). Studies investigating the susceptibility of *F. psychrophilum* to GEN observed varying results of predominately low, moderate (≤ 2 µg/ml) (Van Vliet et al., 2017; Li et al., 2021; Vaibarová et al., 2024), and high (> 4 µg/ml) (Hesami et al., 2010) MICs. GEN is used in aquaculture against gram-negative bacterial infections but not typically for *F. psychrophilum* (Bojarski et al., 2019). To our knowledge, no information is yet available on the susceptibility of *F. psychrophilum* or other flavobacteria to MER. The reduced susceptibility of *F. psychrophilum* to OXY and the quinolones is probably due to the widespread use of these antimicrobials in aquaculture, including for the treatment of *F. psychrophilum* infections (Shao, 2001; Saticioglu et al., 2019). A study from the USA, where the use of quinolones in food animals is restricted, reported a low frequency of NWT isolates for OXY (24%) and the quinolones (0%), supporting this assumption. Although OXY is commonly used in USA aquaculture, this study was the first to report reduced susceptibility, particularly in isolates from farmed rather than wild fish (Van Vliet et al., 2017). According to reports by Slovene fish veterinarians Rosvita Sitar, M.Sc., Marija Seničar, M.Sc., and Dr. Diana Žele Vengušt (personal communication, May 20, 2024), the efficacy of OXY for the treatment of suspected

F. psychrophilum infections is decreasing. Interestingly, a recent study from Italy reported that tested *F. psychrophilum* isolates were sensitive to all antimicrobials, including OXY and the quinolones (Zamparo et al., 2024). Notably, most *F. psychrophilum* isolates in this study, including the reference *F. psychrophilum* strain, displayed high MICs for CTZ and TRS. These results may indicate potential intrinsic resistance or unknown resistance mechanisms. On the other hand, the calculated provisional CO_{WT} value for CTZ (≤ 8 µg/ml) was higher than the tested concentration range, suggesting that the isolates were WT. The distribution of MICs for TRS was variable, as observed in other studies using NRI analysis, resulting in standard deviations greater than the limit of < 1.2 $\log^2$ µg/ml (Smith et al., 2016; Van Vliet et al., 2017; Ngo et al., 2018; Saticioglu et al., 2019) and precluding the establishment of valid ECVs for the interpretation of MICs for this antimicrobial. The NRI analysis conducted by Smith et al. (2016), Van Vliet et al. (2017) (≤ 1/19 µg/ml), and Ngo et al. (2018) (≤ 8/152 µg/ml) generated high provisional CO_{WT} values for TRS, leading to the conclusion that the isolates tested were WT. Considering these results, it is not possible to reliably classify the Slovene isolates as WT or NWT. To our knowledge, no other studies for CTZ are available for comparison, and few studies reported predominantly moderate (Smith et al., 2016; Saticioglu et al., 2019) or low (Van Vliet et al., 2017) MICs for TRS.

Concordance between phenotypic and genotypic AMR profiles of *F. psychrophilum* and *F. branchiophilum* isolates was assessed for AMP, ENR, OXO, and OXY. Reliable classification of isolates as WT or NWT for CTZ, GEN, MER, and TRS was not possible, and only WT isolates were available for FLO and ERY, which precluded the assessment for these antimicrobials. The T83A or T83V substitution in GyrA, previously associated with quinolone resistance in *F. psychrophilum* (Izumi and Aranishi, 2004; Shah et al., 2012; Söderlund et al., 2018; Saticioglu et al., 2019), was observed in five out of seven sequenced *F. psychrophilum* isolates, which was consistent with phenotypic resistance to OXO but not to ENR, indicating possible differences in drug targets or uptake mechanisms. The genotypic AMR profiling revealed no AMR genes or other AMR-associated mutations in the studied *F. psychrophilum* and *F. branchiophilum* genomes. Alvarez et al. (2004) identified the presence of AMR genes, such as

ermF and *tetQ,* in the *F. psychrophilum* genomes, potentially involved in reduced susceptibility to ERY and tetracycline, respectively. However, other studies described the presence of AMR genes, such as *floR*, *sul2,* and various *tet* genes, which were not associated with phenotypic resistance to FLO, sulfonamides, and tetracyclines, respectively (Söderlund et al., 2018; Saticioglu et al., 2019). In addition, the isolates in this study did not have the previously reported six-nucleotide polymorphisms in the 16S rRNA gene associated with tetracycline resistance (Soule et al., 2005). Therefore, the genetic background of tetracycline resistance remains unclear. Furthermore, the β-lactamase gene *bla* is not associated with AMP resistance in *F. psychrophilum* (Alvarez et al., 2004). These discrepancies underline the incomplete understanding of resistance mechanisms in flavobacteria. Notably, AMR genes known in Pseudomonadota are generally not found in Flavobacteriaceae (Verner-Jeffreys et al., 2017), and the inconsistencies between resistance phenotypes and genotypes observed in some studies (Stine et al., 2017; Söderlund et al., 2018; Saticioglu et al., 2019; Kim et al., 2023b) suggest the presence of new or complex resistance mechanisms in flavobacteria. WGS provides an alternative approach to predict AMR profile but requires comprehensive and accurate AMR gene databases, which are currently not available for *Flavobacterium* spp. Recent studies also highlight the importance of efflux pumps in multidrug resistance and pathogenicity of *Flavobacterium* spp. (Clark et al., 2009; Chen et al., 2017; Zhang et al., 2017; Mata et al., 2018; Castillo et al., 2021; Chokmangmeepisarn et al., 2021; Declercq et al., 2021; Park et al., 2021), making them an interesting new area of research and a potential target for new therapeutics (Blair et al., 2014; Declercq et al., 2021).

The two media used for the AST of *F. branchiophilum* gave comparable results. Notably, several lesser-known *Flavobacterium* spp. did not grow using the standardised method recommended for *F. psychrophilum* (CLSI, 2020a) but showed good growth using this method adjusted for AST of *F. branchiophilum* (Appendix 6). Therefore, DCAMHB+C appears to be a suitable growth medium for the AST of *F. branchiophilum* and other more fastidious flavobacteria. It is worth noting that the divalent cations present in DCAMHB+C in higher concentrations are known to reduce the biological activity of quinolones (Pursell et al., 1995) and

tetracyclines (Burka et al., 1997). Consequently, the measured MICs for ENRO, OXO, and OXY may have been underestimated (Appendix 5, Appendix 6).

Classification of *F. branchiophilum* and the lesser-known *Flavobacterium* spp. as WT or NWT was not possible for any of the antimicrobials tested. Slovene *Flavobacterium* isolates of the same species exhibited similar AMR profiles. Notably, some species had MICs at the upper end of the concentration ranges tested for all 10 antimicrobials, namely *F. branchiarum, F. collinsii*, *F. hercynium*, *F. oncorhynchi*, *F. piscis,* and FX23. Additionally, at least one isolate of other tested species demonstrated an MIC above the concentration required for resistance of *F. psychrophilum* to AMP (*F. bernardetii, F. celericrescens, F. erciyesense, F. tiangeerense, F. tructae*, FX2, FX3, FX5, FX9, FX10, FX15, FX18, FX27, FX32, and FX36), ERY (*F. tructae* and FX36), FLO (*F. tructae*), OXY (*F. branchiophilum*, *F. tructae*, FX2, FX5, FX10, and FX36), and the quinolones ENR (*F. branchiophilum, F. celericrescens*, *F. difficile*, *F. erciyesense*, *F. tructae*, FX9, FX10, FX15, FX32, and FX36), and OXO (*F. celericrescens*, FX9, FX15, and FX36). Furthermore, all other tested *Flavobacterium* spp. exceeded the tested CTZ concentration range, all species except *F. branchiophilum* grew at the upper end or above the tested TRS concentration range, and several species grew in the middle or at the upper end of the tested concentration ranges of GEN (*F. erciyesense*, *F. tiangeerense*, *F. tructae,* FX2, FX9, FX10, FX15, FX27, FX32, and FX36) and MER (*F. tructae,* FX27, and FX36). This is consistent with previous studies demonstrating that *Flavobacterium* isolates can grow in high concentrations of various antimicrobials, suggesting the presence of intrinsic and/or acquired resistance mechanisms that contribute to reduced susceptibility to multiple antimicrobials (Hesami et al., 2010; Henríquez-Núñez et al., 2012; Mwega et al., 2020; Heckman et al., 2023). There are no other studies in which the broth microdilution method for AST of *F. branchiophilum* was used for comparison. There is a significant knowledge gap regarding the AMR of this important fish pathogen, likely due in part to its fastidious nature. Additionally, only two studies used the standardised method recommended for *F. psychrophilum* for the AST of lesser-known flavobacteria (Mwega et al., 2020; Heckman et al., 2023). In both studies, 16S rRNA gene sequencing was used for the identification of

Flavobacterium isolates. Heckman et al. (2023) investigated, among others, isolates most closely related to *F. branchiarum*, *F. collinsi*, *F. oncorhynchi*, *F. tiangeerense,* and *F. tructae*, which were also investigated in this study. Mwega et al. (2020) reported combined results for all tested isolates, including *Chryseobacterium* spp. Therefore, a comparison of results for individual species was not possible. As in this study, Heckman et al. (2023) also observed high MICs for amoxicillin, ENR, ERY, FLO, GEN, OXY, and TRS in isolates most closely related to *F. branchiarum*, *F. collinsi*, *F. oncorhynchi,* and *F. tructae*. In contrast to our findings, they reported high MICs for OXY in isolates most closely related to *F. tiangeerense*. Similar to this research, Mwega et al. (2020) reported that all tested isolates showed reduced susceptibility to AMP, GEN, and OXY. They also observed that the isolates were predominantly (97%) susceptible to ENR. However, the given MIC range exceeded the ECV_FP for ENR. Contrary to our results, Mwega et al. (2020) reported that all tested isolates showed reduced susceptibility to ERY and that the isolates were moderately susceptible to FLO (62.7%, MICs 1–8 µg/ml) and TRS (77.6%, MICs 0.5/9.5–2/38 µg/ml). Notably, the MICs reported for TRS were comparable to the results of this study. Both Heckman et al. (2023) and Mwega et al. (2020) observed high MICs for ceftiofur, another third-generation cephalosporin. In this study, the MICs for all tested isolates of *F. branchiophilum* and lesser-known *Flavobacterium* spp. exceeded the highest CTZ concentration tested, further supporting the assumption of intrinsic resistance of flavobacteria to this antimicrobial.

5.3 INFLUENCE OF WATER QUALITY PARAMETERS ON THE ONSET OF FLAVOBACTERIOSIS

Water quality is considered an important factor contributing to the outbreak of flavobacterial diseases (Decostere et al., 1999c; Barnes and Brown, 2011; Strepparava et al., 2014). However, despite thorough database searches aimed at finding studies investigating this relationship, only a few reports were found (Holt et al., 1989; Bullock, 1990; Soltani and Burke, 1995; Nematollahi et al., 2003b; Starliper, 2011; Good et al., 2015; Elmore, 2016). The water quality parameters recorded during this research were within the acceptable range for rainbow trout

aquaculture (Molony, 2001; EFSA, 2008). The water temperature in all sampled fish tanks ranged between 6.6 and 15.8 °C, pH between 7.45 and 8.42, and dissolved oxygen between 7.28 and 12.11 mg/l. We observed a significant seasonal and spatial variability in water quality parameters across the studied fish farms. Seasonal variations were consistent with expected climatic influences, while differences among farms highlight the unique physico-chemical profiles of each site, likely due to different water sources, local environmental conditions, and farm management practices. Significant differences were also found between the water quality parameters at the inlet and outlet of the fish tanks, which is probably due to the influence of the metabolic activity of the fish.

Our results showed a significant association between infection with *F. psychrophilum* and increased water pH at the inlet as well as the winter season. In contrast to our results, Elmore (Elmore, 2016) observed a correlation between *F. psychrophilum* abundance and a lower pH in one farm and an increased temperature in another farm. A literature search revealed no previous studies that confirmed the association we found between water pH and infection risk. The association between *F. psychrophilum* infections and the winter season observed in this study confirms the known cold-adapted nature of the bacterium. However, the pathogen caused disease in all seasons during the study period as water temperatures ranged from 6.6 to 15.8 °C, which is consistent with literature describing that *F. psychrophilum* infections occur most frequently at water temperatures between 4 and 15 °C (Holt et al., 1993). The highest mortality rate and shortest time from infection to death were reported at 15 °C (Holt et al., 1989), which was consistent with the fastest doubling time for this bacterium in culture media (Holt et al., 1993).

In contrast, *F. branchiophilum* infection correlated significantly with increased water temperatures, and a weak but significant negative correlation was observed with dissolved oxygen at the outlet. *F. branchiophilum* was only isolated during summer samplings and strongly correlated with this season. These results suggest that higher water temperatures and possibly lower dissolved oxygen favour the occurrence of *F. branchiophilum* infections. Additionally, prolonged heavy rainfall

was observed before summer sampling in F1 and F2, increasing water turbidity and possibly contributing to *F. branchiophilum* infections. Similarly, other studies also observed that *F. branchiophilum* infections often occur in association with reduced dissolved oxygen (Bullock, 1990; Good et al., 2015), increasing water temperature (15–19 °C) in spring and summer (Bullock et al., 1994; Ostland et al., 1995a) and water turbidity due to sediment disturbance during heavy rainfall, supporting these findings. It is suspected that particulate material irritates the gills and that the sediment may be a reservoir for *F. branchiophilum* (Starliper, 2011).

These results indicate that different environmental conditions influence the odds of infection with *F. psychrophilum* and *F. branchiophilum*. The association between infection and water temperature, increased pH, and decreased dissolved oxygen is probably due to a combination of stress-related immunosuppression in fish and already described environmental preferences of the two pathogens. Inappropriate water temperature, pH, and dissolved oxygen levels are known stressors in fish aquaculture (Rebl et al., 2020). Wagner et al. (1997) found that high water pH (8.4–9.6) and low temperature (7–9 °C) significantly increased stress in rainbow trout, especially when occurring concurrently, which may have contributed to the association we found between these parameters and *F. psychrophilum* infections. They also investigated the influence of high-water temperatures (19 and 22 °C) on fish stress but found no significant association. On the other hand, Uiuiu et al. (2021) found that elevated water temperatures in combination with low dissolved oxygen levels in the summer season significantly increased physiological stress in rainbow trout and led to immunosuppression, which may have contributed to the association we found between these parameters and *F. branchiophilum* infections. Additionally, when water temperature and pH (> 7.5) increase, the ammonia equilibrium shifts towards undissociated ammonia (NH_3), which is highly toxic to fish (Ferguson, 2006; Roberts, 2012). It is important to recognise that other unstudied water quality parameters may also influence the occurrence of flavobacterial diseases. In this study, we found that water quality parameters showed significant differences between seasons and individual fish farms, highlighting the need for customised management depending on the season and the specific conditions of each location. Therefore, regular monitoring of water

quality parameters, namely temperature, pH, and dissolved oxygen, while considering seasonal associations, can help predict and control the increased risk of infection with *F. psychrophilum* and *F. branchiophilum*.

5.4 CONCURRENT INFECTIONS

Concurrent infections in fish are common, including with *F. psychrophilum* (Starliper, 2011), and it is important to consider the influence of their synergistic or antagonistic interactions on the occurrence and severity of fish diseases. However, the available data on this subject in fish are sparse (Kotob et al., 2016; Chowdhury et al., 2021).

The findings of this research show a high prevalence of concurrent infections in the assessed fish. We observed multiple different fish pathogens, most frequently parasites, namely *Acanthocephalus* spp., amoebae, *Apiosoma* spp., *Chilodonella* spp., *Dermocystidium* spp., *Epistylis* spp., *I. necator, I. multifiliis,* Monogenea (*Gyrodactylus* spp. or *Dactylogyrus* spp.), *T. bryosalmonae, Trichodina* spp., and *Riboscyphidia* spp. Other bacterial pathogens, namely *A. salmonicida* subsp. *salmonicida*, *Aeromonas* spp., *C. maltaromaticum*, *C. gillenii*, *L. garvieae, Pseudomonas* spp., and *R. salmoninarum,* as well as lesions consistent with epitheliocystis disease and the fungus *Saprolegnia* spp., were also observed. The Pearson chi-squared test showed that infection with *F. psychrophilum* was associated with infestation of gills and skin with Monogenea. No other pathogens showed association with *F. psychrophilum* infections. Monogeneans can damage the skin and gill epithelium of fish and create entry points for bacterial invasion. Busch et al. (2003) found a weak indication of a possible enhancement of *F. psychrophilum* invasion by the presence of *Gyrodactylus derjavini* in rainbow trout, while mortality was associated with gyrodactylid infestation. Zhang et al. (2015) demonstrated that parasitism in goldfish (*Carassius auratus*) with *Dactylogyrus intermedius* can suppress the host's immune functions and enhance invasion of *F. columnare*. The exact mechanisms by which parasitic infections can promote bacterial invasion remain unclear, but may include direct damage to the skin or gill epithelium, creating entry points for bacteria (Buchmann and Bresciani,

1997), the parasite acting as a vector (Xu et al., 2012), or suppression of the host's immune response (Zhang et al., 2015).

6 CONCLUSIONS

Hypothesis 1: Flavobacteria are widespread in rainbow trout from Slovene fish farms and affect fish health.

This study confirmed the presence of the known fish pathogens *F. psychrophilum* and *F. branchiophilum* in Slovene farmed rainbow trout and described the gross and histopathological lesions associated with the infection. *F. psychrophilum* was isolated from fish across all studied farms and in all seasons, while *F. branchiophilum* was isolated only at LONG samplings in summer. Analysis of the histopathological lesions for the lesser-known *Flavobacterium* spp. showed that *F. hercynium* and *F. hydatis* can indeed be pathogenic for fish. Numerous other lesser-known flavobacteria were also isolated at each sampling. However, further studies are needed to elucidate their pathogenic potential. As only four farms were studied, which may not be representative of all Slovene fish farms, further investigations are needed to confirm the widespread presence of flavobacteria in Slovene farmed rainbow trout.

Hypothesis 2: Environmental factors, such as water temperature, pH, and dissolved oxygen in the water, influence the dynamics of flavobacterial infections.

F. psychrophilum infections were significantly associated with increased pH at the inlet and the winter season, although the pathogen caused disease in all seasons during the study. In contrast, *F. branchiophilum* infections correlated significantly with increased water temperatures, decreased dissolved oxygen at the outlet, and the summer season. These results suggest that different environmental conditions influence the odds of infection with the two pathogens. Monitoring temperature, pH, and dissolved oxygen, considering seasonal associations, can help to predict and control the increased risk of infection with *F. psychrophilum* and *F. branchiophilum*.

Hypothesis 3: Flavobacteria isolated from rainbow trout in Slovene fish farms are multidrug-resistant.

The Slovene *Flavobacterium* spp. isolates were predominantly multidrug-resistant, with *F. psychrophilum* showing high NWT frequencies for ENR, OXO, and OXY, as well as high MICs for CTZ and TRS. Six lesser-known *Flavobacterium* spp. had MICs at the upper end of the tested concentration ranges for all 10 antimicrobials. Most other *Flavobacterium* isolates tested also exhibited high MICs across at least three antimicrobial classes. The consistently high MICs for CTZ and TRS indicate potential intrinsic resistance of most flavobacteria. In *F. psychrophilum*, a substitution in GyrA was associated with reduced susceptibility to OXO. No other AMR genes or AMR-associated mutations were detected in the genomes of *F. psychrophilum* and *F. branchiophilum* isolates.

Additional scientific contribution:

This research is the first to confirm the presence of known pathogens, *F. psychrophilum* and *F. branchiophilum*, in Slovene rainbow trout aquaculture. It is also one of the few studies that analysed the association between the isolation of flavobacteria from naturally infected fish and the presence of semiquantitatively assessed gross and histopathological lesions. This study identified 41 putative new *Flavobacterium* species and contributed to the expansion of the in-house *Flavobacterium* MALDI-TOF MS database at the Veterinary Faculty, University of Ljubljana, thus promoting research and routine diagnostic applications. This is one of a limited number of studies using the standardised AST method for *F. psychrophilum,* providing important information for the selection of appropriate antimicrobial treatment. Additionally, we adjusted the standardised AST method to support the growth of *F. branchiophilum* and other more fastidious flavobacteria. This is the first study to use the broth microdilution method for AST of *F. branchiophilum* and one of the few to investigate AMR of *F. branchiophilum* and lesser-known flavobacteria. This research provides the foundation for further studies on biodiversity and the role of flavobacteria in fish health and disease.

7 SUMMARY

Pathogenic *Flavobacterium* spp., namely *F. psychrophilum*, *F. branchiophilum,* and species of the *F. columnare* group, are recognised worldwide as major causative agents of bacterial diseases in freshwater fish, especially rainbow trout. Additionally, a growing number of lesser-known *Flavobacterium* spp. have been implicated in disease outbreaks, often based on their isolation from clinically diseased fish, yet little is known about their pathogenicity. Despite the worldwide importance of *Flavobacterium* spp. as fish pathogens, their occurrence in Slovene farmed fish has not been investigated so far. This research aimed to address several important knowledge gaps in fish-associated *Flavobacterium* spp. We investigated the biodiversity of flavobacteria in farmed rainbow trout and the pathological lesions associated with flavobacterial disease. A standardised method was used for AST of *F. psychrophilum*, and AST was also performed for *F. branchiophilum* and lesser-known *Flavobacterium* spp. because such studies are limited or lacking. Additionally, this study investigated the association between flavobacterial infections and selected water quality parameters, because water quality is considered an important factor contributing to the occurrence of flavobacterial diseases, but few studies have investigated this relationship.

We sampled a total of 540 rainbow trout, namely longitudinally across all seasons in two farms (F1 and F2) and in two other farms (F3 and F4) following mortality reports. At each sampling, 27 diseased and 27 apparently healthy fish were necropsied, and samples were taken for bacteriological, histopathological, and parasitological investigations. At necropsy, we measured the body weight and total length of the fish, calculated the K for each fish, and semiquantitatively assessed the gross lesions in the skin, fins, opercula, eyes, gills, spleen, kidney, liver, stomach with pyloric caeca, and intestine. The necropsy fish index was calculated from these data. The histopathological lesions in the skin, gills, heart, spleen, kidney, liver, pancreas, stomach with pyloric caeca, and intestine were also assessed semiquantitatively, and for each organ index and reaction indices were calculated. For the isolation of flavobacteria, skin, gill, spleen, kidney, and liver samples were inoculated on AO agar and MS agar supplemented with tobramycin.

Using MALDI-TOF MS, we confirmed the presence of the known fish pathogens *F. psychrophilum* and *F. branchiophilum*. Notably, no species belonging to the *F. columnare* group were detected. At least one *Flavobacterium* spp. was isolated from one or more organs in 89.3% of the sampled fish. Our results suggest that MALDI-TOF MS can reliably identify certain *Flavobacterium* species if corresponding MSPs are available in the database. WGS and ANI analyses of selected isolates (n = 81) revealed that 74.1% of isolates represented 41 putative new *Flavobacterium* species. The data also showed that species-level identification based on 16S rRNA and *gyrB* gene sequencing is not highly reliable for this genus because many isolates (56.7%) representing putative new species exceeded the 99% 16S rRNA gene sequence identity threshold commonly used for species-level classification of *Flavobacterium*. These results confirm that ANI analysis is the most reliable method for the identification of *Flavobacterium* spp. Further comparative studies are required to assess the accuracy and limitations of MALDI-TOF MS in identifying lesser-known or closely related species within this diverse genus.

F. psychrophilum and *F. branchiophilum* were isolated mainly from diseased fish (77.3% and 72.73%, respectively) and also from apparently healthy fish, and various gross lesions were observed in both groups. Therefore, we defined a necropsy fish index threshold of ≥ 5.5 for the statistical analyses to distinguish between INF fish and NH individuals. Our results show that *F. psychrophilum* was the most prevalent (128/540; 23.7%) pathogenic *Flavobacterium* in Slovene farmed rainbow trout because it was isolated across all fish farms and in all seasons. We observed significant differences in infection prevalence between farms, with F3 and F4 having higher infection rates, suggesting that *F. psychrophilum* likely played a role or contributed to the increased mortality observed in these fish farms. *F. psychrophilum*-INF fish had lower body weight and total length, which was consistent with the established understanding that younger fish are more susceptible to infection, although fish at different life stages were affected. The difference in K between *F. psychrophilum*-INF and NH fish was not significant, suggesting that infection did not affect the body condition of the fish. *F. psychrophilum* infection was associated with a significantly higher necropsy

fish index, as well as score values of all organs except the opercula, stomach with pyloric caeca, and intestine. We documented a wide range of gross lesions in the POS fish, many of which were consistent with previous literature, such as skin and fin erosion and ulceration, gill anaemia and thickening, splenomegaly, and other lesions consistent with systemic infection. Notably, we also observed lesions that have not yet been reported in *F. psychrophilum* infections, including gross enlargement of the liver and kidney in association with histopathological lesions. The histopathological analysis confirmed significant differences between INF and NH fish in all organs examined except the pancreas. The regressive, inflammatory, and circulatory reaction indices were significantly higher in most of the organs examined in *F. psychrophilum*-INF fish, while the progressive indices were significantly different only in the gills, spleen, and kidney. Lesions in the skin, gills, and liver included haemorrhage, necrosis, degeneration, and infiltration by lymphocytes, macrophages, and/or neutrophils, many of which have already been described in the literature. In the kidney and spleen, we found hyperplasia of the haematopoietic and white pulp tissues, respectively, as well as white pulp atrophy, lesions that have rarely or never been described in previous studies and indicate immune activation during the infection. Although the spleen is often reported as the most affected organ, we found no significant differences in haemorrhage, necrosis, and inflammation of the splenic capsule or parenchyma. Some gastrointestinal lesions, such as intercellular oedema, vacuolar degeneration and necrosis in the gastric glands, and lymphocytic infiltration in the mucosa, showed significant differences between INF and NH fish, despite being rarely or never reported in previous studies. Our findings contribute new insights into the histopathological lesions caused by *F. psychrophilum* in naturally infected rainbow trout.

F. branchiophilum was isolated from the gills of 11 fish (11/540; 2.04%) from F1 and F2 during summer sampling. Due to its fastidious growth, the bacterium may have been underdetected. The infected fish showed a wide range of body sizes, indicating susceptibility to infection at different life stages. As with *F. psychrophilum*, no significant difference in K was found between *F. branchiophilum*-INF fish and NH fish, while INF fish had significantly higher

necropsy fish indices, as well as score values for gills and spleen. Gross lesions included anaemia, gill filament clubbing, and shortened opercula. Analysis of histopathological variables showed that only the fusion of gill filaments showed a significant difference between INF and NH fish, suggesting severe epithelial hyperplasia, although other gill lesions, such as haemorrhage, necrosis, and inflammation, were also observed. These findings are consistent with previous descriptions of pathological lesions in BGD.

A total of 2652 *Flavobacterium* isolates were identified by MALDI-TOF MS, of which 2105 were preserved. However, due to the high species diversity within the genus and the limited number of *Flavobacterium* MSPs available in the Bruker MALDI-TOF MS reference database, 75.98% of all *Flavobacterium* isolates could not be identified to species level during this research. This was the main limitation of the study because the low number of isolates that could be identified to species level prevented the analysis of pathological lesions for most of the lesser-known *Flavobacterium* spp. Analysis of histopathological lesions was only possible for *F. hercynium, F. hydatis,* and *F. frigidimaris* isolated from gills and skin of diseased and apparently healthy fish. The results showed that the score values for skin ulceration and gill epithelium hyperplasia and hypertrophy were significantly higher in fish INF with *F. hercynium* and *F. hydatis*, respectively, indicating that these species may indeed be pathogenic to fish as previously proposed. However, the analysis for *F. frigidimaris* did not show any significantly different lesions, indicating that this species was not pathogenic to the fish in this research. Several other lesser-known and putative new species were isolated from internal organs or in greater numbers from the gills and/or skin of fish showing gross and/or histopathological lesions, suggesting pathogenic potential. The extensive collection of stored isolates allows us to conduct future studies to determine their species identity, evaluate their pathogenic potential, and investigate their role as pathogens, commensals, or mutualists. It is important to note that the fish used in this research were naturally infected and that some of the lesions observed may have been influenced by co-infection with other pathogens.

AST was performed for 10 antimicrobials representing seven different antimicrobial classes using the standardised broth microdilution method recommended for *F. psychrophilum*. Most of the antimicrobials tested are commonly used in aquaculture, including FLO and OXY, which are approved for use in Slovenia. A total of 106 *Flavobacterium* isolates representing 24 different species were subjected to AST. The results showed that *Flavobacterium* spp. were predominantly multidrug-resistant. *F. psychrophilum* showed high NWT frequencies for OXY (81.5%) and quinolones (87.0% for ENR and 90.7% for OXO), as well as high MICs for CTZ and TRS. However, all isolates were WT for FLO, which remains a suitable drug of choice for the treatment of *F. psychrophilum* infections. The lack of a standardised AST method and ECVs for *F. branchiophilum* and lesser-known flavobacteria prevented differentiation between WT and NWT phenotypes. Nevertheless, most of the lesser-known *Flavobacterium* spp. showed high MICs across at least three antimicrobial classes, and some had high MICs for all 10 antimicrobials tested. The adjustment of the standardised method recommended for *F. psychrophilum* enabled a successful AST of *F. branchiophilum* and other fastidious flavobacteria and may contribute to the development of a standardised method for *F. branchiophilum*. The species tested with the adjusted method showed relatively low MICs for OXY and quinolones, which may be due to the higher concentration of divalent cations in the growth medium, which are known to reduce the biological activity of these antimicrobials. The consistently high MICs for CTZ and TRS indicate potential intrinsic resistance of most flavobacteria. In *F. psychrophilum*, a substitution in GyrA, previously associated with quinolone resistance, was consistent with reduced susceptibility to OXO but not ENR. No other AMR genes or AMR-associated mutations were detected in the genomes of *F. psychrophilum* and *F. branchiophilum* isolates. The multidrug resistance observed in flavobacteria, combined with the limited understanding of resistance mechanisms, highlights the importance of performing AST before treatment and the need for further studies comparing phenotypic and genotypic resistance.

Selected water quality parameters, namely water temperature, pH, and dissolved oxygen, were measured at the inlet and outlet of each sampled fish tank. The

water quality parameters showed significant differences between seasons and fish farms. We investigated the relationship between *F. psychrophilum* and *F. branchiophilum* infections and water quality parameters and found a significant association between *F. psychrophilum* and increased water pH at the inlet and the winter season. The latter finding supports the known cold-adapted nature of this bacterium. However, the association between *F. psychrophilum* infection and increased pH has not been reported previously. In contrast, *F. branchiophilum* was only isolated in summer, and infections correlated significantly with increased water temperatures and decreased dissolved oxygen at the outlet. Heavy rainfall and increased water turbidity were also noted before summer samplings. These findings are consistent with the literature. The observed association between infection risk and water quality parameters is probably due to a combination of stress-related immunosuppression in fish and environmental preferences of pathogens. Previous studies have shown that high pH and low temperatures or high temperatures and low dissolved oxygen increase physiological stress in rainbow trout. Moreover, high water pH and temperature can increase toxic ammonia levels. Seasonal and farm-specific differences in water quality emphasise the need for tailored, site-specific monitoring. Regular monitoring of water temperature, pH, and dissolved oxygen, while considering seasonal associations, can help predict the increased risk of *F. psychrophilum* and *F. branchiophilum* infection, facilitating timely preventive management strategies.

We found a high prevalence of concurrent infections in fish, particularly involving parasites and bacterial pathogens. Infections with *F. psychrophilum* were significantly associated with infestation of the gills and skin with Monogenea, possibly indicating a predisposing interaction. Monogenea may facilitate bacterial invasion by damaging epithelial barriers, suppressing host immunity, or acting as a vector. No other pathogens were associated with *F. psychrophilum* infections. These findings highlight the importance of considering co-infections in the dynamics of fish diseases.

This research provides information on the biodiversity of fish-associated flavobacteria in Slovene rainbow trout aquaculture, provides detailed information

and new insights into the pathological lesions associated with *F. psychrophilum* and *F. branchiophilum* infections, and supports the development of targeted treatment and preventive strategies. It also provides the foundation for further studies on the biodiversity of flavobacteria, their characteristics, and their impact on fish health, including the pathological lesions associated with infections by lesser-known *Flavobacterium* species.

8 POVZETEK

Flavobakterije so po Gramu negativne, aerobne, rumeno pigmentirane paličaste ali filamentozne bakterije. Rod *Flavobacterium* trenutno vsebuje več kot 320 vrst, vendar število novo odkritih in opisanih vrst še vedno hitro narašča. Flavobakterije so ubikvitarne v naravi in del normalne mikroflore rib in ribjih iker, nekatere vrste pa so oportunistični patogeni, ki lahko kažejo različno virulenco. Patogene vrste iz rodu *Flavobacterium*, predvsem *F. psychrophilum*, *F. branchiophilum* ter vrste iz skupine *F. columnare*, so po vsem svetu poznane kot pomembni povzročitelji bakterijskih bolezni pri sladkovodnih ribah. Flavobakterije lahko povzročajo bolezni pri različnih vrstah rib, vendar so postrvi, zlasti šarenke, najbolj občutljive za okužbo. V zadnjem času čedalje več raziskav opisuje manj poznane vrste flavobakterij v povezavi z izbruhi bolezni, pogosto zgolj na podlagi njihove izolacije iz klinično bolnih rib. Po drugi strani pa je o patogenosti manj poznanih vrst flavobakterij in z okužbami povezanih patomorfoloških spremembah le malo znanega. Glede na globalno razširjenost flavobakterij ter klinične znake in patoanatomske spremembe, opažene pri gojenih ribah v Sloveniji, so verjetno flavobakterijske bolezni pogoste tudi v slovenskih ribogojnicah. V Sloveniji doslej ni bilo opravljene raziskave na to temo, zato podatki o vrstah iz rodu *Flavobacterium* pri ribah niso na voljo. Čeprav je bilo v zadnjem stoletju v svetu opravljenih veliko raziskav, katerih namen je bil preprečevanje in obvladovanje izbruhov flavobakterioz, je bil napredek omejen zaradi hitrega taksonomskega razvoja rodu in zahtevnosti izolacije patogenih flavobakterij. Namen naše raziskave je bil obravnavati več pomembnih vrzeli v znanju o flavobakterijah. V raziskavi smo preučevali biodiverziteto flavobakterij pri gojenih šarenkah ter patološke spremembe, ki so povezane s flavobakterijskimi okužbami. Za testiranje protimikrobne občutljivosti (AST) *F. psychrophilum* smo uporabili standardizirano metodo; poleg tega smo testirali tudi izolate *F. branchiophilum* in manj znanih vrst *Flavobacterium*, saj so tovrstne raziskave redke ali pa še niso bile opravljene. Analizirali smo tudi povezavo med flavobakterijskimi okužbami in izbranimi parametri kakovosti vode. Kakovost vode se na splošno razume kot pomemben dejavnik pri pojavu flavobakterijskih okužb, vendar pa je raziskav na to temo malo.

Skupno smo vzorčili 540 šarenk, in sicer longitudinalno skozi vse letne čase v dveh ribogojnicah (F1 in F2) ter v dveh drugih ribogojnicah (F3 in F4) po poročanju o povišanem poginu rib. Pri longitudinalnih vzorčenjih smo ribe odvzeli iz treh različnih bazenov, pri individualnih pa le iz enega bazena. Ob vsakem vzorčenju smo opravili raztelesbo 27 klinično bolnih in 27 na videz zdravih rib ter odvzeli vzorce za bakteriološko, histopatološko in parazitološko preiskavo. Ob vsakem vzorčenju smo pregledali vse bazene v ribogojnici in za nadaljnjo analizo izbrali tiste, v katerih so bile ribe z najbolj izraženimi kliničnimi znaki bolezni. Ribe smo opredelili kot bolne, če so kazale klinične znake, kot so letargija, neješčnost, plavanje v bližini vodne gladine ali na iztoku, pospešeno in poudarjeno gibanje škržnih poklopcev, nepravilno plavanje ter patoanatomske spremembe, kot so napihnjenost trebuha, krvavitve, erozije in ulceracije kože in/ali plavuti, hipopigmentacija ali hiperpigmentacija kože ter izbočene oči (eksoftalmus). Ribe smo opredelili kot na videz zdrave, če so kazale normalno obnašanje in niso imele zunanjih patoanatomskih sprememb. Pri raztelesbi smo izmerili telesno maso in celotno telesno dolžino rib, izračunali Fultonov kondicijski faktor (K) ter semikvantitativno ocenili patoanatomske spremembe kože, plavuti, oči, škržnih poklopcev, škrg, vranice, ledvic, jeter, želodca s piloričnimi podaljški in črevesja ter izračunali indeks raztelesbe rib. Histopatološke spremembe kože, škrg, srca, vranice, ledvic, jeter, trebušne slinavke, želodca s piloričnimi podaljški in črevesja smo prav tako ocenili semikvantitativno. Na podlagi pridobljenih rezultatov smo izračunali indekse organov ter cirkulatorni, regresivni, progresivni in vnetni reakcijski indeks za vsak organ. Za izolacijo flavobakterij smo vzorce kože, škrg, vranice, ledvic in jeter nasadili na Anackerjevo in Ordalovo (AO) gojišče ter modificirano Shiehovo (MS) gojišče z dodanim tobramicinom; za splošno bakteriološko preiskavo pa smo vzorčili zgolj notranje organe in uporabili krvno gojišče.

Vzorčili smo ribe različnih starostnih kategorij, starosti od 4 do 33 mesecev ter z razponom telesne mase od 3 do 650 g, celotne telesne dolžine od 6,5 do 40 cm in K od 0,5 do 2,1. Z uporabo metode masne spektrometrije, ionizacija v matriksu z lasersko desorpcijo – čas preleta ionov (MALDI-TOF MS), smo potrdili znana patogena rib, *F. psychrophilum* in *F. branchiophilum*. Vrst iz skupine *F. columnare*

med raziskavo nismo izolirali, verjetno zaradi prenizke temperature vode, saj ta vrsta povzroča bolezen pri temperaturah nad 15 °C. Sekvenciranje multilokusnih zaporedij (MLST) je pokazalo, da so izolati *F. psychrophilum* (n = 7) pripadali različnim klonskim kompleksom, in sicer CC-ST1 in CC-ST10, ki sta zelo razširjena v Evropi in visoko virulentna za šarenko, ter več novo opredeljenim sekvenčnim tipom. Zaradi majhnega števila sekvenciranih izolatov bodo za boljšo opredelitev genetske raznolikosti slovenskih izolatov *F. psychrophilum* potrebne nadaljnje raziskave. Iz enega ali več organov smo pri 89,3 % vseh vzorčenih rib izolirali vsaj eno vrsto rodu *Flavobacterium*; najpogosteje smo jih izolirali iz škrg (70,2 % vzorčenih rib) in kože (81,1 %), redkeje pa iz notranjih organov (16,9 %). Pri večini rib smo s škrg in kože izolirali več različnih flavobakterij. Med drugim smo ugotovili tudi nekatere manj znane vrste, in sicer *F. araucananum, F. bernardetii, F. collinsii, F. frigidimaris, F. hercynium, F. hydatis, F. oncorhynchi, F. piscis, F. plurextorum* in *F. tructae*, ki so bile v predhodnih raziskavah ocenjene kot potencialno patogene, saj so bile izolirane iz zunanjih sprememb ali iz notranjih organov. Od vseh naštetih manj znanih flavobakterij je bila patogenost eksperimentalno dokazana le za vrsto *F. tructae*. Morfologija kolonij flavobakterij je bila zelo raznolika, tudi znotraj posamezne vrste, medtem ko so si bile nekatere različne vrste morfološko zelo podobne, zato je natančna identifikacija izolatov vedno priporočljiva. V histoloških preparatih škrg in kože nekaterih rib, pri katerih nismo ugotovili *F. psychrophilum* in *F. branchiophilum*, smo opazili filamentozne bakterije, kar nakazuje, da izolacija ni bila vedno uspešna ali pa so bile prisotne bakterije morda predstavnice manj znanih vrst rodu *Flavobacterium*. Naši rezultati kažejo, da je zanesljiva identifikacija določenih vrst *Flavobacterium* z uporabo MALDI-TOF MS mogoča, če so v MALDI-TOF MS podatkovni bazi na voljo ustrezni MSP. Sekvencirali smo celotni genom (WGS) 81 izolatov, izbranih na podlagi izolacije iz notranjih organov ali izolacije v večjem številu iz škrg in kože, ter pogosto opažene morfologije kolonij in/ali rezultatov MALDI-TOF MS ter izvedli analizo povprečne nukleotidne identitete (ANI) izolatov. Ugotovili smo, da je 74,1 % izolatov predstavljalo 41 domnevno novih vrst *Flavobacterium*, vendar so za njihovo priznanje kot veljavno opisane nove vrste potrebne nadaljnje raziskave. Ugotovili smo tudi, da identifikacija na nivoju vrste na podlagi zaporedij genov 16S rRNA in *gyrB* ni zelo zanesljiva za ta rod, saj je 56,7 % izolatov, ki so predstavljali

domnevno nove vrste, imelo 99–100 % identičnost zaporedja gena 16S rRNA z najbližjimi ujemanji, kar presega mejno vrednost (99 %), ki se običajno uporablja za razlikovanje med vrstami znotraj rodu *Flavobacterium*. Ti rezultati potrjujejo, da je ANI analiza trenutno najzanesljivejši pristop za določanje vrst znotraj rodu *Flavobacterium*. Za oceno natančnosti in omejitev metode MALDI-TOF MS pri določanju manj znanih ali sorodnih vrst flavobakterij bodo potrebne dodatne raziskave v kombinaciji s WGS.

F. psychrophilum in *F. branchiophilum* smo izolirali tako iz klinično bolnih (77,3 % in 72,73 % pozitivnih rib) kot iz na videz zdravih rib. Tako pri klinično bolnih kot pri na videz zdravih ribah smo ugotovili različne patoanatomske spremembe, zato smo za potrebe statistične analize uvedli mejno vrednost indeksa raztelesbe rib ≥ 5,5, na podlagi katere smo razlikovali med okuženimi (INF) in negativnimi zdravimi (NH) ribami.

V okviru naše raziskave je bila *F. psychrophilum* najpogosteje izolirana patogena flavobakterija pri slovenskih gojenih šarenkah (128/540; 23,7 %), saj smo jo ugotovili v vseh proučevanih ribogojnicah in v vseh letnih časih. Ugotovili smo statistično značilne razlike v prevalenci okužb med posameznimi ribogojnicami, pri čemer je bila prevalenca v F3 in F4 višja kot v F1 in F2. To nakazuje, da je okužba s *F. psychrophilum* verjetno pomembno prispevala k povečani smrtnosti rib v teh dveh ribogojnicah. *F. psychrophilum*-INF ribe so imele statistično značilno nižjo telesno maso in celotno telesno dolžino v primerjavi z NH ribami, kar je skladno s podatki iz literature, da je zarod bolj dovzeten za okužbo. Vendar pa smo v raziskavi ugotovili okužbo pri ribah različnih razvojnih stopenj. Razlika v K med INF in NH ribami ni bila statistično značilna, kar kaže, da okužba ni pomembno vplivala na telesno kondicijo rib. *F. psychrophilum*-INF ribe so imele statistično značilno višji indeks raztelesbe ter višjo oceno patoloških sprememb v vseh organih (angl. organ score value), razen škržnih poklopcev, želodca s piloričnimi podaljški in črevesja. Pri INF ribah smo ugotovili različne patoanatomske spremembe, od katerih so bile številne že opisane v literaturi, med drugim erozije in ulceracije kože in plavuti ter pri nekaterih ribah popolno izgubo repne plavuti in globoko ulceracijo repnega stebla (bolezen repnega stebla) ali pa rep v obliki črke

S. Ugotovili smo tudi zadebelitev škrg, anemijo škrg in notranjih organov, povečanje vranice ter druge spremembe, značilne za sistemsko okužbo. Opazili pa smo tudi patoanatomske spremembe, ki do zdaj še niso bile opisane pri okužbi s *F. psychrophilum*, kot sta povečanje jeter in ledvic, v katerih smo s histopatološko preiskavo nato ugotovili cirkulatorne, regresivne in vnetne spremembe.

Z analizo rezultatov histopatološke preiskave smo potrdili statistično značilne razlike med *F. psychrophilum*-INF in NH ribami za vse analizirane organe, razen za trebušno slinavko. Cirkulatorni, regresivni in vnetni reakcijski indeksi so bili pri INF ribah značilno višji v večini pregledanih organov, medtem ko je bila razlika za progresivni reakcijski indeks statistično značilna le za škrge, vranico in ledvica. S histopatološko preiskavo smo pri *F. psychrophilum*-INF ribah ugotovili krvavitve, nekroze, degenerativne spremembe ter infiltracijo limfocitov, makrofagov in nevtrofilcev v večini pregledanih organov. Vendar pa smo statistično značilne razlike med *F. psychrophilum*-INF in NH ribami potrdili le za nekaj sprememb. Največ statistično značilnih sprememb smo ugotovili v koži in škrgah *F. psychrophilum*-INF rib; v koži so te spremembe vključevale hidropsko ali vakuolarno degeneracijo, nekrozo, erozijo, ulceracijo ter infiltracijo epidermisa z nevtrofilci, krvavitve in nekrozo dermisa, izgubo ali resorpcijo lusk ali kosti, krvavitve, hidropsko degeneracijo in Zenkerjevo degeneracijo (nekrozo) skeletne prečno progaste mišičnine, infiltracijo dermisa in skeletne mišičnine z limfociti, makrofagi in nevtrofilci ter prisotnost seroznega ali fibrinskega eksudata. V škrgah *F. psychrophilum*-INF rib smo statistično značilno pogosteje ugotovili krvavitve in nekroze v lamelah in filamentih, hidropsko ali vakuolarno degeneracijo, nekrozo, hipertrofijo in hiperplazijo epitelnih celic, ki vodi v zraščanje lamel, ter infiltracijo lamel in filamentov z limfociti, makrofagi in/ali nevtrofilci. V ledvicah INF rib smo ugotovili statistično značilne razlike za hiperplazijo hematopoetskega tkiva, v vranici pa za aktivacijo retikuloendotelnega sistema (RES) in hiperplazijo ali pa atrofijo bele pulpe, kar nakazuje aktivacijo imunskega sistema ob okužbi. Te spremembe v literaturi še niso bile opisane ali pa so opisane le redko. V ledvicah smo ugotovili tudi hidropsko, vakuolarno in hialino kapljičasto degeneracijo ledvičnih tubulov ter zmanjšano regeneracijo tubulov pri INF ribah. Čeprav so

krvavitve, nekroze in vnetje parenhima in kapsule vranice v literaturi pogosto opisane, v naši raziskavi nismo ugotovili statistično značilnih razlik med INF in NH ribami. V jetrih INF rib smo dokazali statistično značilne razlike za krvavitve, zmanjšano vakuolizacijo hepatocitov, vakuolarno in/ali mikro- ali makrovezikularno maščobno degeneracijo, nekrozo ter infiltracijo parenhima z nevtrofilci. Nekatere spremembe v prebavilih, kot so intercelularni edem, vakuolarna degeneracija in nekroza želodčnih žlez ter limfocitna infiltracija sluznice, za katere smo ugotovili statistično značilne razlike med INF in NH ribami, v preteklih raziskavah še niso bile opisane ali pa so opisane zgolj redko.

F. branchiophilum smo izolirali iz škrg 11 rib (11/540; 2,04 %) iz ribogojnic F1 in F2 med poletnim vzorčenjem. Ob izolaciji smo opazili le posamezne kolonije, poleg tega pa je ta bakterija izredno zahtevna za gojenje, zato obstaja možnost, da izolacija ni bila vedno uspešna. Okužene ribe so bile različnih velikosti, kar kaže, da so šarenke dovzetne za okužbo v različnih življenjskih obdobjih. Podobno kot pri *F. psychrophilum* nismo zaznali značilnih razlik v K med *F. branchiophilum*-INF in NH ribami, medtem ko so imele INF ribe statistično značilno višji indeks raztelesbe ter višjo oceno patoloških sprememb v škrgah in vranici. Pri raztelesbi smo ugotovili anemijo škrg in pri eni ribi tudi anemijo vranice in ledvic, zadebelitev škrg, nekrozo konic škržnih filamentov pri eni ribi ter skrajšane škržne poklopce. Pri analizi podatkov histopatološke preiskave smo statistično značilno razliko med *F. branchiophilum*-INF in NH ribami ugotovili zgolj za zraščanje škržnih filamentov, kar nakazuje močno hiperplazijo epitela. Poleg tega smo ugotovili tudi druge spremembe, kot so krvavitve, nekroze in infiltracijo z limfociti, makrofagi in nevtrofilci. Naše ugotovitve se ujemajo s prejšnjimi opisi patoloških sprememb pri bakteriozi škrg (BGD).

S pomočjo metode MALDI-TOF MS smo identificirali 2652 izolatov iz rodu *Flavobacterium* ter 2105 izolatov shranili pri –70 °C. Zaradi visoke vrstne raznolikosti znotraj rodu *Flavobacterium* in omejenega števila referenčnih profilov masnih spektrov (MSP) v Brukerjevi MALDI-TOF MS bazi podatkov pri 75,98 % izolatih nismo mogli določiti vrste. To je bila tudi glavna omejitev raziskave, saj zaradi majhnega števila izolatov z zanesljivo identifikacijo vrste statistična analiza

patoloških sprememb za večino manj znanih vrst *Flavobacterium* ni bila mogoča. Analizo histopatoloških sprememb smo lahko izvedli le za vrste *F. hercynium*, *F. hydatis* in *F. frigidimaris*, ki smo jih izolirali iz škrg in kože bolnih ter na videz zdravih rib. Rezultati so pokazali, da so bile ocene za ulceracijo kože ter hiperplazijo in hipertrofijo epitela škrg statistično značilno višje pri ribah, okuženih s *F. hercynium* oziroma *F. hydatis*, kar potrjuje domnevo, da sta ti vrsti lahko patogeni za ribe. Glede na predhodne raziskave lahko *F. hydatis* povzroča podobne patološke spremembe, kot so opisane pri BGD. Pri analizi za *F. frigidimaris* v naši raziskavi nismo ugotovili statistično značilnih razlik, kar nakazuje, da ta vrsta ni povzročila patoloških sprememb. V raziskavi smo iz notranjih organov ali pa v večjem številu iz škrg in/ali kože rib s patoanatomskimi in/ali histopatološkimi spremembami izolirali tudi druge manj znane in domnevno nove vrste, kar nakazuje njihovo morebitno patogenost. Obsežna zbirka shranjenih izolatov nam omogoča izvedbo prihodnjih raziskav, s katerimi bomo lahko določili identiteto izolatov do nivoja vrste, ocenili njihov patogeni potencial in raziskali njihov pomen v odnosu do rib, bodisi kot ribjih patogenov, komenzalov ali mutualistov. Pomembno je poudariti, da so bile ribe v tej raziskavi naravno okužene, zato so lahko bile nekatere ugotovljene spremembe posledica sočasnih okužb z drugimi povzročitelji. Hierarhično razvrščanje med vzorčenimi ribami in vrstami rodu *Flavobacterium*, ki smo jih izolirali iz več kot 1 % osebkov, ni pokazalo statistično značilnih povezav med vzorci razvrščanja in zdravstvenim stanjem rib, letnimi časi ali vzorčenji.

Z mikrodilucijsko metodo določanja minimalne inhibitorne koncentracije (MIC) smo izvedli AST za 10 antibiotikov iz sedmih različnih skupin protimikrobnih zdravil. Večina testiranih učinkovin se pogosto uporablja v ribogojstvu, vključno s florfenikolom (FLO) in oksitetraciklinom (OXY), ki sta odobrena za uporabo v Sloveniji. AST smo izvedli za 54 izolatov *F. psychrophilum*, osem izolatov *F. branchiophilum*, dva izolata *F. branchiarum, F. bernardetii, F. collinsii* in *F. celericrescens* ter en izolat *F. difficile, F. erciyesense, F. hercynium, F. oncorhynchi, F. piscis, F. tiangeerense* in *F. tructae*. Testirali smo tudi 29 izolatov, ki so pripadali 11 domnevno novim vrstam *Flavobacterium*. Skupno smo testirali 106 izolatov iz rodu *Flavobacterium*, ki so predstavljali 24 različnih vrst.

Inštitut za klinične in laboratorijske standarde (CLSI) je v letu 2020 izdal vodilo VET03 z najnovejšimi navodili za standardizirano mikrodilucijsko metodo za AST *F. psychrophilum* ter vodilo VET04, ki vsebuje prve epidemiološke mejne vrednosti (ECV) za enrofloksacin (ENR), eritromicin (ERY), FLO, oksolinsko kislino (OXO) in OXY. Z uporabo metode normalizirane interpretacije odpornosti (NRI, angl. normalised resistance interpretation) smo izračunali začasno epidemiološko mejno vrednost za divji tip (CO_{WT}) za ampicillin (AMP), ki se je ujemala z redkimi obstoječimi raziskavami (≤ 0,125 µg/ml). Visok delež izolatov *F. psychrophilum* je bil zunaj območja divjega tipa (NWT) za OXY (81,5 %) in kinolone (87,0 % za ENR in 90,7 % za OXO); poleg tega so izolati imeli visoke MIC za ceftazidim (CTZ) in trimetoprim/sulfametoksazol (TRS). Zmanjšana občutljivost *F. psychrophilum* za OXY in kinolone je verjetno posledica pogoste uporabe teh protimikrobnih zdravil v ribogojstvu. Vsi izolati so bili divjega tipa (WT) za FLO, kar nakazuje, da je FLO primernejša izbira za zdravljenje okužb s *F. psychrophilum* v Sloveniji kot OXY. Večina izolatov je bila tudi WT za AMP in ERY; poleg tega so izolati imeli nizke MIC za gentamicin (GEN) in meropenem (MER). Glede na omejeno število raziskav, v katerih so uporabili standardizirano metodo za AST za *F. psychrophilum,* naši rezultati pomembno prispevajo k razumevanju občutljivosti tega pomembnega patogena na protimikrobna sredstva tako v svetovnem kot še zlasti v evropskem prostoru.

Razlikovanje med fenotipi WT in NWT za *F. branchiophilum* in manj znane flavobakterije ni bilo mogoče zaradi pomanjkanja standardiziranih metod za AST ter ECV. Ker izolati *F. branchiophilum* in nekaterih manj znanih vrst *Flavobacterium* niso rasli, ko smo uporabili standardizirano metodo, priporočeno za *F. psychrophilum*, smo metodo prilagodili tako, da smo v sterilno destilirano vodo, ki smo jo uporabili za pripravo razredčenega Mueller-Hinton bujona s prilagojeno vsebnostjo kationov (DCAMHB, angl. diluted cation-adjusted Mueller-Hinton broth) dodali 50 mg/l kalcijevih, magnezijevih in kalijevih kationov. Prilagoditev standardizirane metode za *F. psychrophilum* je omogočila uspešno AST za *F. branchiophilum* in druge za gojenje zahtevne flavobakterije, kar lahko prispeva k razvoju standardizirane metode za *F. branchiophilum*. Pri AST za *F. branchiophilum* smo z uporabo AO bujona ugotovili podobne rezultate. Pri

uporabi prilagojene metode so bile MIC vrednosti za OXY in kinolone na splošno nižje, kar je morda posledica višje koncentracije dvovalentnih kationov v gojišču, za katere je poznano, da zmanjšujejo biološko aktivnost teh učinkovin. Ugotovili smo, da so imeli izolati iste vrste podobne MIC. Nekatere vrste, kot so *F. branchiarum, F. collinsii, F. hercynium, F. oncorhynchi, F. piscis* in FX23, so imele MIC v zgornji polovici testiranega koncentracijskega območja za vseh deset protimikrobnih učinkovin. Poleg tega je imel vsaj en izolat drugih vrst flavobakterij MIC, ki je presegala ECV, priporočeno za *F. psychrophilum* za AMP (*F. bernardetii, F. celericrescens, F. erciyesense, F. tiangeerense, F. tructae,* FX2, FX3, FX5, FX9, FX10, FX15, FX18, FX27, FX32 in FX36), ERY (*F. tructae* in FX36), FLO (*F. tructae*), OXY (*F. branchiophilum, F. tructae,* FX2, FX5, FX10 in FX36) ter za kinolone ENR (*F. branchiophilum, F. celericrescens, F. difficile, F. erciyesense, F. tructae,* FX9, FX10, FX15, FX32 in FX36) in OXO (*F. celericrescens,* FX9, FX15 in FX36). MIC nekaterih vrst smo ugotovili tudi v srednjem ali zgornjem delu testiranega koncentracijskega območja za GEN (*F. erciyesense, F. tiangeerense, F. tructae*, FX2, FX9, FX10, FX15, FX27, FX32 in FX36) in MER (*F. tructae*, FX27 in FX36). Zanimivo je, da so imeli vsi izolati *F. psychrophilum*, vključno z referenčnim sevom, ter vsi izolati *F. branchiophilum* in manj znanih vrst visoke MIC za CTZ. Visoke MIC smo ugotovili pri večini testiranih izolatov tudi za TRS, razen pri *F. branchiophilum* in nekaterih izolatih *F. psychrophilum*. Ti rezultati kažejo na morebitno intrinzično odpornost ali neznane mehanizme odpornosti večine flavobakterij proti CTZ in TRS. Poleg tega smo ocenili skladnost med fenotipskimi in genotipskimi profili odpornosti proti protimikrobnim zdravilom (AMR) izolatov *F. psychrophilum* in *F. branchiophilum*. Zamenjavo T83A ali T83V v GyrA, ki je bila v literaturi že opisana v povezavi z odpornostjo *F. psychrophilum* proti kinolonom, smo ugotovili pri petih od sedmih sekvenciranih izolatov *F. psychrophilum*, kar se je popolnoma ujemalo s fenotipsko odpornostjo proti OXO, ne pa tudi proti ENR. To nakazuje možne razlike v mehanizmih delovanja teh dveh učinkovin. V preučevanih genomih *F. psychrophilum* in *F. branchiophilum* nismo ugotovili AMR genov ali drugih mutacij, povezanih z odpornostjo. Ugotovljena multirezistenca flavobakterij in omejeno razumevanje mehanizmov odpornosti poudarjata pomen izvajanja AST

pred zdravljenjem in potrebo po nadaljnjih raziskavah, ki bi primerjale fenotipsko in genotipsko odpornost flavobakterij.

Na dotoku in iztoku vsakega vzorčenega bazena smo izmerili izbrane parametre kakovosti vode, in sicer temperaturo vode, pH in raztopljeni kisik. Med raziskavo se je temperatura vode gibala med 6,6 in 15,8 °C, pH med 7,45 in 8,42 ter raztopljeni kisik med 7,28 in 12,11 mg/l. Parametri kvalitete vode so se statistično značilno razlikovali med letnimi časi in med ribogojnicami. Sezonska nihanja so bila skladna s pričakovanimi podnebnimi vplivi, medtem ko razlike med posameznimi ribogojnicami odražajo edinstvene fizikalno-kemijske značilnosti posameznih lokacij, verjetno zaradi različnih vodnih virov, lokalnih okoljskih razmer in praks upravljanja ribogojnic. Statistično značilne razlike smo ugotovili tudi med vhodno in izhodno vodo, kar najverjetneje odraža vpliv presnovne aktivnosti rib. Raziskali smo tudi povezavo med okužbami s *F. psychrophilum* in *F. branchiophilum* ter parametri kakovosti vode ter ugotovili statistično značilno povezavo med okužbami s *F. psychrophilum* in zvišanjem pH vode na dotoku ter z zimsko sezono. Ugotovljena povezava med okužbo in zvišanjem pH v drugih raziskavah še ni bila opisana. Nasprotno smo *F. branchiophilum* izolirali le poleti, okužbe pa so bile statistično značilno povezane s povišano temperaturo vode na dotoku in iztoku ter znižano koncentracijo raztopljenega kisika na iztoku. Poleg tega smo pred vzorčenjem opazili močne padavine in povečano motnost vode, kar lahko glede na podatke iz literature prispeva h okužbi s *F. branchiophilum*. Ugotovljena povezava med tveganjem za okužbo in parametri kakovosti vode je verjetno posledica kombinacije stresnega vpliva okoljskih dejavnikov na ribe, kar vodi v oslabitev imunskega sistema, ter okoljskih dejavnikov, ki ustrezajo patogenom. Spremljanje kakovosti vode, predvsem temperature, pH in koncentracije raztopljenega kisika ob upoštevanju sezonskih vplivov lahko pripomore k napovedi povečanega tveganja za okužbe s *F. psychrophilum* in *F. branchiophilum* ter omogoči pravočasno izvajanje preventivnih ukrepov.

Rezultati naše raziskave kažejo, da so sočasne okužbe oziroma infestacije z različnimi patogeni pri gojenih šarenkah zelo pogoste. Poleg flavobakterij smo ugotovili različne patogene rib, pri čemer so bili najpogostejši paraziti, in sicer

Acanthocephalus spp., amebe, *Apiosoma* spp., *Chilodonella* spp., *Dermocystidium* spp., *Epistylis* spp., *Ichthyobodo necator*, *Ichthyophthirius multifiliis*, monogeni sesači (*Gyrodactylus* spp. ali *Dactylogyrus* spp.), *Tetracapsuloides bryosalmonae*, *Trichodina* spp. in *Riboscyphidia* spp. Za oceno infestacije s paraziti smo odvzeli ostružke kože in škrg in jih po sušenju obarvali po Giemsi. Nekatere parazite smo ugotovili tudi s histopatološko preiskavo. S splošno bakteriološko preiskavo smo izolirali tudi druge patogene bakterije, kot so *Aeromonas salmonicida* subsp. *salmonicida*, *Aeromonas* spp., *Carnobacterium maltaromaticum*, *Citrobacter gillenii*, *Lactococcus garvieae* in *Pseudomonas* spp. ter s histopatološko preiskavo ugotovili *Renibacterium salmoninarum* in spremembe, značilne za bolezen epitelijskih cist (angl. epitheliocystis disease). S histopatološko preiskavo smo ugotovili tudi glive *Saprolegnia* spp. Ob parazitološki in histopatološki preiskavi smo stopnjo infestacije oziroma okužbe ocenili kot blago, zmerno ali močno ter rezultate uporabili za analizo povezave med prisotnostjo drugih patogenov in okužbo s *F. psychrophilum*. Pearsonov hi-kvadrat test je pokazal, da je bila okužba s *F. psychrophilum* povezana z infestacijo škrg in kože z monogeni sesači (*Monogenea*). Za druge patogene nismo ugotovili statistično značilne povezave. Natančni mehanizmi, s katerimi infestacija s paraziti pripomore h okužbi z bakterijami, še niso povsem pojasnjeni. Možno je, da paraziti povzročijo poškodbo epitela kože in škrg rib ter s tem olajšajo vstop bakterij, da delujejo kot vektorji ali pa gostitelju povzročijo stres, ki vodi v zmanjšano imunsko odpornost.

Z raziskavo smo pridobili pomembne podatke o biodiverziteti flavobakterij, povezanih z ribami, ter o pomembnih patogenih vrstah pri šarenki v Sloveniji. Raziskava vsebuje podrobne in nove informacije o patoloških spremembah, povezanih z okužbami s *F. psychrophilum* in *F. branchiophilum;* rezultati prav tako nakazujejo patogenost manj znanih vrst *F. hercynium* in *F. hydatis*. Ta raziskava je tudi ena izmed redkih, v kateri je bila preučena povezava med okužbo rib s flavobakterijami in patoanatomskimi in/ali histopatološkimi spremembami. Raziskava omogoča razvoj ciljno usmerjenih terapevtskih in preventivnih strategij ter predstavlja osnovo za nadaljnje raziskave o biodiverziteti in značilnostih flavobakterij ter njihovega vpliva na zdravje rib.

9 ACKNOWLEDGMENTS

I would like to sincerely thank my mentor, Assoc. Prof. Dr. Tanja Švara, and my co-mentor, Sr. Res. Fell. Dr. Matjaž Ocepek, for giving me the opportunity to pursue this doctoral study and for their invaluable guidance, patience, and support. Tanja, I am deeply grateful to have had you as a mentor and to have been able to learn from you not only during the doctoral programme, but also throughout my professional development in veterinary pathology.

I would also like to thank the members of the Expert Committee, Prof. Dr. Vlasta Jenčič, Prof. Dr. Andrej Pengov, and Prof. Dr. Heike Schmidt-Posthaus, whose insightful suggestions and corrections have greatly improved the quality of this thesis. My special thanks go to Prof. Dr. Schmidt-Posthaus and her team, for hosting me at FIWI, in Bern, and including me in the fish rounds, generously sharing their knowledge and enthusiasm for fish, which further increased my fascination for fish medicine and pathology. Heike, thank you for always being available for my many questions; I am truly grateful to have been able to learn from you.

My sincere thanks go to the fish farmers for their cooperation, without which this research would not have been possible.

I am immensely grateful to Prof. Dr. Irena Zdovc, who was also like a mentor to me. Thank you for your help and guidance in bacteriology, your support and our many valuable discussions.

Special thanks to Res. Assoc. Dr. Bojan Papić for his help with bacterial genomics and for always being available for my questions or when I needed help throughout this doctoral study.

I would like to thank Boshko Koloski, M.Sc., and Asst. Prof. Dr. Tanja Knific for their indispensable help with the statistical analyses.

Special thanks to my co-workers Tch. Asst. Dr. Marko Cvetko and Tch. Asst. Domen Maj Fras for their help with the sampling and work responsibilities, as well as to the (then) students Teodora Nikodinovska, Maja Pogačnik, Ana Gotvanj, Lara Marušič, and Ana Perić, for their valuable help in sampling and processing the samples. I would also like to thank Jurij Omahen, Benjamin Cerk, and Luka Bregar for preparing the histopathological preparations, and to all other co-workers at IPDRČ for their encouragement and support.

Sincere thanks to Res. Assoc. Dr. Igor Gruntar for his guidance and assistance with MALDI-TOF MS, and to Magda Klun for preparing the numerous agar plates for the bacteriological investigation. I would also like to thank all members of the Institute of Microbiology and Parasitology, Veterinary Faculty, especially Olga Kosar and Maja Lepen, for their help and support.

I am grateful to Rosvita Sitar, M.Sc., Marija Seničar, M.Sc., and Dr. Diana Žele Vengušt for their help with the research design and contact with the fish farmers.

My sincere thanks also go to Biljana Grubišić for her guidance through the administrative procedures, and to Stanka Ujc, Slavica Sekulić and Dr. Metka Šimundić for their help with the literature.

I would like to thank Tadej Ian, M.Sc., for the final English and Slovene language editing of this thesis.

Finally, I am grateful to my partner and family for their love, encouragement and support.

10 LITERATURE

Abby SS, Cury J, Guglielmini J, Néron B, Touchon M, Rocha EPC. Identification of protein secretion systems in bacterial genomes. Sci Rep 2016; 6: 23080. doi: 10.1038/srep23080

Abd El-Tawab A, El-Hofy F, Refaat EG, Awad S, El-Mougy E, Mohamed S. Phenotypic and genotypic characterization of antibiotic resistant strains of *Flavobacterium columnare* isolated from Nile tilapia (*Oreochromis niloticus*). Benha Vet Med J 2020; 38(2): 141–5. doi: 10.21608/bvmj.2020.25826.1188

Adamek M, Syakuri H, Harris S, et al. Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.). Vet Microbiol 2013; 162(2/4): 456–70. doi: 10.1016/j.vetmic.2012.10.033

Adamek M, Teitge F, Jung-Schroers V, et al. Flavobacteria as secondary pathogens in carp suffering from koi sleepy disease. J Fish Dis 2018; 41(11): 1631–42. doi: 10.1111/jfd.12872

Adams SM, Brown AM, Goede RW. A quantitative health assessment index for rapid evaluation of fish condition in the field. Trans Am Fish Soc 1993; 122(1): 63–73. doi: 10.1577/1548-8659(1993)122<0063:aqhaif>2.3.co;2

Alcock BP, Raphenya AR, Lau TTY, et al. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res 2020; 48(D1): D517–D25. doi: 10.1093/nar/gkz935

Altinok I, Grizzle JM. Effects of low salinities on *Flavobacterium columnare* infection of euryhaline and freshwater stenohaline fish. J Fish Dis 2001; 24(6): 361–7. doi: 10.1046/j.1365-2761.2001.00306.x

Alvarez B, Secades P, McBride MJ, Guijarro JA. Development of genetic techniques for the psychrotrophic fish pathogen *Flavobacterium psychrophilum*. Appl Environ Microbiol 2004; 70(1): 581–7.

doi: 10.1128/AEM.70.1.581-587.2004

Aly SM, Nouh WG, Atti NA, El-Ashram AM. Pathological and electron microscopic studies on cold water disease among cultured rainbow trout (*Oncorhynchus mykiss* Walbaum). Vet Sci Dev 2013; 3(1): 2.
doi: 10.4081/vsd.2013.e2

Anacker RL, Ordal EJ. Study of a bacteriophage infecting the myxobacterium *Chondrococcus*. J Bacteriol 1955; 70(6): 738–41.
doi: 10.1128/JB.70.6.738-741.1955

Anacker RL, Ordal EJ. Studies on the myxobacterium *Chondrococcus columnaris*. II. Bacteriocins. J Bacteriol 1959; 78(1): 33–40.
doi: 10.1128/JB.78.1.33-40.1959

Anderson RL, Ordal EJ. *Cytophaga succinicans* sp. n, a facultatively anaerobic, aquatic myxobacterium. J Bacteriol 1961; 81(1): 130–8.
doi: 10.1128/jb.81.1.130-138.1961

Arias CR, Lafrentz S, Cai W, Olivares-Fuster O. Adaptive response to starvation in the fish pathogen *Flavobacterium columnare*: cell viability and ultrastructural changes. BMC Microbiol 2012; 12: 266.
doi: 10.1186/1471-2180-12-266

Automatic NRI calculations for MIC distributions, for Windows and MAC (2019 version). http://www.bioscand.se/nri/ (accessed 5 September 2024)

Avendaño-Herrera R, Benavides I, Espina JA, et al. Zebrafish (*Danio rerio*) as an animal model for bath infection by *Flavobacterium psychrophilum*. J Fish Dis 2020; 43(5): 561–70.
doi: 10.1111/jfd.13156

Bader JA, Shotts EB. Identification of *Flavobacterium* and *Flexibacter* species by species-specific polymerase chain reaction primers to the 16S ribosomal RNA gene. J Aquat Anim Health 1998; 10(4): 311–9.
doi: 10.1577/1548-8667(1998)010<0311:IOFAFS>2.0.CO;2

Bader JA, Shoemaker CA, Klesius PH. Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*. J Microbiol Methods 2003; 52(2): 209–20.
doi: 10.1016/s0167-7012(02)00208-7

Bader JA, Shoemaker CA, Klesius PH. Production, characterization and evaluation of virulence of an adhesion defective mutant of *Flavobacterium columnare* produced by beta-lactam selection. Lett Appl Microbiol 2005; 40(2): 123–7.
doi: 10.1111/j.1472-765X.2004.01641.x

Bakhtiyar Y, Yousuf T, Arafat M. Bacterial gill disease and aquatic pollution: a serious concern for the aquaculture industry. In: Dar GH, eds. Bacterial fish diseases. 1st ed. London: Elsevier Academic Press, 2022: 269–78.
doi: 10.1016/B978-0-323-85624-9.00012-9

Bandilla M, Valtonen ET, Suomalainen L-R, Aphalo PJ, Hakalahti T. A link between ectoparasite infection and susceptibility to bacterial disease in rainbow trout. Int J Parasitol 2006; 36(9): 987–91.
doi: 10.1016/j.ijpara.2006.05.001

Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012; 19(5): 455–77.
doi: 10.1089/cmb.2012.0021

Barbier P, Rochat T, Mohammed HH, et al. The type IX secretion system is required for virulence of the fish pathogen *Flavobacterium psychrophilum*. Appl Environ Microbiol 2020; 86(16): e00799–20.
doi: 10.1128/AEM.00799-20

Barker GA, Smith SN, Bromage NR. Effect of oxolinic acid on bacterial flora and hatching success rate of rainbow trout, *Oncorhynchus mykiss*, eggs. Aquaculture 1990; 91(3): 205–22.
doi: 10.1016/0044-8486(90)90189-T

Barnes ME, Brown ML. A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treat. Open Fish Sci J 2011; 4(1): 40–8.
doi: 10.2174/1874401x01104010040

Beck BH, Farmer BD, Straus DL, Li C, Peatman E. Putative roles for a rhamnose binding lectin in *Flavobacterium columnare* pathogenesis in channel catfish *Ictalurus punctatus*. Fish Shellfish Immunol 2012; 33(4): 1008–15.
doi: 10.1016/j.fsi.2012.08.018

Bergey DH, Harrison FC, Breed RS, Hammer BW, Huntoon FM. Bergey's manual of determinative bacteriology. Baltimore: Williams and Wilkins, 1923: 97–117.

Bernardet JF, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P. Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). Int J Syst Bacteriol 1996; 46(1): 128–48. doi: 10.1099/00207713-46-1-128

Bernardet JF, Nakagawa Y, Holmes B. Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. Int J Syst Evol Microbiol 2002; 52(3): 1049–70. doi: 10.1099/00207713-52-3-1049

Bernardet JF, Bowman JP. The genus *Flavobacterium*. In: Dworkin M, eds. The Prokaryotes: a handbook on the biology of bacteria. 3rd ed. Vol. 7. New York: Springer Science & Business Media, 2006: 481–531.

Bernardet JF, Bowman JP. *Flavobacterium*. In: Krieg NR, eds. Bergey's manual of systematics of archaea and bacteria. Hoboken: John Wiley & Sons, 2015: 1–75. doi: 10.1002/9781118960608.gbm00312

Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T. Histopathology in fish: Proposal for a protocol to assess aquatic pollution. J Fish Dis 1999; 22(1): 25–34. doi: 10.1046/j.1365-2761.1999.00134.x

Bertolini J, Rohovec J. Electrophoretic detection of proteases from different *Flexibacter columnaris* strains and assessment of their variability. Dis Aquat Organ 1992; 12: 121–8. doi: 10.3354/dao012121

Bertolini JM, Wakabayashi H, Watral VG, Whipple MJ, Rohovec JS. Electrophoretic detection of proteases from selected strains of *Flexibacter psychrophilus* and assessment of their variability. J Aquat Anim Health 1994; 6(3): 224–33. doi: 10.1577/1548-8667(1994)006<0224:EDOPFS>2.3.CO;2

Bioscand AB. Normalized Resistance Interpretation. European patent No.

1383913, 2002. US Patent No. 7,465,559, 2008. Täby: International Patent Application WO 02/083935 A1.

Blair JMA, Richmond GE, Piddock LJV. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiol 2014; 9(10): 1165–77.
doi: 10.2217/fmb.14.66

Bojarski B, Jakubiak M, Bień M, et al. Assessment of gentamicin effect on oxidoreductive balance and microstructure of trunk kidney in Prussian carp (*Carassius gibelio*). Ann Warsaw Univ Life Sci 2019; 58(2): 115–23.
doi: 10.22630/AAS.2019.58.2.12

Bokan I. Isolation and characterization of the bacteria from the skin of the olm and its aquatic environment. Ljubljana: University of Ljubljana, Biotechnical Faculty, 2013. Master's thesis.
https://repozitorij.uni-lj.si/IzpisGradiva.php?lang=eng&id=126333 (9. 4. 2025).

Bortolaia V, Kaas RS, Ruppe E, et al. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother 2020; 75(12): 3491–500.
doi: 10.1093/jac/dkaa345

Boutin S, Sauvage C, Bernatchez L, Audet C, Derôme N. Inter individual variations of the fish skin microbiota: host genetics basis of mutualism? PLoS One 2014; 9(7): e102649.
doi: 10.1371/journal.pone.0102649

Brenden TO, Ivan LN, Loch TP. Reducing *Flavobacterium psychrophilum* transmission risk via hatchery-rearing practices: an individual-based modeling evaluation. Aquaculture 2023; 563: 738868.
doi: 10.1016/j.aquaculture.2022.738868

Bruno DW. *Cytophaga psychrophila* (= '*Flexibacter psychrophilus*') (Borg), histopathology associated with mortalities among farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum) in the UK. Bull Eur Assoc Fish Pathol 1992; 12(6): 215–6.

Bruno DW, Noguera PA, Poppe TT. A colour atlas of salmonid diseases. 2nd ed. London: Springer Science & Business Media, 2013: 82–4.
doi: 10.1007/978-94-007-2010-7

Bruun MS, Schmidt AS, Madsen L, Dalsgaard I. Antimicrobial resistance patterns

in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture 2000; 187(3/4): 201–12.
doi: 10.1016/S0044-8486(00)00310-0

Buchmann K, Bresciani J. Parasitic infections in pond-reared rainbow trout *Oncorhynchus mykiss* in Denmark. Dis Aquat Organ 1997; 28: 125–38.
doi: 10.3354/dao028125

Bullock GL, Hsu TC, Shotts EB. Columnaris disease of fishes. Fish disease leaflet 72. Washington: United States Fish and Wildlife Service, 1986: 9 str.
https://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1128&context=usfwspubs (10. 5. 2025).

Bullock GL. Bacterial gill disease of freshwater fishes. Fish disease leaflet 84. Washington: United States Fish and Wildlife Service, 1990: 3 str.
https://core.ac.uk/reader/188061010 (10. 5. 2025).

Bullock G, Herman R, Heinen J, Noble A, Weber A, Hankins J. Observations on the occurrence of bacterial gill disease and amoeba gill infestation in rainbow trout cultured in a water recirculation system. J Aquat Anim Health 1994; 6(4): 310–17.
doi: 10.1577/1548-8667(1994)006<0310:OOTOOB>2.3.CO;2

Burka JF, Hammell KL, Horsberg TE, Johnson GR, Rainnie DJ, Speare DJ. Drugs in salmonid aquaculture: a review. J Vet Pharmacol Ther 1997; 20(5): 333–49.
doi: 10.1046/j.1365-2885.1997.00094.x

Burreson EM. Misuse of PCR assay for diagnosis of mollusc protistan infections. Dis Aquat Organ 2008; 80(1): 81–3.
doi: 10.3354/dao01925

Busch S, Dalsgaard I, Buchmann K. Concomitant exposure of rainbow trout fry to *Gyrodactylus derjavini* and *Flavobacterium psychrophilum*: effects on infection and mortality of host. Vet Parasitol 2003; 117(1): 117–22.
doi: 10.1016/j.vetpar.2003.07.018

Cabello FC, Godfrey HP, Tomova A, et al. Antimicrobial use in aquaculture re-examined: Its relevance to antimicrobial resistance and to animal and human health. Environ Microbiol 2013; 15(7): 1917–42.
doi: 10.1111/1462-2920.12134

Cai W. Biofilm formation, virulence attenuation and comparative genomics of the fish pathogen *Flavobacterium columnare*. Auburn: Auburn University, 2018. Doctoral dissertation.

Caldwell CA, Hinshaw J. Physiological and haematological responses in rainbow trout subjected to supplemental dissolved oxygen in fish culture. Aquaculture 1994; 126(1): 183–93.
doi: 10.1016/0044-8486(94)90259-3

Calvez S, Navarro-Gonzalez N, Siekoula-Nguedia C, Fournel C, Duchaud E. High genetic diversity in *Flavobacterium psychrophilum* isolates from healthy rainbow trout (*Oncorhynchus mykiss*) farmed in the same watershed, revealed by two typing methods. Appl Environ Microbiol 2021; 87(2).
doi: 10.1128/AEM.01398-20

Carson J, Schmidtke LM, Munday BL. *Cytophaga johnsonae*: a putative skin pathogen of juvenile farmed barramundi, *Lates calcarifer* Bloch. J Fish Dis 1993; 16(3): 209–18.
doi: 10.1111/j.1365-2761.1993.tb01250.x

Castillo D, Christiansen RH, Dalsgaard I, Madsen L, Espejo R, Middelboe M. Comparative genome analysis provides insights into the pathogenicity of *Flavobacterium psychrophilum*. PLoS One 2016; 11(4): e0152515.
doi: 10.1371/journal.pone.0152515

Castillo D, Donati VL, Jørgensen J, et al. Comparative genomic analyses of *Flavobacterium psychrophilum* isolates reveals new putative genetic determinants of virulence traits. Microorganisms 2021; 9(8): 1658.
doi: 10.3390/microorganisms9081658

Chai J, Wang D, Chen F, Cao Y, Lu T, Li S. Tissue tropism and dynamic distribution of *Flavobacterium psychrophilum* in infected rainbow trout (*Oncorhynchus mykiss*) after intramuscular injection. J Fishery Sci China 2021; 28(11): 1426–35.
doi: 10.12264/JFSC2021-0081

Chen S, Blom J, Loch TP, Faisal M, Walker ED. The emerging fish pathogen *Flavobacterium spartansii* isolated from Chinook salmon: comparative genome analysis and molecular manipulation. Front Microbiol 2017; 8: 2339.
doi: 10.3389/fmicb.2017.02339

Chen Y-C, Davis MA, Lapatra SE, Cain KD, Snekvik KR, Call DR. Genetic diversity of *Flavobacterium psychrophilum* recovered from commercially raised rainbow trout, *Oncorhynchus mykiss* (Walbaum), and spawning coho salmon, *O. kisutch* (Walbaum). J Fish Dis 2008; 31(10): 765–73. doi: 10.1111/j.1365-2761.2008.00950.x

Chinchilla B, Vázquez Fernández E, Rebollada-Merino A, Perez-Sancho M, Domínguez Rodríguez L, Rodriguez-Bertos A. First detection of *Flavobacterium psychrophilum* in juvenile Siberian sturgeons (*Acipenser baerii*) and description of the pathological findings. J Fish Dis 2023; 46(8): 887–94. doi: 10.1111/jfd.13801

Chokmangmeepisarn P, Thangsunan P, Kayansamruaj P, Rodkhum C. Resistome characterization of *Flavobacterium columnare* isolated from freshwater cultured Asian sea bass (*Lates calcarifer*) revealed diversity of quinolone resistance associated genes. Aquaculture 2021; 544: 737149. doi: 10.1016/j.aquaculture.2021.737149

Chowdhury MMR, Roy A, Auvinen K, Pulkkinen K, Suonia H, Taskinen J. Glochidial infection by the endangered *Margaritifera margaritifera* (Mollusca) increased survival of salmonid host (Pisces) during experimental *Flavobacterium* disease outbreak. Parasitol Res 2021; 120(10): 3487–96. doi: 10.1007/s00436-021-07285-7

Cipriano RC, Holt RA. *Flavobacterium psychrophilum*, cause of bacterial cold-water disease and rainbow trout fry syndrome. Fish Disease Leaflet 86. Kearneysville: United States Department of the Interior, 2005: 44 str. https://freshwater-aquaculture.extension.org/wp-content/uploads/2019/08/USFWS_Coldwater_disease.pdf (10. 5. 2025).

Clark SE, Jude BA, Russell Danner G, Fekete FA. Identification of a multidrug efflux pump in *Flavobacterium johnsoniae*. Vet Res 2009; 40(6): 55. doi: 10.1051/vetres/2009038

CLSI VET04-A2. Methods for broth dilution susceptibility testing of bacteria isolated from aquatic animals, approved guideline VET04-A2. Wayne: Clinical and Laboratory Standards Institute, 2014.

CLSI VET03. Methods for antimicrobial broth dilution and disk diffusion

susceptibility testing of bacteria isolated from aquatic animals, approved guideline. Wayne: Clinical and Laboratory Standards Institute, 2020a.

CLSI VET04. Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals, approved guideline. Wayne: Clinical and Laboratory Standards Institute, 2020b.

Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. Hillsdale: Lawrence Erlbaum Associates, 1988.
doi: 10.4324/9780203771587

Dalsgaard I. Virulence mechanisms in *Cytophaga psychrophila* and other *Cytophaga*-like bacteria pathogenic for fish. Annu Rev Fish Dis 1993; 3: 127–44.
doi: 10.1016/0959-8030(93)90032-7

Darwish AM, Mitchell AJ, Hobbs MS. In vitro and in vivo evaluation of potassium permanganate treatment efficacy for the control of acute experimental infection by *Flavobacterium columnare* in channel catfish. N Am J Aquac 2008; 70(3): 314–22.
doi: 10.1577/A06-081.1

Davis HS. A new bacterial disease of fresh-water fishes. Bull US Bur Fish 1922; 38: 261–80.

Davis HS. A new gill disease of trout. Trans Am Fish Soc 1926; 56(1): 156–60.
doi: 10.1577/1548-8659(1926)56[156:ANGDOT]2.0.CO;2

Davis HS. Care and diseases of trout. Research report 12. Washington: Government Printing Office, Fish and Wildlife Service, 1946: 100 str.

de Abreu Reis Ferreira D, Assane IM, Vaneci-Silva D, et al. Morpho-molecular identification, pathogenicity for *Piaractus mesopotamicus*, and antimicrobial susceptibility of a virulent *Flavobacterium columnare* isolated from Nile tilapia cultured in Brazil. Aquaculture 2022; 560: 738486.
doi: 10.1016/j.aquaculture.2022.738486

de Alexandre Sebastião F, LaFrentz BR, Shelley JP, et al. *Flavobacterium inkyongense* isolated from ornamental cichlids. J Fish Dis 2019; 42(9): 1309–13.
doi: 10.1111/jfd.13043

Declercq AM, Haesebrouck F, Van den Broeck W, Bossier P, Decostere A.

Columnaris disease in fish: a review. Vet Res 2013a; 44: 27. doi: 10.1186/1297-9716-44-27

Declercq AM, Boyen F, Van den Broeck W, et al. Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species. J Fish Dis 2013b; 36(1): 45–55. doi: 10.1111/j.1365-2761.2012.01410.x

Declercq AM, Tilleman L, Gansemans Y, et al. Comparative genomics of *Flavobacterium columnare* unveils novel insights in virulence and antimicrobial resistance mechanisms. Vet Res 2021; 52(1): 1–13. doi: 10.1186/s13567-021-00899-w

Decostere A, Haesebrouck F, Devriese LA. Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. J Clin Microbiol 1997; 35(1): 322–24. doi: 10.1128/jcm.35.1.322-324.1997

Decostere A, Haesebrouck F, Charlier G, Ducatelle R. The association of *Flavobacterium columnare* strains of high and low virulence with gill tissue of black mollies (*Poecilia sphenops*). Vet Microbiol 1999a; 67(4): 287–98. doi: 10.1016/s0378-1135(99)00050-4

Decostere A, Haesebrouck F, Van Driessche E, Charlier G, Ducatelle R. Characterization of the adhesion of *Flavobacterium columnare* (*Flexibacter columnaris*) to gill tissue. J Fish Dis 1999b; 22(6): 465–74. doi: 10.1046/j.1365-2761.1999.00198.x

Decostere A, Haesebrouck F, Turnbull JF, Charlier G. Influence of water quality and temperature on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches. J Fish Dis 1999c; 22(1): 1–11. doi: 10.1046/j.1365-2761.1999.00132.x

Decostere A, D'Haese E, Lammens M, Nelis H, Haesebrouck F. In vivo study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss*, spleen phagocytes. J Fish Dis 2001; 24(8): 481–7. doi: 10.1046/j.1365-2761.2001.00322.x

Decostere A. *Flavobacterium columnare* infections in fish: the agent and its adhesion to the gill tissue. Verh K Acad Geneesk Belg 2002; 64(6): 421–30.

Del Cerro A, Márquez I, Prieto JM. Genetic diversity and antimicrobial resistance of *Flavobacterium psychrophilum* isolated from cultured rainbow trout, *Oncorhynchus mykiss*, in Spain. J Fish Dis 2010; 33(4): 285–91. doi: 10.1111/j.1365-2761.2009.01120.x

Delalay G, Berezowski J, Diserens N, Schmidt-Posthaus H. Characteristics of bacterial isolates in Swiss farmed and ornamental fish from a retrospective study from 2000 to 2017. Schweiz Arch Tierheilkd 2018; 161(1): 43–57. doi: 10.17236/sat00193

Deng F, Wang D, Yu Y, Lu T, Li S. Systemic immune response of rainbow trout exposed to *Flavobacterium psychrophilum* infection. Fish Shellfish Immunol 2024; 144: 109305. doi: 10.1016/j.fsi.2023.109305

Dong K, Chen F, Du Y, Wang G. *Flavobacterium enshiense* sp. nov., isolated from soil, and emended descriptions of the genus *Flavobacterium* and *Flavobacterium cauense*, *Flavobacterium saliperosum* and *Flavobacterium suncheonense*. Int J Syst Evol Microbiol 2013; 63(3): 886–92. doi: 10.1099/ijs.0.039974-0

Dong HT, LaFrentz B, Pirarat N, Rodkhum C. Phenotypic characterization and genetic diversity of *Flavobacterium columnare* isolated from red tilapia, *Oreochromis* sp., in Thailand. J Fish Dis 2015; 38(10): 901–13. doi: 10.1111/jfd.12304

Dong H, Nguyen V, Mata W, et al. Diversity of non-*Flavobacterium columnare* bacteria associated with columnaris-like diseased fish. Thai Vet Med 2016; 46: 251–9. doi: 10.56808/2985-1130.2733

do Vale A, Marques F, Silva MT. Apoptosis of sea bass (*Dicentrarchus labrax*) neutrophils and macrophages induced by experimental infection with *Photobacterium damselae* subsp. *piscicida*. Fish Shellfish Immunol 2003; 15(2): 129–44. doi: 10.1016/s1050-4648(02)00144-4

Duchaud E, Rochat T, Habib C, et al. Genomic diversity and evolution of the fish pathogen *Flavobacterium psychrophilum*. Front Microbiol 2018; 9: 138. doi: 10.3389/fmicb.2018.00138

Eckroat TJ, Greguske C, Hunnicutt DW. The Type 9 secretion system is required for *Flavobacterium johnsoniae* biofilm formation. Front Microbiol 2021; 12: 660887.
doi: 10.3389/fmicb.2021.660887

Edgar RC. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics 2018; 34(14): 2371–5.
doi: 10.1093/bioinformatics/bty113

European Food Safety Authority. Animal welfare aspects of husbandry systems for farmed trout: scientific opinion of the panel on animal health and welfare. EFSA J 2008; 796: 1–22.
doi: 10.2903/j.efsa.2008.796

Einarsdottir T, Guttormsdottir G, Connaghan D, Hjartardottir S. Longitudinal survey of *Flavobacterium* species in Icelandic salmonid fish farms. Dis Aquat Organ 2020; 141: 15–24.
doi: 10.3354/dao03508

Eissa AE, Zaki MM, Aziz AA. *Flavobacterium columnare*/*Myxobolus tilapiae* concurrent infection in the earthen pond reared Nile tilapia (*Oreochromis niloticus*) during the early summer. Interdiscip Bio Cent 2010; 2: 5.
doi: 10.4051/ibc.2010.2.2.0005

Ekman E, Norrgren L. Pathology and immunohistochemistry in three species of salmonids after experimental infection with *Flavobacterium psychrophilum*. J Fish Dis 2003; 26(9): 529–38.
doi: 10.1046/j.1365-2761.2003.00487.x

Ekman E. Natural and experimental infections with *Flavobacterium psychrophilum* in salmonid fish. Uppsala: Swedish University of Agricultural Sciences, 2008. Doctoral dissertation.

Elkamel AA, Ahmed SHM, Mohammed HH. Pathogenesis of columnaris disease in African sharptooth catfish, *Clarias gariepinus*. Assiut Vet Med J 2010; 56(127): 1–19.
doi: 10.21608/avmj.2010.174203

Elmore D. Studies on water quality of aquaculture farms with an emphasis on *Flavobacterium psychrophilum* and UV treatment. Thunder Bay: Lakehead University, The Faculty of Graduate Studies, 2016. Master's thesis.

https://knowledgecommons.lakeheadu.ca/handle/2453/4197

Eržen S. Microbial community analysis of karst cave waters using high-throughput sequencing. Ljubljana: University of Ljubljana, Biotechnical Faculty, 2024. Master's thesis. https://repozitorij.uni-lj.si/IzpisGradiva.php?lang=sl&vid=155594 (10. 5. 2025).

Evenhuis JP, LaPatra SE, Marancik D. Early life stage rainbow trout (*Oncorhynchus mykiss*) mortalities due to *Flavobacterium columnare* in Idaho, USA. Aquaculture 2014; 418/419: 126–31. doi: 10.1016/j.aquaculture.2013.09.044

Evenhuis JP, LaFrentz BR. Virulence of *Flavobacterium columnare* genomovars in rainbow trout (*Oncorhynchus mykiss*). Dis Aquat Organ 2016; 120(3): 217–24. doi: 10.3354/dao03027

Faisal M, Loch TP, Fujimoto M, et al. Characterization of novel *Flavobacterium* spp. involved in the mortality of coho salmon (*Oncorhynchus kisutch*) in their early life stages. J Aquac Res Dev 2011; S2: 1–8. doi: 10.4172/2155-9546.S2-005

FAO. The State of World Fisheries and Aquaculture: blue transformation and action. Rome: Food and Agriculture Organization, 2024. https://openknowledge.fao.org/items/06690fd0-d133-424c-9673-1849e414543d (accessed 10 March 2025)

Farmer BD, Mitchell AJ, Straus DL. The effect of high total ammonia concentration on the survival of channel catfish experimentally infected with *Flavobacterium columnare*. J Aquat Anim Health 2011; 23(3): 162–8. doi: 10.1080/08997659.2011.616836linkid

Ferguson HW, Ostland VE, Byrne P, Lumsden JS. Experimental production of bacterial gill disease in trout by horizontal transmission and by bath challenge. J Aquat Anim Health 1991; 3(2): 118–23. doi: 10.1577/1548-8667(1991)003<0118:EPOBGD>2.3.CO;2

Ferguson HW. Systemic pathology of fish: a text and atlas of normal tissues in teleosts and their responses in disease. 2nd ed. London: Scottian Press, 2006.

Fernández-Álvarez C, Gijón D, Álvarez M, Santos Y. First isolation of *Aeromonas salmonicida* subsp. *salmonicida* from diseased sea bass, *Dicentrarchus*

labrax (L.), cultured in Spain. Aquac Rep 2016; 4: 36–41. doi: 10.1016/j.aqrep.2016.05.006

Fernández-Álvarez C, Torres-Corral Y, Saltos-Rosero N, Santos Y. MALDI-TOF mass spectrometry for rapid differentiation of *Tenacibaculum* species pathogenic for fish. Appl Microbiol Biotechnol 2017; 101(13): 5377–90. doi: 10.1007/s00253-017-8324-3

Fernández-Álvarez C, Torres-Corral Y, Santos Y. Use of ribosomal proteins as biomarkers for identification of *Flavobacterium psychrophilum* by MALDI-TOF mass spectrometry. J Proteomics 2018; 170: 59–69. doi: 10.1016/j.jprot.2017.09.007

Ferreira EO, Schutt V, Stein M, Burdz T, Bernard K, Stefanovici C. First case of intrauterine fetal demise by *Flavobacterium oncorhynchi* diagnosed with placental microscopy. J Obstet Gynaecol Can 2024; 46(5): 101975. doi: 10.1016/j.jogc.2022.04.020

Figueiredo HCP, Klesius PH, Arias CR, et al. Isolation and characterization of strains of *Flavobacterium columnare* from Brazil. J Fish Dis 2005; 28(4): 199–204. doi: 10.1111/j.1365-2761.2005.00616.x

Finlay BB, McFadden G. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell 2006; 124(4): 767–82. doi: 10.1016/j.cell.2006.01.034

Fisher RA. Statistical methods for research workers. 3rd ed. Edinburgh: Oliver and Boyd, 1925.

Flemming L, Rawlings D, Chenia H. Phenotypic and molecular characterisation of fish-borne *Flavobacterium johnsoniae*-like isolates from aquaculture systems in South Africa. Res Microbiol 2007; 158(1): 18–30. doi: 10.1016/j.resmic.2006.09.001

Foscarini R. Induction and development of bacterial gill disease in the eel (*Anguilla japonica*) experimentally infected with *Flexibacter columnaris*: pathological changes in the gill vascular structure and in cardiac performance. Aquaculture 1989; 78(1): 1–20. doi: 10.1016/0044-8486(89)90002-1

Frasca S, Wolf JC, Kinsel MJ, Camus AC, Lombardini ED. Osteichthyes. In: Terio

KA, eds. Pathology of wildlife and zoo animals. Academic Press, 2018: 953–1001.
doi: 10.1016/B978-0-12-805306-5.00039-0

Fujihara MP, Nakatani RE. Antibody production and immune responses of rainbow trout and coho salmon to *Chondrococcus columnaris*. J Fish Res Board Can 1971; 28(9): 1253–8.
doi: 10.1139/f71-191

Galloway-Peña J, Sahasrabhojane P, Tarrand J, Han XY, Shelburne SA. *GyrB* polymorphisms accurately assign invasive viridans group streptococcal species. J Clin Microbiol 2014; 52(8): 2905–12.
doi: 10.1128/jcm.01068-14

García-López M, Meier-Kolthoff JP, Tindall BJ, et al. Analysis of 1,000 type-strain genomes improves taxonomic classification of *Bacteroidetes*. Front Microbiol 2019; 10: 2083.
doi: 10.3389/fmicb.2019.02083

GBC. The List of Prokaryotic names with Standing in Nomenclature (LPSN), Genus *Flavobacterium*. Global Biodata Coalition, 2025. https://lpsn.dsmz.de/genus/Flavobacterium (accessed 13 June 2025)

Gélinas V, Paquet VE, Paquet MF, Vincent AT, Charette SJ. Whole-genome-based taxonomy as the most accurate approach to identify *Flavobacterium* species. FEMS Microbiol Lett 2024; 371: fnae089.
doi: 10.1093/femsle/fnae089

Gieseker CM, Crosby TC, Mayer TD, Bodeis SM, Stine CB. Development of similar broth microdilution methods to determine the antimicrobial susceptibility of *Flavobacterium columnare* and *F. psychrophilum*. J Aquat Anim Health 2016; 28(1): 27–38.
doi: 10.1080/08997659.2015.1105878

Good CM, Thorburn MA, Stevenson RMW. Factors associated with the incidence of bacterial gill disease in salmonid lots reared in Ontario, Canada government hatcheries. Prev Vet Med 2008; 83(3/4): 297–307.
doi: 10.1016/j.prevetmed.2007.08.007

Good C, Davidson J, Wiens GD, Welch TJ, Summerfelt S. *Flavobacterium branchiophilum* and *F. succinicans* associated with bacterial gill disease in

rainbow trout *Oncorhynchus mykiss* (Walbaum) in water recirculation aquaculture systems. J Fish Dis 2015; 38(4): 409–13.
doi: 10.1111/jfd.12249

Grabowski LD, LaPatra SE, Cain KD. Systemic and mucosal antibody response in tilapia, *Oreochromis niloticus* (L.), following immunization with *Flavobacterium columnare*. J Fish Dis 2004; 27(10): 573–81.
doi: 10.1111/j.1365-2761.2004.00576.x

Gupta E, Bhalla P, Khurana N, Singh T. Histopathology for the diagnosis of infectious diseases. Indian J Med Microbiol 2009; 27(2): 100–6.
doi: 10.4103/0255-0857.49423

Hawke JP, Thune RL. Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish. J Aquat Anim Health 1992; 4(2): 109–13.
doi: 10.1577/1548-8667(1992)004<0109:SIAASO>2.3.CO;2

Heath R, Du Preez H, Genthe B, Avenant-Oldewage A. Freshwater fish and human health reference guide: report no. TT213/04. Pretoria: Water Research Commission, 2004.

Heckman TI, Yazdi Z, Pomaranski EK, et al. Atypical flavobacteria recovered from diseased fish in the Western United States. Front Cell Infect Microbiol 2023; 13: 1149032.
doi: 10.3389/fcimb.2023.1149032

Henríquez-Núñez H, Evrard O, Kronvall G, Avendaño-Herrera R. Antimicrobial susceptibility and plasmid profiles of *Flavobacterium psychrophilum* strains isolated in Chile. Aquaculture 2012; 354/5: 38–44.
doi: 10.1016/j.aquaculture.2012.04.034

Hesami S, Parkman J, MacInnes JI, Gray JT, Gyles CL, Lumsden JS. Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from Ontario. J Aquat Anim Health 2010; 22(1): 39–49.
doi: 10.1577/H09-008.1

Hoare R, Ngo TPH, Bartie KL, Adams A. Efficacy of a polyvalent immersion vaccine against *Flavobacterium psychrophilum* and evaluation of immune response to vaccination in rainbow trout fry (*Oncorhynchus mykiss* L.). Vet Res 2017; 48: 1–13.

doi: 10.1186/s13567-017-0448-z

Holt RA. *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease in salmonid fish. Corvallis: Oregon State University, 1987. Doctoral dissertation.

Holt RA, Amandi A, Rohovec JS, Fryer JL. Relation of water temperature to bacterial cold-water disease in coho salmon, chinook salmon, and rainbow trout. J Aquat Anim Health 1989; 1(2): 94–101.
doi: 10.1577/1548-8667(1989)001<0094:ROWTTB>2.3.CO;2

Holt RA, Rohovec JS, Fryer JL. Bacterial cold-water disease. In: Inglis V, eds. Bacterial diseases of fish. New York: Halsted Press; 1993: 3–22.

Hunter JD. Matplotlib: a 2D graphics environment. Comput Sci Eng 2007; 9(3): 90–5.
doi: 10.1109/MCSE.2007.55

Ilardi P, Valdes S, Rivera J, Irgang R, Avendaño-Herrera R. Co-occurrence of heterogeneous *Flavobacterium psychrophilum* isolates within the same Chilean farm and during the same infectious outbreak. J Fish Dis 2023; 46(10): 1085–96.
doi: 10.1111/jfd.13828

Izumi S, Aranishi F. Relationship between *gyrA* mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. Appl Environ Microbiol 2004; 70(7): 3968–72.
doi: 10.1128/AEM.70.7.3968-3972.2004

Jarau M, Di Natale A, Huber PE, MacInnes JI, Lumsden JS. Virulence of *Flavobacterium psychrophilum* isolates in rainbow trout *Oncorhynchus mykiss* (Walbaum). J Fish Dis 2018; 41(10): 1505–14.
doi: 10.1111/jfd.12861

Jarau M, MacInnes JI, Lumsden JS. Erythromycin and florfenicol treatment of rainbow trout *Oncorhynchus mykiss* (Walbaum) experimentally infected with *Flavobacterium psychrophilum*. J Fish Dis 2019; 42(3): 325–34.
doi: 10.1111/jfd.12944

Jarčič M. Isolation and identification of psychrophilic and psychrotolerant bacteria and fungi from Triglav and Skuta glaciers. Maribor: University of Maribor, Faculty of Natural Sciences and Mathematics, 2021. Master's thesis.

https://dk.um.si/IzpisGradiva.php?lang=engid=78707 (10. 5. 2025).

JAZMP. Seznam zdravil za uporabo v veterinarski medicini. Ljubljana: Javna agencija Republike Slovenije za zdravila in medicinske pripomočke, 2025. https://www.jazmp.si/veterinarska-zdravila/podatki-o-zdravilih/seznam-zdravil/2025-2/ (accessed 10 March 2025)

Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res 2018; 3: 124. doi: 10.12688/wellcomeopenres.14826.1

Kämpfer P, Lodders N, Martin K, Avendaño-Herrera R. *Flavobacterium chilense* sp. nov. and *Flavobacterium araucananum* sp. nov, isolated from farmed salmonid fish. Int J Syst Evol Microbiol 2012; 62(6): 1402–8. doi: 10.1099/ijs.0.033431-0

Kämpfer P, Irgang R, Glaeser SP, et al. *Flavobacterium salmonis* sp. nov. isolated from Atlantic salmon (*Salmo salar*) and formal proposal to reclassify *Flavobacterium spartansii* as a later heterotypic synonym of *Flavobacterium tructae*. Int J Syst Evol Microbiol 2020; 70(12): 6147–54. doi: 10.1099/ijsem.0.004510

Kang JY, Chun J, Jahng KY. *Flavobacterium aciduliphilum* sp. nov, isolated from freshwater, and emended description of the genus *Flavobacterium*. Int J Syst Evol Microbiol 2013; 63(5): 1633–8. doi: 10.1099/ijs.0.044495-0

Kapur V, Topouzis S, Majesky MW, et al. A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. Microb Pathog 1993; 15(5): 327–46. doi: 10.1006/mpat.1993.1083

Kayansamruaj P, Dong HT, Hirono I, Kondo H, Senapin S, Rodkhum C. Comparative genome analysis of fish pathogen *Flavobacterium columnare* reveals extensive sequence diversity within the species. Infect Genet Evol 2017; 54: 7–17. doi: 10.1016/j.meegid.2017.06.012

Kent ML, Groff JM, Morrison JK, Yasutake WT, Holt RA. Spiral swimming behavior due to cranial and vertebral lesions associated with *Cytophaga psychrophila*

infections in salmonid fishes. Dis Aquat Organ 1989; 6(1): 11–6. doi: 10.3354/dao006011

Kim KK, Kim MK, Lim JH, Park HY, Lee ST. Transfer of *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* to *Elizabethkingia* gen. nov. as *Elizabethkingia meningoseptica* comb. nov. and *Elizabethkingia miricola* comb. nov. Int J Syst Evol Microbiol 2005; 55(3): 1287–93. doi: 10.1099/ijs.0.63541-0

Kim M, Oh HS, Park SC, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol 2014; 64(2): 346–51. doi: 10.1099/ijs.0.059774-0

Kim M, Cha IT, Lee KE, Li M, Park SJ. Pangenome analysis provides insights into the genetic diversity, metabolic versatility, and evolution of the genus *Flavobacterium*. Microbiol Spectr 2023a; 11(5): e0100323. doi: 10.1128/spectrum.01003-23

Kim YS, Hwang EM, Jeong CM, Cha CJ. *Flavobacterium psychrotrophum* sp. nov. and *Flavobacterium panacagri* sp. nov, isolated from freshwater and soil. J Microbiol 2023b; 61(10): 891–901. doi: 10.1007/s12275-023-00081-1

Klesius PH, Shoemaker CA, Evans JJ. *Flavobacterium columnare* chemotaxis to channel catfish mucus. FEMS Microbiol Lett 2008; 288(2): 216–20. doi: 10.1111/j.1574-6968.2008.01348.x

Knupp C, Wiens GD, Faisal M, et al. Large-scale analysis of *Flavobacterium psychrophilum* multilocus sequence typing genotypes recovered from North American salmonids indicates that both newly identified and recurrent clonal complexes are associated with disease. Appl Environ Microbiol 2019; 85(6): e02305-18. doi: 10.1128/AEM.02305-18

Knupp C, Loch TP. Immersion challenge of three salmonid species (family Salmonidae) with three multilocus sequence typing variants of *Flavobacterium psychrophilum* provides evidence of differential host specificity. J Fish Dis 2025; 48(8): e13889.

doi: 10.1111/jfd.13889

Ko YM, Heo GJ. Characteristics of *Flavobacterium branchiophilum* isolated from rainbow trout in Korea. Fish Pathol 1997; 32(2): 97–102.
doi: 10.3147/jsfp.32.97

Kondo M, Kawai K, Kurohara K, Oshima S. Adherence of *Flavobacterium psychrophilum* on the body surface of the ayu *Plecoglossus altivelis*. Microbes Infect 2002; 4(3): 279–83.
doi: 10.1016/s1286-4579(02)01539-3

Kotob M, Menanteau-Ledouble S, Kumar G, Abdelzaher M, El-Matbouli M. The impact of co-infections on fish: a review. Vet Res 2016; 47: 98.
doi: 10.1186/s13567-016-0383-4

Králová S, Busse HJ, Bezdíček M, et al. *Flavobacterium flabelliforme* sp. nov. and *Flavobacterium geliluteum* sp. nov, two multidrug-resistant psychrotrophic species isolated from Antarctica. Front Microbiol 2021; 12: 729977.
doi: 10.3389/fmicb.2021.729977

Kronvall G. Normalized resistance interpretation as a tool for establishing epidemiological MIC susceptibility breakpoints. J Clin Microbiol 2010; 48(12): 4445–52.
doi: 10.1128/JCM.01101-10

Kruskal WH, Wallis WA. Use of Ranks in One-Criterion Variance Analysis. J Am Stat Assoc 1952; 47(260): 583–621.
doi: 10.2307/2280779

Kumagai A, Nakayasu C, Oseko N. Effect of tobramycin supplementation to medium on isolation of *Flavobacterium psychrophilum* from ayu *Plecoglossus altivelis*. Fish Pathol 2004; 39(2): 75–8.
doi: 10.3147/jsfp.39.75

Kumru S, Tekedar HC, Gulsoy N, Waldbieser GC, Lawrence ML, Karsi A. Comparative analysis of the *Flavobacterium columnare* genomovar I and II genomes. Front Microbiol 2017; 8: 1375.
doi: 10.3389/fmicb.2017.01375

Kumru S, Tekedar HC, Blom J, Lawrence ML, Karsi A. Genomic diversity in flavobacterial pathogens of aquatic origin. Microb Pathog 2020; 142: 104053.
doi: 10.1016/j.micpath.2020.104053

Kunttu H. Characterizing the bacterial fish pathogen *Flavobacterium columnare*, and some factors affecting its pathogenicity. Jyväskylä: University of Jyväskylä, Faculty of Mathematics and Science, 2010. Doctoral dissertation.

Kunttu HMT, Jokinen EI, Valtonen ET, Sundberg L-R. Virulent and nonvirulent *Flavobacterium columnare* colony morphologies: characterization of chondroitin AC lyase activity and adhesion to polystyrene. J Appl Microbiol 2011; 111(6): 1319–26.
doi: 10.1111/j.1365-2672.2011.05149.x

Kunttu HMT, Sundberg L-R, Pulkkinen K, Valtonen ET. Environment may be the source of *Flavobacterium columnare* outbreaks at fish farms. Environ Microbiol Rep 2012; 4(4): 398–402.
doi: 10.1111/j.1758-2229.2012.00342.x

Kuo I, Saw J, Kapan DD, Christensen S, Kaneshiro KY, Donachie SP. *Flavobacterium akiainvivens* sp. nov, from decaying wood of *Wikstroemia oahuensis*, Hawai'i, and emended description of the genus *Flavobacterium*. Int J Syst Evol Microbiol 2013; 63(9): 3280–6.
doi: 10.1099/ijs.0.047217-0

LaFrentz BR, Králová S, Burbick CR, et al. The fish pathogen *Flavobacterium columnare* represents four distinct species: *Flavobacterium columnare*, *Flavobacterium covae* sp. nov., *Flavobacterium davisii* sp. nov. and *Flavobacterium oreochromis* sp. nov., and emended description of *Flavobacterium columnare*. Syst Appl Microbiol 2022; 45(2): 126293.
doi: 10.1016/j.syapm.2021.126293

Lee BH, Nicolas P, Saticioglu IB, et al. Investigation of the genus *Flavobacterium* as a reservoir for fish-pathogenic bacterial species: the case of *Flavobacterium collinsii*. Appl Environ Microbiol 2023; 89(4): e0216222.
doi: 10.1128/aem.02162-22

Lee KB, Heo GJ. First isolation and identification of *Cytophaga psychrophila* from cultured ayu in Korea. Fish Pathol 1998; 33(1): 37–8.
doi: 10.3147/jsfp.33.37

Li N, Zhu Y, LaFrentz BR, et al. The Type IX secretion system is required for virulence of the fish pathogen *Flavobacterium columnare*. Appl Environ Microbiol 2017; 83(23): e01769-17.

doi: 10.1128/AEM.01769-17

Li S, Chai J, Knupp C, et al. Phenotypic and genetic characterization of *Flavobacterium psychrophilum* recovered from diseased salmonids in China. Microbiol Spectr 2021; 9(2): e0033021.
doi: 10.1128/spectrum.00330-21

Liang K-Y, Zeger SL. Longitudinal data analysis using generalized linear models. Biometrika 1986; 73(1): 13–22.
doi: 10.1093/biomet/73.1.13

Liu H, Izumi S, Wakabayashi H. Detection of *Flavobacterium psychrophilum* in various organs of ayu *Plecoglossus altivelis* by in situ hybridization. Jpn Soc Fish Pathol 2001; 36(1): 7–11.
doi: 10.3147/jsfp.36.7

Liu J, Li Y, Ma F, Su S, Yao W, Wu Z. Spatiotemporal variation of microbial community structure and physicochemical parameters in grass carp culture ponds. J Freshwater Ecol 2019; 34(1): 123–33.

Loch TP. Identification of novel flavobacteria from Michigan and assessment of their impacts on fish health. East Lansing: Michigan State University, 2012. Doctoral dissertation.

Loch TP, Fujimoto M, Woodiga SA, Walker ED, Marsh TL, Faisal M. Diversity of fish-associated flavobacteria of Michigan. J Aquat Anim Health 2013; 25(3): 149–64.
doi: 10.1080/08997659.2012.758189

Loch TP, Faisal M. Deciphering the biodiversity of fish-pathogenic *Flavobacterium* spp. recovered from the Great Lakes basin. Dis Aquat Organ 2014; 112(1): 45–57.
doi: 10.3354/dao02791

Loch TP, Faisal M. Emerging flavobacterial infections in fish: a review. J Adv Res 2015; 6(3): 283–300.
doi: 10.1016/j.jare.2014.10.009

Loch TP, Faisal M. *Flavobacterium spartansii* induces pathological changes and mortality in experimentally challenged Chinook salmon *Oncorhynchus tshawytscha* (Walbaum). J Fish Dis 2016; 39(4): 483–8.
doi: 10.1111/jfd.12363

Loch TP, Faisal M. *Flavobacterium* spp. In: Woo PTK, eds. Fish viruses and bacteria: pathobiology and protection. 1st ed. Wallingford: CABI, 2017: 211–32.
doi: 10.1079/9781780647784.0000

Loch TP, Faisal M. Flavobacteria colonizing the early life stages of hatchery-incubated Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) are markedly diverse. J Fish Dis 2018; 41(5): 829–45.
doi: 10.1111/jfd.12795

Lorenzen E, Dalsgaard I, From J, et al. Preliminary investigations of fry mortality syndrome in rainbow trout. Bull Eur Assoc Fish Pathol 1991; 11(2): 77–9.

Lorenzen E, Dalsgaard I, Bernardet J-F. Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome I: phenotypic and genomic studies. Dis Aquat Organ 1997; 31: 197–208.
doi: 10.3354/dao031197

Ma J, Bruce TJ, Sudheesh PS, et al. Assessment of cross-protection to heterologous strains of *Flavobacterium psychrophilum* following vaccination with a live-attenuated coldwater disease immersion vaccine. J Fish Dis 2019; 42(1): 75–84.
doi: 10.1111/jfd.12902

Mabrok M, Chokmangmeepisarn P, LaFrentz BR, Kayansamruaj P, Dong HT, Rodkhum C. Development of a species-specific polymerase chain reaction for highly sensitive detection of *Flavobacterium columnare* targeting chondroitin AC lyase gene. Aquaculture 2020; 521: 734597.
doi: 10.1016/j.aquaculture.2019.734597

Madetoja J, Nyman P, Wiklund T. *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 2000; 43(1): 27–38.
doi: 10.3354/dao043027

Madetoja J, Dalsgaard I, Wiklund T. Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. Dis Aquat Organ 2002; 52(2): 109–18.
doi: 10.3354/dao052109

Madetoja J, Nystedt S, Wiklund T. Survival and virulence of *Flavobacterium*

psychrophilum in water microcosms. FEMS Microbiol Ecol 2003; 43(2): 217–23.
doi: 10.1111/j.1574-6941.2003.tb01061.x

Madsen L, Dalsgaard I. Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 1999; 36(3): 169–76.
doi: 10.3354/dao036169

Madsen L, Arnbjerg J, Dalsgaard I. Radiological examination of the spinal column in farmed rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquac Res 2001; 32(3): 235–41.
doi: 10.1046/j.1365-2109.2001.00552.x

Mallik S, Singh S, Shahi N, et al. Characterization and pathological analysis of *Flavobacterium tructae* recovered from farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), in the Indian Himalayan Region. Aquac Int 2023; 31: 2399–420.
doi: 10.1007/s10499-023-01089-5

Mann HB, Whitney DR. On a test of whether one of two random variables is stochastically larger than the other. Ann Math Stat 1947; 18(1): 50–60.
doi: 10.1214/aoms/1177730491

Mantareva V, Kussovski V, Orozova P, Angelov I, Durmuş M, Najdenski H. Palladium phthalocyanines varying in substituents position for photodynamic inactivation of *Flavobacterium hydatis* as sensitive and resistant species. Curr Issues Mol Biol 2022; 44(5): 1950–9.
doi: 10.3390/cimb44050133

Marancik DP, Leeds TD, Wiens GD. Histopathologic changes in disease-resistant-line and disease-susceptible-line juvenile rainbow trout experimentally infected with *Flavobacterium psychrophilum*. J Aquat Anim Health 2014; 26(3): 181–9.
doi: 10.1080/08997659.2014.920735

Mata W, Putita C, Dong HT, Kayansamruaj P, Senapin S, Rodkhum C. Quinolone-resistant phenotype of *Flavobacterium columnare* isolates harbouring point mutations both in *gyrA* and *parC* but not in *gyrB* or *parE*. J Glob Antimicrob Resist 2018; 15: 55–60.

doi: 10.1016/j.jgar.2018.05.014

McBride MJ, Zhu Y. Gliding motility and Por secretion system genes are widespread among members of the phylum *Bacteroidetes*. J Bacteriol 2013; 195(2): 270–8.
doi: 10.1128/JB.01962-12

McBride MJ, Nakane D. *Flavobacterium* gliding motility and the type IX secretion system. Curr Opin Microbiol 2015; 28: 72–7.
doi: 10.1016/j.mib.2015.07.016

McDaniel LD, Young E, Delaney J, Ruhnau F, Ritchie KB, Paul JH. High frequency of horizontal gene transfer in the oceans. Science 2010; 330(6000): 50.
doi: 10.1126/science.1192243

Miranda CD, Smith P, Rojas R, Contreras-Lynch S, Vega JMA. Antimicrobial susceptibility of *Flavobacterium psychrophilum* from Chilean salmon farms and their epidemiological cut-off values using agar dilution and disk diffusion methods. Front Microbiol 2016; 7: 1880.
doi: 10.3389/fmicb.2016.01880

Miwa S, Nakayasu C. Pathogenesis of experimentally induced bacterial cold water disease in ayu *Plecoglossus altivelis*. Dis Aquat Organ 2005; 67(1/2): 93–104.
doi: 10.3354/dao067093

Møller JD, Barnes AC, Dalsgaard I, Ellis AE. Characterisation of surface blebbing and membrane vesicles produced by *Flavobacterium psychrophilum*. Dis Aquat Organ 2005; 64(3): 201–9.
doi: 10.3354/dao064201

Molony B. Environmental requirements and tolerances of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) with special reference to Western Australia. Fish Res Rep West Aust 2001; 130: 1–28.
https://library.dpird.wa.gov.au/fr_rr/196

Mühle E, Abry C, Leclerc P, et al. *Flavobacterium bizetiae* sp. nov, isolated from diseased freshwater fish in Canada at the end of the 1970s. Int J Syst Evol Microbiol 2021; 71(1): 004576.
doi: 10.1099/ijsem.0.004576

Mulec J, Oarga-Mulec A, Skok S, et al. Emerging ecotone and microbial

community of a sulfidic spring in the Reka River near Škocjanske Jame, Slovenia. Diversity 2021a; 13(12): 655.
doi: 10.3390/d13120655

Mulec J, Oarga-Mulec A, Holko L, et al. Microbiota entrapped in recently-formed ice: Paradana Ice Cave, Slovenia. Sci Rep 2021b; 11(1): 1993.
doi: 10.1038/s41598-021-81528-6

Mwega E, Colquhoun DJ, Tuntufye H, et al. Isolation and characterization of Flavobacteriaceae from farmed and wild Nile tilapia in Tanzania. J Aquat Anim Health 2019; 31(1): 23–30.
doi: 10.1002/aah.10048

Mwega E, Chengula A, Colquhoun D, Mutoloki S, Evensen Ø, Wasteson Y. Antimicrobial susceptibility of Flavobacteriaceae isolates from Nile tilapia (*Oreochromis niloticus*) in Tanzania. Afr J Microbiol Res 2020; 14(1): 42–50.
doi: 10.5897/AJMR2019.9240

Nematollahi A, Decostere A, Pasmans F, Haesebrouck F. *Flavobacterium psychrophilum* infections in salmonid fish. J Fish Dis 2003a; 26(10): 563–74.
doi: 10.1046/j.1365-2761.2003.00488.x

Nematollahi A, Decostere A, Pasmans F, Ducatelle R, Haesebrouck F. Adhesion of high and low virulence *Flavobacterium psychrophilum* strains to isolated gill arches of rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 2003b; 55(2): 101–7.
doi: 10.3354/dao055101

Nematollahi A, Pasmans F, Haesebrouck F, Decostere A. Early interactions of *Flavobacterium psychrophilum* with macrophages of rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 2005; 64(1): 23–8.
doi: 10.3354/dao064023

Newton JC, Wood TM, Hartley MM. Isolation and partial characterization of extracellular proteases produced by isolates of *Flavobacterium columnare* derived from channel catfish. J Aquat Anim Health 1997; 9(2): 75–85.
doi: 10.1577/1548-8667(1997)009<0075:IAPCOE>2.3.CO;2

Ngo TPH, Bartie KL, Thompson KD, Verner-Jeffreys DW, Hoare R, Adams A. Genetic and serological diversity of *Flavobacterium psychrophilum* isolates from salmonids in United Kingdom. Vet Microbiol 2017; 201: 216–24.

doi: 10.1016/j.vetmic.2017.01.032

Ngo TPH, Smith P, Bartie KL, et al. Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from the United Kingdom. J Fish Dis 2018; 41(2): 309–20.
doi: 10.1111/jfd.12730

Nicolas P, Mondot S, Achaz G, Bouchenot C, Bernardet J-F, Duchaud E. Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. Appl Environ Microbiol 2008; 74(12): 3702–9.
doi: 10.1128/AEM.00244-08

Nikaido H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 1994; 264(5157): 382–88.
doi: 10.1126/science.8153625

Nilsen H, Olsen AB, Vaagnes Ø, et al. Systemic *Flavobacterium psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. J Fish Dis 2011; 34(5): 403–8.
doi: 10.1111/j.1365-2761.2011.01249.x

Nilsen H, Sundell K, Duchaud E, et al. Multilocus sequence typing identifies epidemic clones of *Flavobacterium psychrophilum* in Nordic countries. Appl Environ Microbiol 2014; 80(9): 2728–36.
doi: 10.1128/AEM.04233-13

Noble C, Gismervik K, Iversen MH, et al. Welfare indicators for farmed rainbow trout: tools for assessing fish welfare. Tromsø: Nofima, 2020.
https://nofima.no/fishwell/trout/

Ostland VE, Ferguson HW, Prescott J, Stevenson R, Barker I. Bacterial gill disease of salmonids: relationship between the severity of gill lesions and bacterial recovery. Dis Aquat Organ 1990; 9: 5–14.
doi: 10.3354/dao009005

Ostland VE, Lumsden JS, MacPhee DD, Ferguson HW. Characteristics of *Flavobacterium branchiophilum*, the cause of salmonid bacterial gill disease in Ontario. J Aquat Anim Health 1994; 6(1): 13–26.
doi: 10.1577/1548-8667(1994)006<0013:COFBTC>2.3.CO;2

Ostland VE, MacPhee DD, Lumsden JS, Ferguson HW. Virulence of *Flavobacterium branchiophilum* in experimentally infected salmonids. J Fish

Dis 1995a; 18(3): 249–62.
doi: 10.1111/j.1365-2761.1995.tb00300.x

Ostland VE, Byrne PJ, Speare DJ, et al. Comparison of formalin and Chloramine-T for control of a mixed gill infection (bacterial gill disease and ichthyobodiasis) in rainbow trout. J Aquat Anim Health 1995b; 7: 118–23.
doi: 10.1577/1548-8667(1995)007<0118:COFACT>2.3.CO;2

Ostland VE, Byrne PJ, Hoover G, Ferguson HW. Necrotic myositis of rainbow trout, *Oncorhynchus mykiss* (Walbaum): proteolytic characteristics of a crude extracellular preparation from *Flavobacterium psychrophilum*. J Fish Dis 2000; 23(5): 329–36.
doi: 10.1046/j.1365-2761.2000.00251.x

Otis EJ. Lesions of coldwater disease in steelhead trout (*Salmo gairdneri*): the role of *Cytophaga psychrophila* extracellular products. Kingston: University of Rhode Island, 1984. Doctoral dissertation.

Ototake M, Wakabayashi H. Characteristics of extracellular products of *Flavobacterium* sp., a pathogen of bacterial gill disease. Fish Pathol 1985; 20: 167–71.
doi: 10.3147/JSFP.20.167

Ourth DD, Bachinski LM. Bacterial sialic acid modulates activation of the alternative complement pathway of channel catfish (*Ictalurus punctatus*). Dev Comp Immunol 1987; 11(3): 551–64.
doi: 10.1016/0145-305x(87)90044-9

Pacha RE, Ordal EJ. Histopathology of experimental columnaris disease in young salmon. J Comp Pathol 1967; 77(4): 419–23.
doi: 10.1016/0021-9975(67)90027-8

Papadopoulou A, Dalsgaard I, Lindén A, Wiklund T. In vivo adherence of *Flavobacterium psychrophilum* to mucosal external surfaces of rainbow trout (*Oncorhynchus mykiss*) fry. J Fish Dis 2017; 40(10): 1309–20.
doi: 10.1111/jfd.12603

Park J, Roh H, Lee Y, Park J, Kim D-H. Complete genome analysis of *Flavobacterium psychrophilum* strain FPRT1, isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in South Korea. Microbiol Resource Announc 2021; 10(12): e00151-21.

doi: 10.1128/MRA.00151-21

Park J, Roh HJ, Lee Y, et al. Characterization and pathogenicity of *Flavobacterium psychrophilum* isolated from rainbow trout (*Oncorhynchus mykiss*) in Korea. Microorganisms 2023; 11(10): 2546.
doi: 10.3390/microorganisms11102546

Pate JL, Ordal EJ. The fine structure of *Chondrococcus columnaris*. 3. The surface layers of *Chondrococcus columnaris*. J Cell Biol 1967; 35(1): 37–51.
doi: 10.1083/jcb.35.1.37

Pearson K. Note on Regression and Inheritance in the Case of Two Parents. Proc R Soc Lond 1895; 58: 240–2.
doi: 10.1098/rspl.1895.0041

Pearson K. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. Philos Mag 1900; 50(302): 157–75.
doi: 10.1080/14786440009463897

Pedersen CL. Energy budgets for juvenile rainbow trout at various oxygen concentrations. Aquaculture 1987; 62(3): 289–98.
doi: 10.1016/0044-8486(87)90171-2

Peeters K, Willems A. The *gyrB* gene is a useful phylogenetic marker for exploring the diversity of *Flavobacterium* strains isolated from terrestrial and aquatic habitats in Antarctica. FEMS Microbiol Lett 2011; 321(2): 130–40.
doi: 10.1111/j.1574-6968.2011.02326.x

Pérez-Sancho M, Vela AI, Wiklund T, Kostrzewa M, Domínguez L, Fernández-Garayzábal JF. Differentiation of *Flavobacterium psychrophilum* from *Flavobacterium psychrophilum*-like species by MALDI-TOF mass spectrometry. Res Vet Sci 2017; 115: 345–52.
doi: 10.1016/j.rvsc.2017.06.022

Pravilnik o zahtevah za zdravstveno varstvo živali in proizvodov iz akvakulture ter o ukrepih za ugotavljanje, preprečevanje in obvladovanje določenih bolezni vodnih živali. Uradni list Republike Slovenije, št. 6/14, 10/19 in 16/19. Ur List RS 2014; 24(6): 431–9. https://www.uradni-list.si/_pdf/2014/Ur/u2014006.pdf (accessed 10 March 2025)

Pritchard L, Glover RH, Humphris S, Elphinstone JG, Toth IK. Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. Anal Methods 2016; 8(1): 12–24.
doi: 10.1039/C5AY02550H

Pulkkinen K, Suomalainen L-R, Read AF, Ebert D, Rintamäki P, Valtonen ET. Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc Biol Sci 2010; 277(1681): 593–600.
doi: 10.1098/rspb.2009.1659

Pursell L, Samuelsen OB, Smith P. Reduction in the in-vitro activity of flumequine against *Aeromonas salmonicida* in the presence of Mg^{2+} and Ca^{2+} ions found in seawater. Aquaculture 1995; 135(4): 245–55.
doi: 10.1016/0044-8486(95)01028-9

Raetz CRH, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem 2002; 71(1): 635–700.
doi: 10.1146/annurev.biochem.71.110601.135414

Ramesh D, Souissi S. Antibiotic resistance and virulence traits of bacterial pathogens from infected freshwater fish, *Labeo rohita*. Microb Pathog 2018; 116: 113–9.
doi: 10.1016/j.micpath.2018.01.019

Ramšak A, Avguštin G. Phylogenetic diversity of bacterial population in rumen. Acta Agric Slov 1998; 72(1): 77–83.
doi: 10.14720/aas.1998.72.1.17076

Rangdale RE, Richards RH, Alderman DJ. Histopathological and electron microscopical observations on rainbow trout fry syndrome. Vet Rec 1999; 144(10): 251–4.
doi: 10.1136/vr.144.10.251

Reasoner DJ, Blannon JC, Geldreich EE. Rapid seven-hour fecal coliform test. Appl Environ Microbiol 1979; 38: 229–36.
doi: 10.1128/aem.38.2.229-236.1979

Rebl A, Korytář T, Borchel A, et al. The synergistic interaction of thermal stress coupled with overstocking strongly modulates the transcriptomic activity and immune capacity of rainbow trout (*Oncorhynchus mykiss*). Sci Rep 2020; 10(1): 14913.

doi: 10.1038/s41598-020-71852-8

Reichenbach H. Order I. *Cytophagales* Leadbetter 1974. In: Staley JT, eds. Bergey's Manual of Systematic Bacteriology. 1st ed. Vol. 3. Baltimore: Williams & Wilkins, 1989: 2011–3.

Reichley SR, Ware C, Steadman J, et al. Comparative phenotypic and genotypic analysis of *Edwardsiella* isolates. J Clin Microbiol 2017; 55(12): 3466–91. doi: 10.1128/jcm.00970-17

Riesco R, Trujillo ME. Update on the proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int J Syst Evol Microbiol 2024; 74(3): 006300. doi: 10.1099/ijsem.0.006300

Rintamäki-Kinnunen P, Bernardet J-F, Bloigu A. Yellow pigmented filamentous bacteria connected with farmed salmonid fish mortality. Aquaculture 1997; 149(1/2): 1–14. doi: 10.1016/S0044-8486(96)01431-7

Roberts RJ. Fish pathology. 4th ed. Oxford: Wiley-Blackwell, 2012.

Rochat T, Fujiwara-Nagata E, Calvez S, et al. Genomic characterization of *Flavobacterium psychrophilum* serotypes and development of a multiplex PCR-based serotyping scheme. Front Microbiol 2017; 8: 1752. doi: 10.3389/fmicb.2017.01752

Ross AJ, Smith CA. Effect of temperature on survival of *Aeromonas liquefaciens*, *Aeromonas salmonicida*, *Chondrococcus columnaris*, and *Pseudomonas fluorescens*. Prog Fish Cult 1974; 36(1): 51–2. doi: 10.1577/1548-8659(1974)36[51:EOTOSO]2.0.CO;2

Rucker RR, Earp BJ, Ordal EJ. Infectious diseases of Pacific salmon. Trans Am Fish Soc 1954; 83(1): 297–312. doi: 10.1577/1548-8659(1953)83[297:IDOPS]2.0.CO;2

Saraiva A, Costa J, Serrão J, Cruz C, Eiras JC. A histology-based fish health assessment of farmed seabass (*Dicentrarchus labrax* L.). Aquaculture 2015; 448: 375–81. doi: 10.1016/j.aquaculture.2015.06.028

Saticioglu IB, Duman M, Smith P, Wiklund T, Altun S. Antimicrobial resistance and resistance genes in *Flavobacterium psychrophilum* isolates from Turkey.

Aquaculture 2019; 512: 734293.
doi: 10.1016/j.aquaculture.2019.734293

Saticioglu IB, Ay H, Altun S, Sahin N, Duman M. *Flavobacterium bernardetii* sp. nov, a possible emerging pathogen of farmed rainbow trout (*Oncorhynchus mykiss*) in cold water. Aquaculture 2021a; 540: 736717.
doi: 10.1016/j.aquaculture.2021.736717

Saticioglu IB, Ay H, Altun S, Duman M, Sahin N. *Flavobacterium turcicum* sp. nov. and *Flavobacterium kayseriense* sp. nov. isolated from farmed rainbow trout in Turkey. Syst Appl Microbiol 2021b; 44(2): 126186.
doi: 10.1016/j.syapm.2021.126186

Saticioglu IB, Duman M, Ajmi N, Altun S, Rochat T, Duchaud E. Phylogenomic characterization of *Flavobacterium psychrophilum* isolates retrieved from Turkish rainbow trout farms. J Fish Dis 2024; 48(8): e13961.
doi: 10.1111/jfd.13961

Sato K, Naito M, Yukitake H, et al. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. Proc Natl Acad Sci USA 2010; 107(1): 276–81.
doi: 10.1073/pnas.0912010107

Schurmann H, Steffensen J, Lomholt JP. The influence of hypoxia on the preferred temperature of rainbow trout *Oncorhynchus mykiss*. J Exp Biol 1991; 157: 75–86.
doi: 10.1242/jeb.157.1.75

Seabold S, Perktold J. Statsmodels: Econometric and Statistical Modeling with Python. In: Van der Walt A, eds. Proceedings of the 9th python in science conference. Austin: SciPy, 2010: 92–6.
doi: 10.25080/Majora-92bf1922-011

Secades P, Alvarez B, Guijarro JA. Purification and characterization of a psychrophilic, calcium-induced, growth-phase-dependent metalloprotease from the fish pathogen *Flavobacterium psychrophilum*. Appl Environ Microbiol 2001; 67(6): 2436–44.
doi: 10.1128/AEM.67.6.2436-2444.2001

Secades P, Alvarez B, Guijarro JA. Purification and properties of a new psychrophilic metalloprotease (Fpp2) in the fish pathogen *Flavobacterium*

psychrophilum. FEMS Microbiol Lett 2003; 226(2): 273–9. doi: 10.1016/S0378-1097(03)00599-8

Semple SL, Bols NC, Lumsden JS, Dixon B. Understanding the pathogenesis of *Flavobacterium psychrophilum* using the rainbow trout monocyte/macrophage-like cell line, RTS11, as an infection model. Microb Pathog 2020; 139: 103910. doi: 10.1016/j.micpath.2019.103910

Shah SQA, Nilsen H, Bottolfsen K, Colquhoun DJ, Sørum H. DNA gyrase and topoisomerase IV mutations in quinolone-resistant *Flavobacterium psychrophilum* isolated from diseased salmonids in Norway. Microb Drug Resist 2012; 18(2): 207–14. doi: 10.1089/mdr.2011.0142

Shamsudin MN, Plumb JA. Morphological, biochemical, and physiological characterization of *Flexibacter columnaris* isolates from four species of fish. J Aquat Anim Health 1996; 8(4): 335–9. doi: 10.1577/1548-8667(1996)008<0335:MBAPCO>2.3.CO;2

Shao ZJ. Aquaculture pharmaceuticals and biologicals: current perspectives and future possibilities. Adv Drug Deliv Rev 2001; 50(3): 229–43. doi: 10.1016/s0169-409x(01)00159-4

Shapiro SS, Wilk MB. An analysis of variance test for normality (complete samples). Biometrika 1965; 52(3/4): 591–611. doi: 10.2307/2333709

Shoemaker CA, LaFrentz BR. Growth and survival of the fish pathogenic bacterium, *Flavobacterium columnare*, in tilapia mucus and porcine gastric mucin. FEMS Microbiol Lett 2015; 362(4): fnu060. doi: 10.1093/femsle/fnu060

Shoemaker CA, LaFrentz BR, Peatman E, Beck BH. Influence of native catfish mucus on *Flavobacterium columnare* growth and proteolytic activity. J Fish Dis 2018; 41(9): 1395–402. doi: 10.1111/jfd.12833

Shovill (1.18 version). https://github.com/tseemann/shovill (accessed 5 July 2023)

Silley P. Susceptibility testing methods, resistance and breakpoints: what do these terms really mean? Rev Sci Tech 2012; 31(1): 33–41.

doi: 10.20506/rst.31.1.2097

Singh S, Mallik SK, Kala K, et al. Characterization of *Flavobacterium columnare* from farmed infected rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) of Central Indian Himalayan region, India. Aquaculture 2021; 544: 737118. doi: 10.1016/j.aquaculture.2021.737118

Siwicki AK, Pozet F, Morand M, Kazuń K, Głąbski E, Trapkowska S. Atypical bacterial gill disease in rainbow trout (*Oncorhynchus mykiss*): influence on non specific humoral defence mechanisms. Arch Pol Fish 2004; 12(1): 5–12.

Skulska IV. Culture of the bacterial gill disease organism, *Flavobacterium branchiophilum* and strain differences relevant to epizoology. Guelph: University of Guelph,
2014. Master's thesis.

Smith P, Endris R, Kronvall G, et al. Epidemiological cut-off values for *Flavobacterium psychrophilum* MIC data generated by a standard test protocol. J Fish Dis 2016; 39(2): 143–54.
doi: 10.1111/jfd.12336

Smith SA. Fish diseases and medicine. 1st ed. Boca Raton: CRC Press, 2019: 107–10, 135.

Soares SMC, Walker A, Elwenn SA, et al. First isolation of *Flavobacterium psychrophilum* associated with reports of moribund wild European eel (*Anguilla anguilla*) in Scotland. J Fish Dis 2019; 42(11): 1509–21.
doi: 10.1111/jfd.13069

Söderlund R, Hakhverdyan M, Aspan A, Jansson E. Genome analysis provides insights into the epidemiology of infection with *Flavobacterium psychrophilum* among farmed salmonid fish in Sweden. Microb Genom 2018; 4(12): e000241.
doi: 10.1099/mgen.0.000241

Soltani M, Munday B, Carson J. Susceptibility of some freshwater species of fish to infection by *Cytophaga johnsonae*. Bull Eur Assoc Fish Pathol 1994; 14(4): 133–5.

Soltani M, Burke C. Responses of fish-pathogenic *Cytophaga*/*Flexibacter*-like bacteria (CFLB) to environmental conditions. Bull Eur Assoc Fish Pathol 1994; 14(6): 185–7.

Song YL, Fryer JL, Rohovec JS. Comparison of six media for the cultivation of *Flexibacter columnaris*. Fish Pathol 1988; 23(2): 91–4. doi: 10.3147/jsfp.23.91

Soule M, LaFrentz S, Cain K, LaPatra S, Call DR. Polymorphisms in 16S rRNA genes of *Flavobacterium psychrophilum* correlate with elastin hydrolysis and tetracycline resistance. Dis Aquat Organ 2005; 65(3): 209–16. doi: 10.3354/dao065209

Speare DJ, Ferguson HW, Beamish F, Yager JA, Yamashiro S. Pathology of bacterial gill disease: sequential development of lesions during natural outbreaks of disease. J Fish Dis 1991; 14(1): 21–32. doi: 10.1111/j.1365-2761.1991.tb00573.x

Spearman C. The proof and measurement of association between two things. Am J Psychol 1904; 15(1): 72–101. doi: 10.2307/1412159

Starliper CE. Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. J Adv Res 2011; 2(2): 97–108. doi: 10.1016/j.jare.2010.04.001

Staroscik AM, Nelson DR. The influence of salmon surface mucus on the growth of *Flavobacterium columnare*. J Fish Dis 2008; 31(1): 59–69. doi: 10.1111/j.1365-2761.2007.00867.x

Statistical Office of the Republic of Slovenia. SiStat Database. Available online: https://www.stat.si/StatWeb/News/Index/13038 (accessed on 6 March 2025)

Steinbach C, Kroupová HK, Wahli T, Klicnarová J, Schmidt-Posthaus H. Histopathological alterations of the heart in fish: proposal for a standardized assessment. Dis Aquat Organ 2016; 118(3): 185–94. doi: 10.3354/dao02971

Stine CB, Li C, Crosby TC, Hasbrouck NR, Lam C, Tadesse DA. Draft whole-genome sequences of 18 *Flavobacterium* spp. Genome Announc 2017; 5(46): e00865-17. doi: 10.1128/genomeA.00865-17

Straus DL, Farmer BD, Beck BH, Bosworth BG, Torrans EL, Tucker CS. Water hardness influences *Flavobacterium columnare* pathogenesis in channel catfish. Aquaculture 2015; 435: 252–6.

doi: 10.1016/j.aquaculture.2014.10.003

Strepparava N, Wahli T, Segner H, Polli B, Petrini O. Fluorescent in situ hybridization: A new tool for the direct identification and detection of *F. psychrophilum*. PLoS One 2012a; 7(11): e49280.
doi: 10.1371/journal.pone.0049280

Strepparava N, Segner H, Petrini O, Wahli T. MALDI–TOF MS for the identification and characterization of *Flavobacterium* isolates from water and the environment. In: *Flavobacterium* 2012: programme and abstracts. Turku: Åbo Akademi University, 5–7 June, 2012b.

Strepparava N, Wahli T, Segner H, Petrini O. Detection and quantification of *Flavobacterium psychrophilum* in water and fish tissue samples by quantitative real time PCR. BMC Microbiol 2014; 14: 105.
doi: 10.1186/1471-2180-14-105

Strohl WR, Tait LR. *Cytophaga aquatilis* sp. nov. a facultative anaerobe isolated from the gills of freshwater fish. Int J Syst Bacteriol 1978; 28(2): 293–303.
doi: 10.1099/00207713-28-2-293

Suebsing R, Kim J-H. Isolation and Characterization of *Flavobacterium johnsoniae* from Farmed Rainbow Trout *Oncorhynchus mykiss*. J Fish Aquat Sci 2012; 15: 83–9.
doi: 10.5657/FAS.2012.0083

Sun F, Peatman E, Li C, et al. Transcriptomic signatures of attachment, NF-κB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection. Dev Comp Immunol 2012; 38(1): 169–80.
doi: 10.1016/j.dci.2012.05.006

Sundell K, Wiklund T. Effect of biofilm formation on antimicrobial tolerance of *Flavobacterium psychrophilum*. J Fish Dis 2011; 34(5): 373–83.
doi: 10.1111/j.1365-2761.2011.01250.x

Sundell K, Heinikainen S, Wiklund T. Structure of *Flavobacterium psychrophilum* populations infecting farmed rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 2013; 103(2): 111–9.
doi: 10.3354/dao02573

Sundell K, Landor L, Nicolas P, et al. Phenotypic and Genetic Predictors of Pathogenicity and Virulence in *Flavobacterium psychrophilum*. Front Microbiol

2019; 10: 1711.
doi: 10.3389/fmicb.2019.01711

Suomalainen L-R, Tiirola MA, Valtonen ET. Influence of rearing conditions on *Flavobacterium columnare* infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 2005; 28(5): 271–7.
doi: 10.1111/j.1365-2761.2005.00631.x

Taylor PW. Detection of *Flavobacterium psychrophilum* in eggs and sexual fluids of Pacific salmonids by a polymerase chain reaction assay: Implications for vertical transmission of bacterial coldwater disease. J Aquat Anim Health 2004; 16(2): 104–8.
doi: 10.1577/H03-053.1

Teufel R, Mascaraque V, Ismail W, et al. Bacterial phenylalanine and phenylacetate catabolic pathway revealed. Proc Natl Acad Sci USA 2010; 107(32): 14390–5.
doi: 10.1073/pnas.1005399107

Thunes NC, Conrad RA, Mohammed HH, et al. Type IX secretion system effectors and virulence of the model *Flavobacterium columnare* strain MS-FC-4. Appl Environ Microbiol 2022; 88(3): e0170521.
doi: 10.1128/AEM.01705-21

Thunes NC, Evenhuis JP, Lipscomb RS, et al. Gliding motility proteins GldJ and SprB contribute to *Flavobacterium columnare* virulence. J Bacteriol 2024; 206(4): e0006824.
doi: 10.1128/jb.00068-24

Timur G, Akayli T, Korun J, Yardimci RE. A study on bacterial haemorrhagic septicemia in farmed young Russian sturgeon (*Acipenser gueldenstaedtii*) in Turkey. İstanbul Univ J Fish Aquat Sci 2010; 25(1): 19–26.

Tinta T, Kogovšek T, Malej A, Turk V. Jellyfish modulate bacterial dynamic and community structure. PLoS One 2012; 7(6): e39274.
doi: 10.1371/journal.pone.0039274

Touchon M, Barbier P, Bernardet JF, et al. Complete genome sequence of the fish pathogen *Flavobacterium branchiophilum*. Appl Environ Microbiol 2011; 77(21): 7656–62.
doi: 10.1128/AEM.05625-11

Toyama T, Kita-Tsukamoto K, Wakabayashi H. Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. Fish Pathol 1994; 29(4): 271–5.
doi: 10.3147/jsfp.29.271

Toyama T, Kita-Tsukamoto K, Wakabayashi H. Identification of *Flexibacter maritimus*, *Flavobacterium branchiophilum* and *Cytophaga columnaris* by PCR targeted 16S ribosomal DNA. Fish Pathol 1996; 31(1): 25–31.
doi: 10.3147/jsfp.31.25

Turk V, Tinta T. Short-term changes in microbial communities in the water column around the fish farm in the Bay of Piran. Acta Biol Slov 2021; 64(2): 9–23.
doi: 10.14720/abs.64.2.15817

Uiuiu P, Latiu C, Păpuc T, et al. Multi-approach assessment for stress evaluation in rainbow trout females, *Oncorhynchus mykiss* (Walbaum, 1792) from three different farms during the summer season. Animals 2021; 11(6):1810.
doi: 10.3390/ani11061810

Vaibarová V, Čížek A. Supposed virulence factors of *Flavobacterium psychrophilum*: A review. Fishes 2024; 9(5): 163.
doi: 10.3390/fishes9050163

Vaibarová V, Kralová S, Palíková M, et al. Genetic and phenotypic diversity of *Flavobacterium psychrophilum* isolates from Czech salmonid fish farms. BMC Microbiol 2024; 24(1): 352.
doi: 10.1186/s12866-024-03490-3

Vandamme P, Bernardet J-F, Segers P, Kersters K, Holmes B. New perspectives in the classification of the flavobacteria: Description of *Chryseobacterium* gen. nov., *Bergeyella* gen. nov., and *Empedobacter* nom. rev. Int J Syst Bacteriol 1994; 44(4): 827–48.
doi: 10.1099/00207713-44-4-827

Vanden Broeck D, Horvath C, De Wolf MJS. *Vibrio cholerae*: Cholera toxin. Int J Biochem Cell Biol 2007; 39(10): 1771–5.
doi: 10.1016/j.biocel.2007.07.005

Van Rossum G, Drake FL. Python 3 reference manual. Scotts Valley, CA: CreateSpace, 2009.

Varga Z, Sellyei B, Paulus P, Papp M, Molnár K, Székely C. Isolation and

characterisation of flavobacteria from wild and cultured freshwater fish species in Hungary. Acta Vet Hung 2016; 64(1): 13–25. doi: 10.1556/004.2016.002

Verma V, Prasad Y, Singh BR. Effect of pH and salinity on pathogenicity of *Flavobacterium columnare* and *Myxobacterium* sp. in Indian catfish, *Clarias batrachus* (Linn.) and *Heteropneustes fossilis* (Bloch.). J Environ Biol 2011; 32(5): 573–7.

Verma DK, Rathore G. New host record of five *Flavobacterium* species associated with tropical freshwater farmed fishes from North India. Braz J Microbiol 2015; 46(4): 969–76. doi: 10.1590/S1517-838246420131081

Verner-Jeffreys DW, Brazier T, Perez RY, et al. Detection of the florfenicol resistance gene *floR* in *Chryseobacterium* isolates from rainbow trout. Exception to the general rule? FEMS Microbiol Ecol 2017; 93(4). doi: 10.1093/femsec/fix015

Van Vliet D, Loch TP, Smith P, Faisal M. Antimicrobial susceptibilities of *Flavobacterium psychrophilum* isolates from the Great Lakes Basin, Michigan. Microb Drug Resist 2017; 23(6): 791–8. doi: 10.1089/mdr.2016.0103

Wagner E, Bosakowski T, Intelmann S. Combined effects of temperature and high pH on mortality and the stress response of rainbow trout after stocking. Trans Am Fish Soc 1997; 126(6): 985–98. doi: 10.1577/1548-8659(1997)126<0985:CEOTAH>2.3.CO;2

Wahli T, Madsen L. *Flavobacteria*, a never-ending threat for fish: A review. Curr Clin Microbiol Rep 2018; 5(1): 26–37. doi: 10.1007/s40588-018-0086-x

Wakabayashi H, Huh GJ, Kimura N. *Flavobacterium branchiophilum* sp. nov., a causative agent of bacterial gill disease of freshwater fishes. Int J Syst Bacteriol 1989; 39(3): 213–6. doi: 10.1099/00207713-39-3-213

Wakabayashi H. Columnaris disease. In: Inglis V, eds. Bacterial diseases of fish. Oxford: Blackwell, 1993: 23–39.

Ward JH Jr. Hierarchical grouping to optimize an objective function. J Am Stat

Assoc 1963; 58(301): 236–44.
doi: 10.1080/01621459.1963.10500845

Wiklund T, Dalsgaard I. Association of *Flavobacterium psychrophilum* with rainbow trout (*Oncorhynchus mykiss*) kidney phagocytes *in vitro*. Fish Shellfish Immunol 2003; 15(5): 387–95.
doi: 10.1016/s1050-4648(03)00009-3

Wu AK, Kropinski AM, Lumsden JS, Dixon B, MacInnes JI. Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum* ATCC 49418^{T}. Stand Genomic Sci 2015; 10: 3.
doi: 10.1186/1944-3277-10-3

Xu D-H, Shoemaker CA, Klesius PH. *Ichthyophthirius multifiliis* as a potential vector of *Edwardsiella ictaluri* in channel catfish. FEMS Microbiol Lett 2012; 329(2): 160–7.
doi: 10.1111/j.1574-6968.2012.02518.x

Yi H, Chun J. *Flavobacterium weaverense* sp. nov. and *Flavobacterium segetis* sp. nov., novel psychrophiles isolated from the Antarctic. Int J Syst Evol Microbiol 2006; 56(6): 1239–44.
doi: 10.1099/ijs.0.64164-0

Yoon S-H, Ha S-M, Kwon S, et al. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 2017; 67(5): 1613–7.
doi: 10.1099/ijsem.0.001755

Zamora L, Fernández-Garayzábal JF, Svensson-Stadler LA, et al. *Flavobacterium oncorhynchi* sp. nov., a new species isolated from rainbow trout (*Oncorhynchus mykiss*). Syst Appl Microbiol 2012; 35(2): 86–91.
doi: 10.1016/j.syapm.2011.11.007

Zamora L, Fernández-Garayzábal JF, Sánchez-Porro C, et al. *Flavobacterium plurextorum* sp. nov. isolated from farmed rainbow trout (*Oncorhynchus mykiss*). PLoS One 2013; 8(6): e67741.
doi: 10.1371/journal.pone.0067741

Zamora L, Vela AI, Sánchez-Porro C, et al. *Flavobacterium tructae* sp. nov. and *Flavobacterium piscis* sp. nov., isolated from farmed rainbow trout (*Oncorhynchus mykiss*). Int J Syst Evol Microbiol 2014; 64(2): 392–9.

doi: 10.1099/ijs.0.056341-0

Zamparo S, Orioles M, Brocca G, et al. Novel insights on microbiome dynamics during a gill disease outbreak in farmed rainbow trout (*Oncorhynchus mykiss*). Sci Rep 2024; 14(1): 17791.
doi: 10.1038/s41598-024-68287-w

Zhang C, Li D, Chi C, Ling F, Wang G. *Dactylogyrus intermedius* parasitism enhances *Flavobacterium columnare* invasion and alters immune-related gene expression in *Carassius auratus*. Dis Aquat Organ 2015; 116(1): 11–21.
doi: 10.3354/dao02902

Zhang Y, Zhao L, Chen W, et al. Complete genome sequence analysis of the fish pathogen *Flavobacterium columnare* provides insights into antibiotic resistance and pathogenicity-related genes. Microb Pathog 2017; 111: 203–11.
doi: 10.1016/j.micpath.2017.08.035

Zhu Y, Lechardeur D, Bernardet J-F, et al. Two functionally distinct heme/iron transport systems are virulence determinants of the fish pathogen *Flavobacterium psychrophilum*. Virulence 2022; 13(1): 1221–41.
doi: 10.1080/21505594.2022.2101197

Zurbuchen R, de Roche M, Galimanis A, et al. First case of meningoencephalitis and bacteraemia with *Flavobacterium lindanitolerans*. Eur J Case Rep Intern Med 2023; 10(8): 003980.
doi: 10.12890/2023_003980

APPENDICES

Appendix 1: Owner's informed consent form

First and last name of the owner: _____
Fish farm (location, G-MID farm identification number): _____
Animal information: rainbow trout (*Oncorhynchus mykiss*)

Owner's declaration:

I, the undersigned, as the owner of the above-mentioned animals, have understood the procedures to be carried out as part of the research entitled "Biodiversity of pathogenic flavobacteria in rainbow trout (*Oncorhynchus mykiss*) and dynamics of infections in selected Slovene fish farms" and consent to the use of my animals in this research.

With my signature, I permit the collection of samples for the following purposes:
☒ necropsy, histopathological, and bacteriological examination

My participation in the research is entirely voluntary, and I can refuse or terminate my participation at any time during the research.
I have been informed that the results of this research will contribute to the advancement of veterinary medicine. I am aware that the methodology of this research has been reviewed and approved by the Ethical Committee for experiments on animals.
Associate Professor Dr. Tanja Švara, who is responsible for the safe conduct of the research, has clearly explained to me the benefits, risks, and potential discomforts to which my animals may be exposed.

Therefore, I voluntarily and knowingly consent to the use of my animals in this research and agree that any samples collected during the research may be used for research purposes. I also consent to the publication of the research results and the use of photographic material, provided that the identity of the owner is not disclosed.

Signature of the animal owner: _____
Date: _____

Researcher's declaration:

I, the undersigned, have clearly explained to the animal owner the benefits, risks, and potential discomforts of participating in the research.

Signature of the researcher: _____

Date: _____

Appendix 2: Questionnaire for fish farmers

Note: The questionnaire was completed directly by the fish farmers, but inconsistencies were noted in several responses, particularly regarding the dimensions of the fish tanks, the number of fish per tank, and the body weight of the fish, which prevented an accurate calculation of the fish density per tank.

NA, answer not available.

A) QUESTIONNAIRE: FIRST LONGITUDINAL SAMPLING AND INDIVIDUAL SAMPLINGS

I. GENERAL INFORMATION

1. What is the annual production of fish (kg or tons)?

FISH FARM	Answer
F1_autumn 2022	**8 tons**
F2_autumn 2022	**200 tons**
F3_March 2023	**15 tons**
F4_June 2024	**5 tons**

2. Which system(s) do you use?

ponds/parallel tanks/flow-through tanks/partial recirculation systems/recirculation systems/other

FISH FARM	Answer
F1_autumn 2022	**parallel and flow-through tanks**
F2_autumn 2022	**parallel and flow-through tanks**
F3_March 2023	**parallel and flow-through tanks**
F4_June 2024	**parallel tanks**

3. What type of substrate is used in the tanks?

soil/soil with gravel base/concrete with gravel base/concrete/plastic/other

FISH FARM	Answer
F1_autumn 2022	**concrete and plastic**
F2_autumn 2022	**concrete**
F3_March 2023	**concrete**
F4_June 2024	**concrete**

4. What water source do you use?

spring/river/stream/lake/other

Water source name:

FISH FARM	Answer

F1_autumn 2022	**spring**
F2_autumn 2022	**river**
F3_March 2023	**river**
F4_June 2024	**stream**

5. Does the water supplying your fish farm pass through upstream fish farms? YES/NO

If yes, how many?

FISH FARM	Answer
F1_autumn 2022	**no**
F2_autumn 2022	**yes, two**
F3_March 2023	**yes, probably one**
F4_June 2024	**no**

6. Is the water treated at the inflow and/or outflow (sedimentation, filtration, disinfection, etc.)? YES/NO

FISH FARM	Answer
F1_autumn 2022	**no**
F2_autumn 2022	**no**
F3_March 2023	**no**
F4_June 2024	**yes, sedimentation**

7. What is the current water flow rate (liters/second)?

FISH FARM	Answer
F1_autumn 2022	**40 l/s**
F2_autumn 2022	**1500 l/s**
F3_March 2023	**50 l/s**
F4_June 2024	**30 l/s**

8. Do you monitor the water quality? YES/NO

If yes, how often?

How do you perform the measurements? Probes/test strips/other

What parameters do you monitor, and what are the average values during the different seasons?

a) temperature
b) dissolved oxygen
c) pH
d) other

FISH FARM	Answer
F1_autumn 2022	**no, water temperature is 9 °C throughout the year**

F2_autumn 2022	**yes, daily temperature measurements using a thermometer, on average 8–20 °C (minimum 3 °C and maximum 23 °C)**
F3_March 2023	**no, water temperature around 2–14 °C**
F4_June 2024	**no**

9. Do you also farm other fish species besides rainbow trout? YES/NO

If yes, please list the species:

FISH FARM	Answer
F1_autumn 2022	**no**
F2_autumn 2022	**yes, sturgeons**
F3_March 2023	**no**
F4_June 2024	**no**

10. What age categories of rainbow trout do you have in your farm?

Eggs (____%)/sac fry (____%)/fry and fingerlings (____%)/one-year-old juveniles (____%)/two-year-old juveniles (____%)/adult fish or broodstock (____%)

FISH FARM	Answer
F1_autumn 2022	**fry and fingerlings 70%, one-year-old juveniles 20%, adult fish or broodstock 10%**
F2_autumn 2022	**fry and fingerlings 50%, one-year-old juveniles 50%**
F3_March 2023	**fry and fingerlings 75%, one-year-old juveniles 25%**
F4_June 2024	**fry and fingerlings 90%, one-year-old juveniles 10%**

11. Do you farm sterile triploid female rainbow trout? YES/NO

FISH FARM	Answer
F1_autumn 2022	**yes**
F2_autumn 2022	**yes**
F3_March 2023	**yes**
F4_June 2024	**yes**

12. Do you hatch your own eggs, or do you buy the fish?

If you buy fish:

What age category(ies) do you purchase?

Do the purchased fish have any certificates (disease-free, organic farming, etc.)?

FISH FARM	Answer
F1_autumn 2022	**hatching their own eggs in a separate facility**
F2_autumn 2022	**purchasing eyed-stage eggs with a disease-free (VHS and IHN) certificate**
F3_March 2023	**purchasing juveniles weighing 10 g (100 fish/kg), NA**

F4_June 2024	**purchasing eyed-stage eggs with a disease-free (VHS and IHN) certificate**

II. INFORMATION ABOUT THE SAMPLED TANKS AND FISH

1. What is the size of the tank (m^3)?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**20 m^3 (outside, concrete)**	**27 m^3 (outside, concrete)**	**1.5 m^3 (inside, plastic)**
F2_autumn 2022	**256 m^3 (outside, concrete)**	**256 m^3 (outside, concrete)**	**256 m^3 (outside, concrete)**
F3_March 2023	**56 m^3 (outside, concrete)**	**/**	**/**
F4_June 2024	**50 m^3 (outside, concrete)**	**/**	**/**

2. How many fish are in the tank, and what is their average weight?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**15000 fish, 40 g**	**2000 fish, 82 g**	**2000 fish, 84 g**
F2_autumn 2022	**4500 fish, 50 g**	**77556 fish, 55 g**	**5000 fish, 180 g**
F3_March 2023	**32730 fish, 55 g**	**/**	**/**
F4_June 2024	**141000 fish, 8.3 g**	**/**	**/**

3. What is the approximate age of the fish (in days/months/years)?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**8 months**	**9 months**	**9 months**
F2_autumn 2022	**17–18 months**	**20 months**	**20 months**
F3_March 2023	**20 months**	**/**	**/**
F4_June 2024	**7 months**	**/**	**/**

4. What is the current water flow rate in the tank?

High/satisfactory/low

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**satisfactory**	**satisfactory**	**satisfactory**
F2_autumn 2022	**satisfactory**	**satisfactory**	**satisfactory**
F3_March 2023	**satisfactory**	**/**	**/**
F4_June 2024	**satisfactory**	**/**	**/**

5. Does the water supplying the tank also flow through upstream tanks? YES/NO

If yes, how many tanks?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3

F1_autumn 2022	**no**	**yes**	**no**
F2_autumn 2022	**yes, two tanks**	**yes, two tanks**	**yes, two tanks**
F3_March 2023	**yes, two tanks**	**/**	**/**
F4_June 2024	**no**	**/**	**/**

6. What feed do you use? How much (kg feed/kg fish) and how often per day do you feed the fish? Do you use manual feeding or automatic feeding systems?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**Biomar, 1% feed per 1 kg of fish, twice daily, manual and automatic feeding**	**Biomar, 1% feed per 1 kg of fish, twice daily, manual and automatic feeding**	**Biomar, 1% feed per 1 kg of fish, twice daily, manual and automatic feeding**
F2_autumn 2022	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**
F3_March 2023	**NA, 13.5 kg of feed per 1000 kg of fish, NA, manual feeding**	**/**	**/**
F4_June 2024	**Biomar, 2% feed per 1 kg of fish, once daily, manual feeding**	**/**	**/**

7. Do you currently use other food products/supplements (probiotics, vitamins, minerals, etc.)?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**no**	**no**	**no**
F2_autumn 2022	**no**	**no**	**no**
F3_March 2023	**no**	**/**	**/**
F4_June 2024	**no**	**/**	**/**

8. Have the fish been treated with antibiotics in the last month? YES/NO

If yes, which antibiotic(s) and when?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**no**	**no**	**no**
F2_autumn 2022	**no**	**no**	**no**
F3_March 2023	**no**	**/**	**/**
F4_June 2024	**no**	**/**	**/**

9. Have you observed any changes in the behaviour or appearance of the fish in the last month? YES/NO

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**no**	**no**	**no**
F2_autumn 2022	**no**	**no**	**no**

F3_March 2023	**increased mortality**	/	/
F4_June 2024	**increased mortality**	/	/

10. What was the daily mortality rate in the last two weeks?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**normal**	**normal**	**normal**
F2_autumn 2022	**normal**	**mildly increased, 4–5 kg daily (normal 1 kg daily)**	**mildly increased, 3 kg daily (normal 1 kg daily)**
F3_March 2023	**severely increased**	/	/
F4_June 2024	**severely increased**	/	/

11. Have the fish been exposed to stress in the last two weeks (handling, transportation, sorting, etc.)? YES/NO

If yes, please explain:

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_autumn 2022	**no**	**no**	**no**
F2_autumn 2022	**no**	**no**	**no**
F3_March 2023	**no**	/	/
F4_June 2024	**no**	/	/

III. DISEASES, PREVENTION, AND TREATMENT

1. Is the fish farm positive for any of the mandatory reportable infectious diseases?

Viral hemorrhagic septicemia (VHS)/infectious hematopoietic necrosis (IHN)/disease-free status/other

FISH FARM	Answer
F1_autumn 2022	**disease-free status**
F2_autumn 2022	**IHN positive**
F3_March 2023	**IHN positive**
F4_June 2024	**disease-free status**

2. Are the fish vaccinated? YES/NO

If yes, against which pathogens/diseases?

FISH FARM	Answer
F1_autumn 2022	**no**
F2_autumn 2022	**no**
F3_March 2023	**no**
F4_June 2024	**no**

3. How often do you inspect the tanks for the health and well-being of the fish?

FISH FARM	Answer
F1_autumn 2022	**daily**
F2_autumn 2022	**daily**
F3_March 2023	**daily**
F4_June 2024	**daily**

4. How often do you remove dead fish?

FISH FARM	Answer
F1_autumn 2022	**daily**
F2_autumn 2022	**daily**
F3_March 2023	**daily**
F4_June 2024	**daily**

5. In your opinion, which diseases (bacterial, viral, parasitic, fungal, or other) occur frequently on your farm?

FISH FARM	Answer
F1_autumn 2022	***Flavobacterium psychrophilum* (RTFS)**
F2_autumn 2022	**furunculosis, yersiniosis, flavobacteriosis, IHN**
F3_March 2023	**NA**
F4_June 2024	***Flavobacterium psychrophilum*, *Aeromonas***

6. Has a veterinarian confirmed any disease in the last month? YES/NO

If yes, which one?

FISH FARM	Answer
F1_autumn 2022	**no**
F2_autumn 2022	**no**
F3_March 2023	**no**
F4_June 2024	**no**

7. Have you heard of flavobacterial diseases (including rainbow trout fry syndrome/cold water disease, columnaris disease, bacterial gill disease)?

YES/NO

If yes, do you think they occur in your fish farm?

FISH FARM	Answer
F1_autumn 2022	**yes, yes**

F2_autumn 2022	**yes, yes**
F3_March 2023	**yes, no**
F4_June 2024	**yes, yes**

8. Are the fish selectively bred for increased resistance to any of the flavobacterial diseases? YES/NO

If yes, which one?

FISH FARM	Answer
F1_autumn 2022	**no**
F2_autumn 2022	**no**
F3_March 2023	**NA**
F4_June 2024	**no**

9. What preventive measures do you take to avoid diseases in fish?

FISH FARM	Answer
F1_autumn 2022	**appropriate stocking density and feeding, hygiene, if needed, bath/immersion treatment**
F2_autumn 2022	**hygiene**
F3_March 2023	**hygiene, salt, lime**
F4_June 2024	**hygiene**

B) QUESTIONNAIRE: FOLLOW-UP LONGITUDINAL SAMPLINGS

INFORMATION ABOUT THE SAMPLED TANKS AND FISH

1. What is the size of the tank (m^3)?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**25 m^3 (outside, concrete)**	**7.5 m^3 (outside, concrete)**	**1.5 m^3 (inside, plastic)**
F2_winter 2023	**256 m^3 (outside, concrete)**	**121 m^3 (outside, concrete)**	**121 m^3 (outside, concrete)**
F1_spring 2023	**140 m^3 (outside, concrete)**	**27 m^3 (outside, concrete)**	**30 m^3 (outside, concrete)**
F2_spring 2023	**256 m^3 (outside, concrete)**	**121 m^3 (outside, concrete)**	**498 m^3 (outside, concrete)**
F1_summer 2023	**25 m^3 (outside, concrete)**	**23 m^3 (outside, concrete)**	**1.5 m^3 (inside, plastic)**
F2_summer 2023	**256 m^3 (outside, concrete)**	**140 m^3 (outside, concrete)**	**256 m^3 (outside, concrete)**

2. How many fish are in the tank, and what is their average weight?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**10000 fish, 86 g**	**2000 fish, 81 g**	**8000 fish, 1.8 g**

F2_winter 2023	**7600 fish, 500 g**	**35000 fish, 83 g**	**35000 fish, 83 g**
F1_spring 2023	**10500 fish, 185 g**	**15000 fish, 89 g**	**2500 fish, 166 g**
F2_spring 2023	**39732 fish, 100 g**	**26000 fish, 71 g**	**84220 fish, 33 g**
F1_summer 2023	**5700 fish, 225 g**	**10000 fish, 65 g**	**1000 fish, 14.7 g**
F2_summer 2023	**6000 fish, 30 g**	**63000 fish, 120 g**	**70000 fish, 50 g**

3. What is the approximate age of the fish (in days/months/years)?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**9 months**	**9 months**	**4 months**
F2_winter 2023	**33 months**	**23 months**	**23 months**
F1_spring 2023	**9 months**	**7 months**	**10 months**
F2_spring 2023	**23 months**	**20 months**	**20 months**
F1_summer 2023	**13 months**	**9 months**	**7 months**
F2_summer 2023	**20 months**	**23 months**	**20 months**

4. What is the current water flow rate in the tank?

If possible, in liters/second:

or describe: high/satisfactory/low.

If the flow rate is low, do you currently apply measures such as water reuse or aeration/oxygenation?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**satisfactory**	**satisfactory**	**satisfactory**
F2_winter 2023	**satisfactory**	**satisfactory**	**satisfactory**
F1_spring 2023	**satisfactory**	**satisfactory**	**satisfactory**
F2_spring 2023	**satisfactory**	**satisfactory**	**satisfactory**
F1_summer 2023	**satisfactory**	**satisfactory**	**satisfactory**
F2_summer 2023	**satisfactory**	**satisfactory**	**satisfactory**

5. Does the water supplying the tank also flow through upstream tanks? YES/NO

If yes, how many tanks?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**yes, NA**	**yes, NA**	**no**
F2_winter 2023	**yes, two tanks**	**no**	**no**
F1_spring 2023	**yes, NA**	**yes, NA**	**yes, NA**
F2_spring 2023	**yes, two tanks**	**no**	**no**
F1_summer 2023	**yes, NA**	**yes, NA**	**no**
F2_summer 2023	**yes, two tanks**	**yes, three tanks**	**yes, two tanks**

6. What feed do you use? How much (kg feed/kg fish) and how often per day do you feed the fish? Do you use manual feeding or automatic feeding systems?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**Biomar, 1% of fish weight, twice daily, manual and automatic feeding**	**Biomar, 1% of fish weight, twice daily, manual and automatic feeding**	**Biomar, 1.5% of fish weight, twice daily, manual and automatic feeding**
F2_winter 2023	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**
F1_spring 2023	**Biomar, diameter 4.5 mm, 25 kg/day, five times daily, automatic feeding**	**Biomar, diameter 2 mm, 18 kg/day, NA, manual feeding**	**Biomar, diameter 4.5 mm, 12 kg/day, NA, manual feeding**
F2_spring 2023	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**
F1_summer 2023	**Biomar, 15 kg feed per 1425 kg biomass, twice daily, manual and automatic feeding**	**Biomar, 7 kg feed per 650 kg biomass, twice daily, manual and automatic feeding**	**Biomar, 1.5 kg feed per 15 kg biomass, NA, manual feeding**
F2_summer 2023	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**	**Biomar, 1% feed of biomass (1 kg feed per 100 kg fish), NA, manual feeding**

7. Do you currently use other food products/supplements (probiotics, vitamins, minerals, etc.)?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**no**	**no**	**no**
F2_winter 2023	**no**	**no**	**no**
F1_spring 2023	**no**	**no**	**no**
F2_spring 2023	**no**	**no**	**no**
F1_summer 2023	**no**	**no**	**no**
F2_summer 2023	**no**	**no**	**no**

8. Have the fish been treated with antibiotics in the last month? YES/NO

If yes, which antibiotic(s) and when?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**no**	**no**	**no**
F2_winter 2023	**no**	**no**	**no**
F1_spring 2023	**no**	**no**	**no**
F2_spring 2023	**no**	**no**	**no**
F1_summer 2023	**no**	**no**	**no**
F2_summer 2023	**no**	**no**	**no**

9. Have you observed any changes in the behavior or appearance of the fish in the last month? YES/NO

If yes, please describe (when/duration, what were the first signs, how did they progress, what percentage of fish was affected, mortalities, etc.):

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**no**	**no**	**no**
F2_winter 2023	**no**	**no**	**no**
F1_spring 2023	**no**	**no**	**no**
F2_spring 2023	**no**	**no**	**no**
F1_summer 2023	**no**	**no**	**no**
F2_summer 2023	**no**	**no**	**no**

10. Has a veterinarian confirmed any disease in the last month? YES/NO

If yes, which one?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**no**	**no**	**no**
F2_winter 2023	**no**	**no**	**no**
F1_spring 2023	**no**	**no**	**no**
F2_spring 2023	**no**	**no**	**no**
F1_summer 2023	**no**	**no**	**no**
F2_summer 2023	**no**	**no**	**no**

11. What was the daily mortality rate in the last two weeks?

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**normal**	**normal**	**normal**
F2_winter 2023	**very mildly increased, 2 kg daily (normal 1 kg daily)**	**normal**	**normal**
F1_spring 2023	**normal**	**normal**	**normal**
F2_spring 2023	**normal**	**normal**	**normal**
F1_summer 2023	**normal**	**normal**	**normal**
F2_summer 2023	**mildly increased, 5 kg daily (normal 1 kg daily)**	**mildly increased, 5 kg daily (normal 1 kg daily)**	**mildly increased, 4 kg daily (normal 1 kg daily)**

12. Have the fish been exposed to stress in the last two weeks (handling, transportation, sorting, etc.)? YES/NO

If yes, please explain:

FISH FARM	FISH TANK 1	FISH TANK 2	FISH TANK 3
F1_winter 2023	**no**	**no**	**no**

F2_winter 2023	**no**	**no**	**no**
F1_spring 2023	**no**	**no**	**no**
F2_spring 2023	**no**	**no**	**no**
F1_summer 2023	**no**	**no**	**no**
F2_summer 2023	**no**	**no**	**no**

Appendix 3: Information on the *Flavobacterium* isolates subjected to whole genome sequencing and/or antimicrobial susceptibility testing, including sampling time, fish farm, fish tank, health status of the fish, source of isolation, and other positive organs

ISOLATE NAME	*FLAVOBACTERIUM* SPECIES	SAMPLING TIME	FISH FARM, TANK, HEALTH#	ISOLATION SOURCE	OTHER POSITIVE ORGANS
F21	*F. psychrophilum*	Autumn 2022	F1T1D	kidney	gills, skin
F23	*F. psychrophilum*	Autumn 2022	F1T1D	skin	-
F27	*F. psychrophilum*	Autumn 2022	F1T2D	gill	skin, liver
F29	*F. psychrophilum*	Autumn 2022	F1T2D	kidney	-
F35	*F. psychrophilum*	Autumn 2022	F1T3D	gill	-
F36	*F. psychrophilum*	Autumn 2022	F1T3D	kidney	liver
F38	*F. psychrophilum*	Autumn 2022	F1T2H	skin	-
F82	*F. psychrophilum*	Autumn 2022	F2T2D	kidney	gills, skin, spleen, liver
F88	*F. psychrophilum*	Autumn 2022	F2T2D	skin	-
F202	*F. psychrophilum*	Winter 2023	F1T1D	spleen	-
F206	*F. psychrophilum*	Winter 2023	F1T1D	spleen	gill, kidney, liver
F211	*F. psychrophilum*	Winter 2023	F1T2D	kidney	gill, spleen, liver
F328	*F. psychrophilum*	Winter 2023	F1T3D	liver	-
F332	*F. psychrophilum*	Winter 2023	F1T3D	kidney	gill, spleen
F296	*F. psychrophilum*	Winter 2023	F1T1H	gill	-
F255	*F. psychrophilum*	Winter 2023	F1T2H	spleen	gill, kidney, liver
F341	*F. psychrophilum*	Winter 2023	F1T3H	spleen	-
F415	*F. psychrophilum*	Winter 2023	F2T1D	skin	-
F418	*F. psychrophilum*	Winter 2023	F2T2D	skin	-
F421	*F. psychrophilum*	Winter 2023	F2T2D	skin	-
F444	*F. psychrophilum*	Winter 2023	F2T3D	skin	spleen, liver
F427	*F. psychrophilum*	Winter 2023	F2T3D	skin	-
F1125	*F. psychrophilum*	Spring 2023	F1T1D	skin	-
F1126	*F. psychrophilum*	Spring 2023	F1T1D	skin	-
F937	*F. psychrophilum*	Spring 2023	F1T2D	skin	kidney
F1127	*F. psychrophilum*	Spring 2023	F1T3D	skin	-
F1070	*F. psychrophilum*	Spring 2023	F1T3D	liver	-
F1146	*F. psychrophilum*	Spring 2023	F1T2H	gill	-
F1147	*F. psychrophilum*	Spring 2023	F1T3H	skin	-
F1184	*F. psychrophilum*	Spring 2023	F2T1D	spleen	gills, skin, kidney, liver
F1186	*F. psychrophilum*	Spring 2023	F2T1D	spleen	kidney
F1187	*F. psychrophilum*	Spring 2023	F2T2D	spleen	skin, kidney, liver
F1188	*F. psychrophilum*	Spring 2023	F2T2D	spleen	skin, kidney, liver
F1266	*F. psychrophilum*	Spring 2023	F2T3D	spleen	skin, kidney, liver

F1267	*F. psychrophilum*	Spring 2023	F2T3D	kidney	spleen, liver
F1268	*F. psychrophilum*	Spring 2023	F2T3H	liver	-
F1560	*F. psychrophilum*	Summer 2023	F1T1D	skin	-
F1516	*F. psychrophilum*	Summer 2023	F1T1D	skin	-
F1518	*F. psychrophilum*	Summer 2023	F1T2D	kidney	liver
F1562	*F. psychrophilum*	Summer 2023	F1T3D	skin	-
F1519	*F. psychrophilum*	Summer 2023	F1T3D	liver	gills, skin, spleen, kidney
F1595	*F. psychrophilum*	Summer 2023	F1T1H	gill	-
F1719	*F. psychrophilum*	Summer 2023	F2T1D	liver	skin
F1723	*F. psychrophilum*	Summer 2023	F2T1D	skin	gill, kidney
F1828	*F. psychrophilum*	Summer 2023	F2T2D	spleen	skin, kidney, liver
F1819	*F. psychrophilum*	Summer 2023	F2T2D	gill	skin, spleen, kidney, liver
F1824	*F. psychrophilum*	Summer 2023	F2T1H	gill	-
F1823	*F. psychrophilum*	Summer 2023	F2T2H	gill	-
F551	*F. psychrophilum*	March 2023	F3D	liver	gills, skin, spleen, kidney
F621	*F. psychrophilum*	March 2023	F3D	skin	gill
F896	*F. psychrophilum*	March 2023	F3H	gill	-
F2125	*F. psychrophilum*	June 2024	F4D	spleen	kidney, liver
F2130	*F. psychrophilum*	June 2024	F4D	liver	skin, spleen, kidney
F2123	*F. psychrophilum*	June 2024	F4D	kidney	spleen, liver
F1517	*F. branchiophilum*	Summer 2023	F1T2D	gill	-
F1628	*F. branchiophilum*	Summer 2023	F1T3D	gill	-
F1629	*F. branchiophilum*	Summer 2023	F1T3D	gill	-
F1727	*F. branchiophilum*	Summer 2023	F2T1D	gill	-
F1728	*F. branchiophilum*	Summer 2023	F2T2D	gill	-
F1862	*F. branchiophilum*	Summer 2023	F2T3D	gill	-
F1822	*F. branchiophilum*	Summer 2023	F2T2H	gill	-
F1730	*F. branchiophilum*	Summer 2023	F2T3H	gill	-
F1040	*F. bernardetii*	Spring 2023	F1T1D	skin	-
F609	*F. bernardetii*	Spring 2023	F1T1D	skin	-
F324	*F. branchiarum*	Winter 2023	F1T2D	skin	-
F963	*F. branchiarum*	Spring 2023	F1T2D	gill	-
F1856	*F. celericrescens*	Summer 2023	F2T2H	gill	-
F1959	*F. celericrescens*	Summer 2023	F2T1H	gill	-
F1673	*F. collinsii*	Summer 2023	F2T1D	gill	-
F1548	*F. collinsii*	Summer 2023	F1T1D	skin	-
F2097	*F. cupriresistens*	June 2024	F4D	gill	-
F1633	*F. difficile*	Summer 2023	F1T2D	gill	-
F562	*F. erciyesense*	March 2023	F3D	skin	-
F2050	*F. hercynium*	June 2024	F4D	gill	-
F1942	*F. oncorhynchi*	Summer 2023	F2T1D	liver	gill

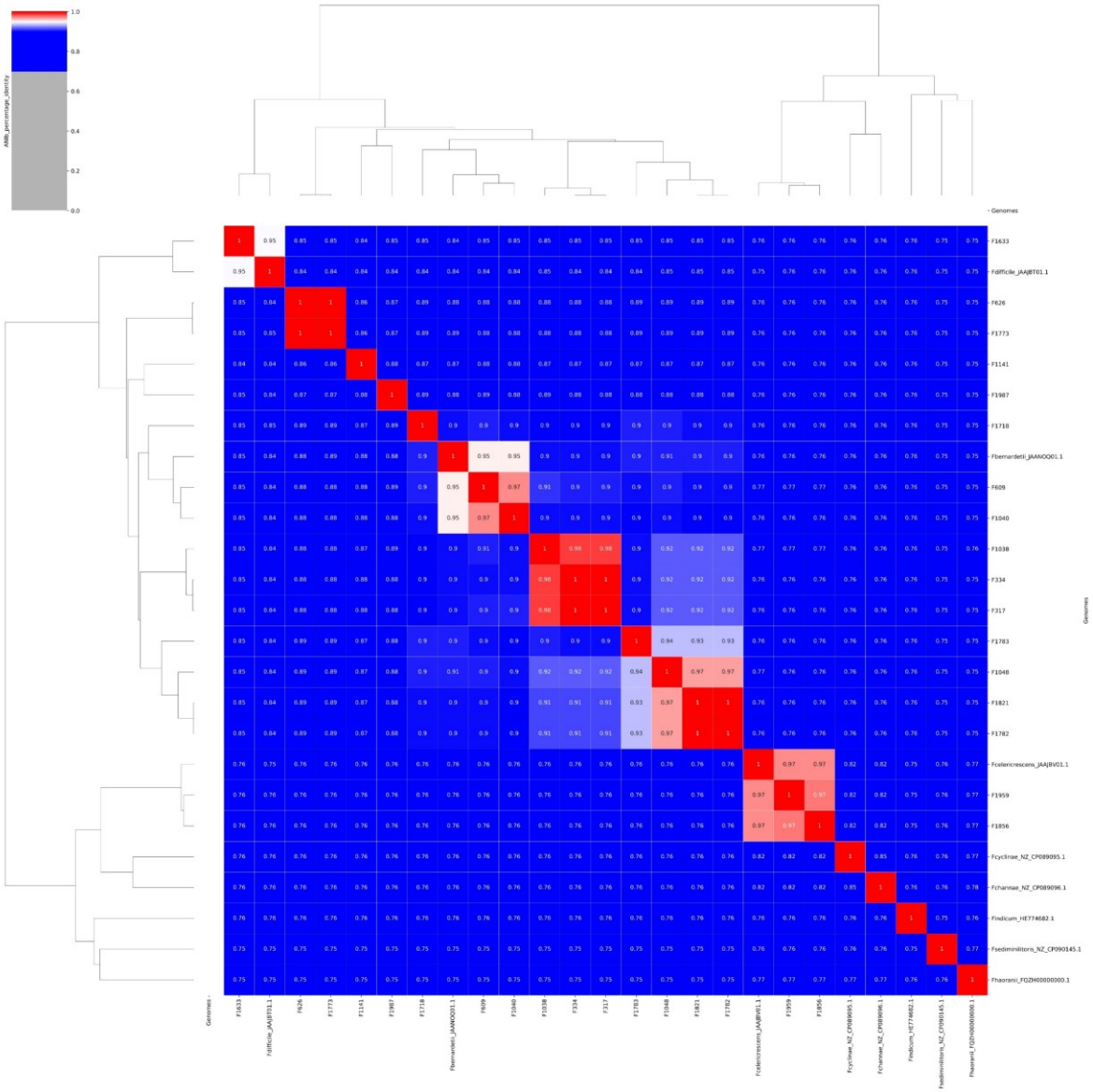
F1228	*F. piscis*	Spring 2023	F2T1D	gill	-
F1485	*F. tiangeerense*	Summer 2023	F1T1D	gill	-
F1900	*F. tructae*	Summer 2023	F2T3D	gill	-
F1141	FX1	Spring 2023	F1T2D	gill	-
F334	FX2	Winter 2023	F1T2H	skin	-
F317	FX2	Winter 2023	F1T3H	skin	-
F1038	FX2	Spring 2023	F1T1D	skin	-
F1048	FX3	Spring 2023	F1T3D	gill	-
F1821	FX3	Summer 2023	F2T2D	liver	gills, skin,
F1782	FX3	Summer 2023	F2T2D	skin	gill, liver
F1100	FX4	Spring 2023	F1T2H	gill	-
F1933	FX5	Summer 2023	F2T3H	gill	-
F1140	FX5	Spring 2023	F1T3D	gill	liver
F1142	FX5	Spring 2023	F1T1D	gill	-
F1466	FX6	Spring 2023	F2T3D	skin	-
F1987	FX7	Summer 2023	F2T1D	gill	-
F1718	FX8	Summer 2023	F2T1D	spleen	skin
F1616	FX9	Summer 2023	F1T3D	gill	-
F945	FX9	Spring 2023	F1T1D	gill	-
F944	FX10	Spring 2023	F1T1D	skin	-
F1511	FX10	Summer 2023	F1T2D	liver	gill
F1498	FX11	Summer 2023	F1T1D	gill	-
F62	FX12	Autumn 2022	F2T1H	liver	gill
F65	FX13	Autumn 2022	F2T2H	spleen	-
F77	FX14	Autumn 2022	F2T1D	liver	-
F2167	FX15	June 2024	F4D	gill	-
F2043	FX15	June 2024	F4D	gill	-
F1859	FX15	Summer 2023	F2T1D	spleen	-
F1692	FX15	Summer 2023	F2T1D	gill	-
F106	FX15	Autumn 2022	F2T1H	spleen	-
F1677	FX15	Summer 2023	F2T1D	liver	-
F283	FX16	Winter 2023	F1T3D	gill	-
F616	FX17	March 2023	F3H	skin	-
F1773	FX18	Summer 2023	F2T1D	skin	-
F626	FX18	March 2023	F3H	spleen	gill
F656	FX19	March 2023	F3D	gill	-
F666	FX20	March 2023	F3D	skin	-
F799	FX21	March 2023	F3D	skin	-
F1112	FX22	Spring 2023	F1T2D	skin	-
F1259	FX23	Spring 2023	F2T2D	skin	-
F1213	FX23	Spring 2023	F2T2D	skin	-
F1252	FX24	Spring 2023	F2T1D	liver	-
F1418	FX25	Spring 2023	F2T2D	skin	gill
F1486	FX26	Summer 2023	F1T2D	skin	-
F1593	FX27	Summer 2023	F1T1D	liver	-

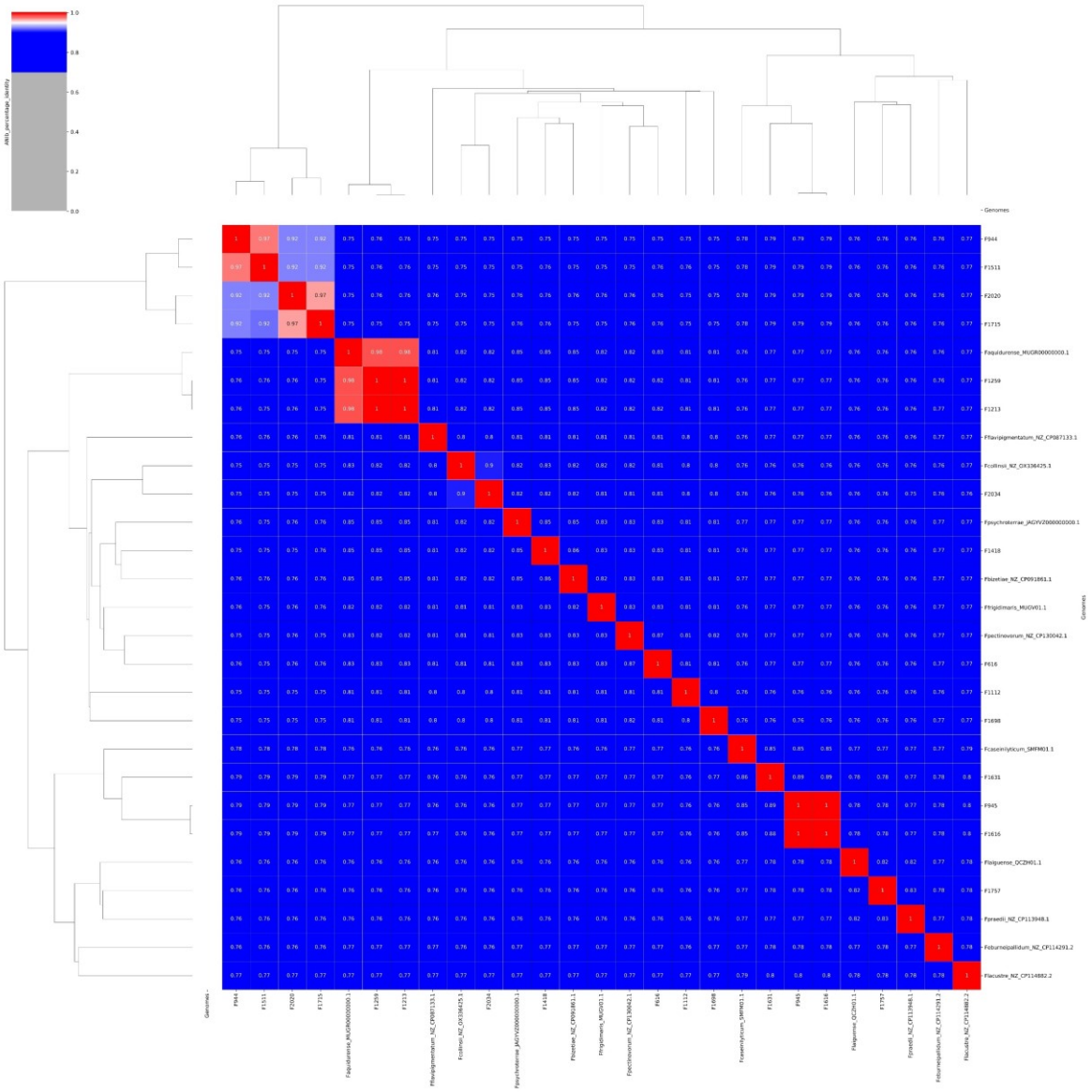
F1527	FX27	Summer 2023	F1T1D	liver	-
F1631	FX28	Summer 2023	F1T3D	skin	-
F1678	FX29	Summer 2023	F2T1D	liver	-
F1683	FX30	Summer 2023	F2T1D	skin	gill
F1698	FX31	Summer 2023	F2T2D	skin	-
F1715	FX32	Summer 2023	F2T1D	kidney	-
F2020	FX32	Summer 2023	F2T2D	skin	-
F1797	FX33	Summer 2023	F2T1D	gill	-
F1849	FX34	Summer 2023	F2T2D	skin	-
F1902	FX35	Summer 2023	F2T1H	gill	-
F1996	FX36	Summer 2023	F2T1H	gill	-
F2035	FX36	Summer 2023	F2T1H	gill	-
F2009	FX37	Summer 2023	F2T1D	gill	-
F2034	FX38	Summer 2023	F2T1D	kidney	-
F2063	FX39	June 2024	F4D	skin	-
F2074	FX40	June 2024	F4D	gill	-
F1757	FX41	Summer 2023	F2T2D	gill	-

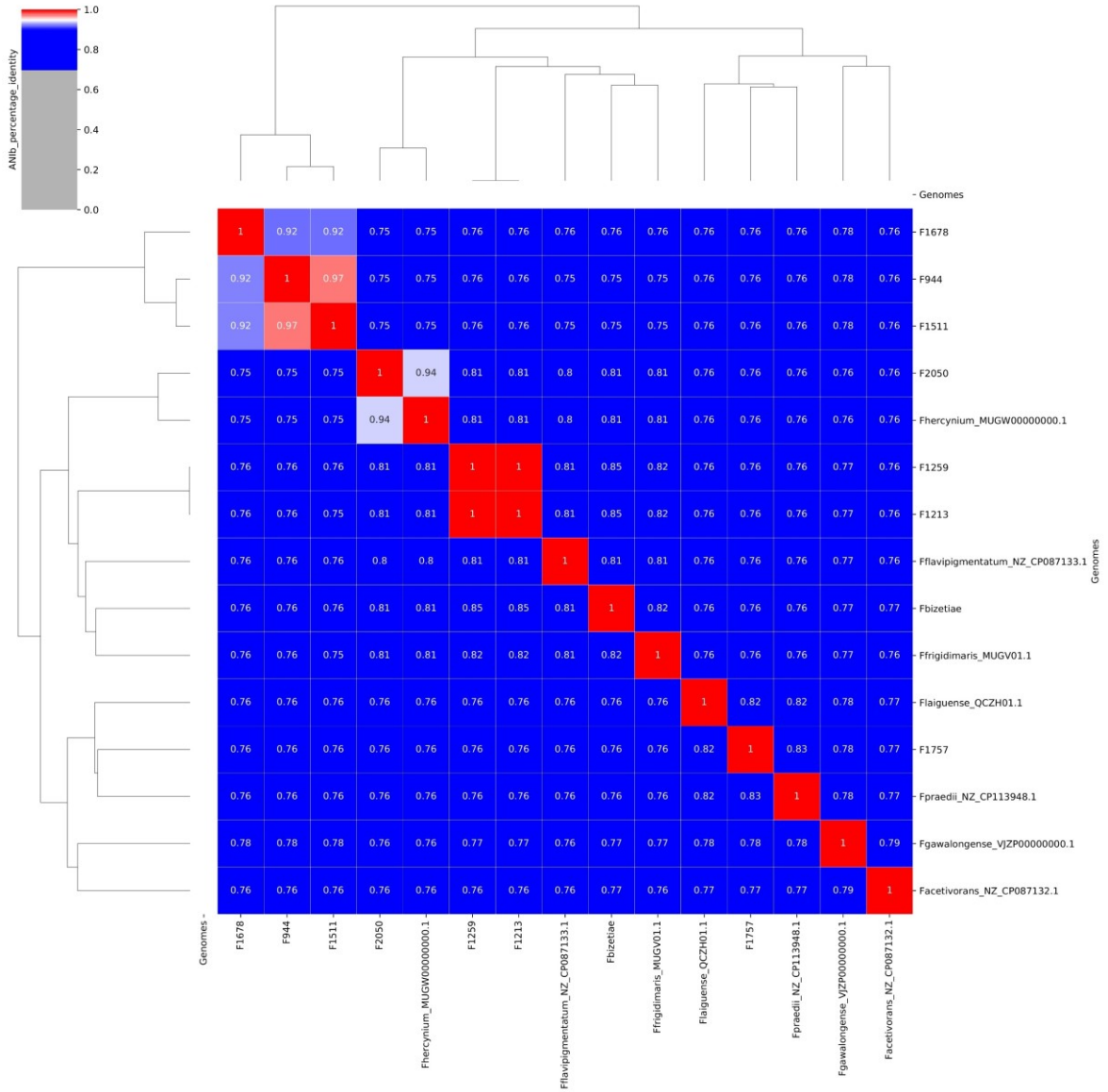
#F, fish farm; T, fish tank; D, diseased fish; H, apparently healthy fish.

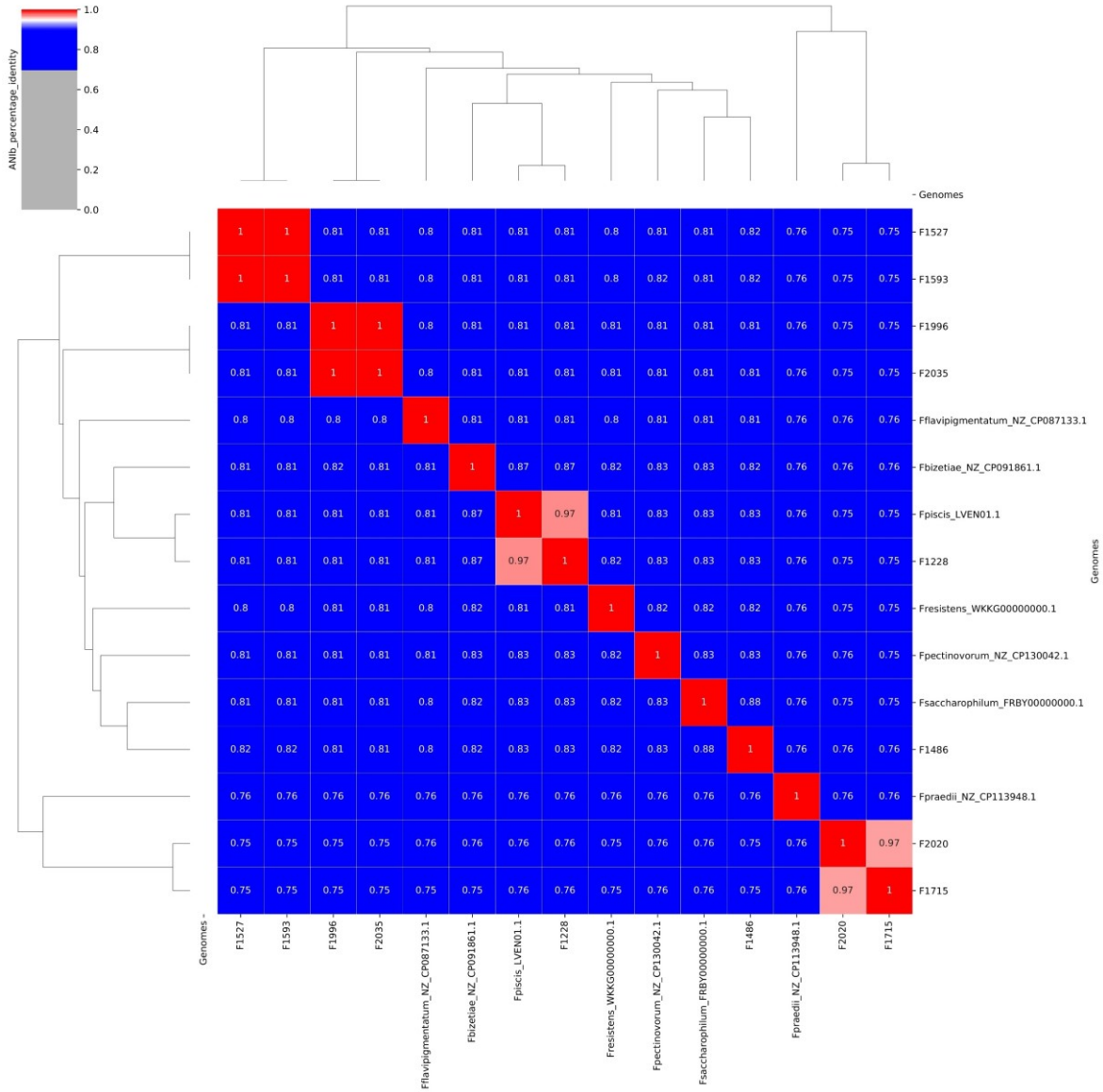
Isolates that were further characterised using whole-genome sequencing (WGS) are marked in bold, isolates that underwent antimicrobial susceptibility testing (AST) are marked in italic, and isolates that underwent both WGS and AST are marked in bold and italic.

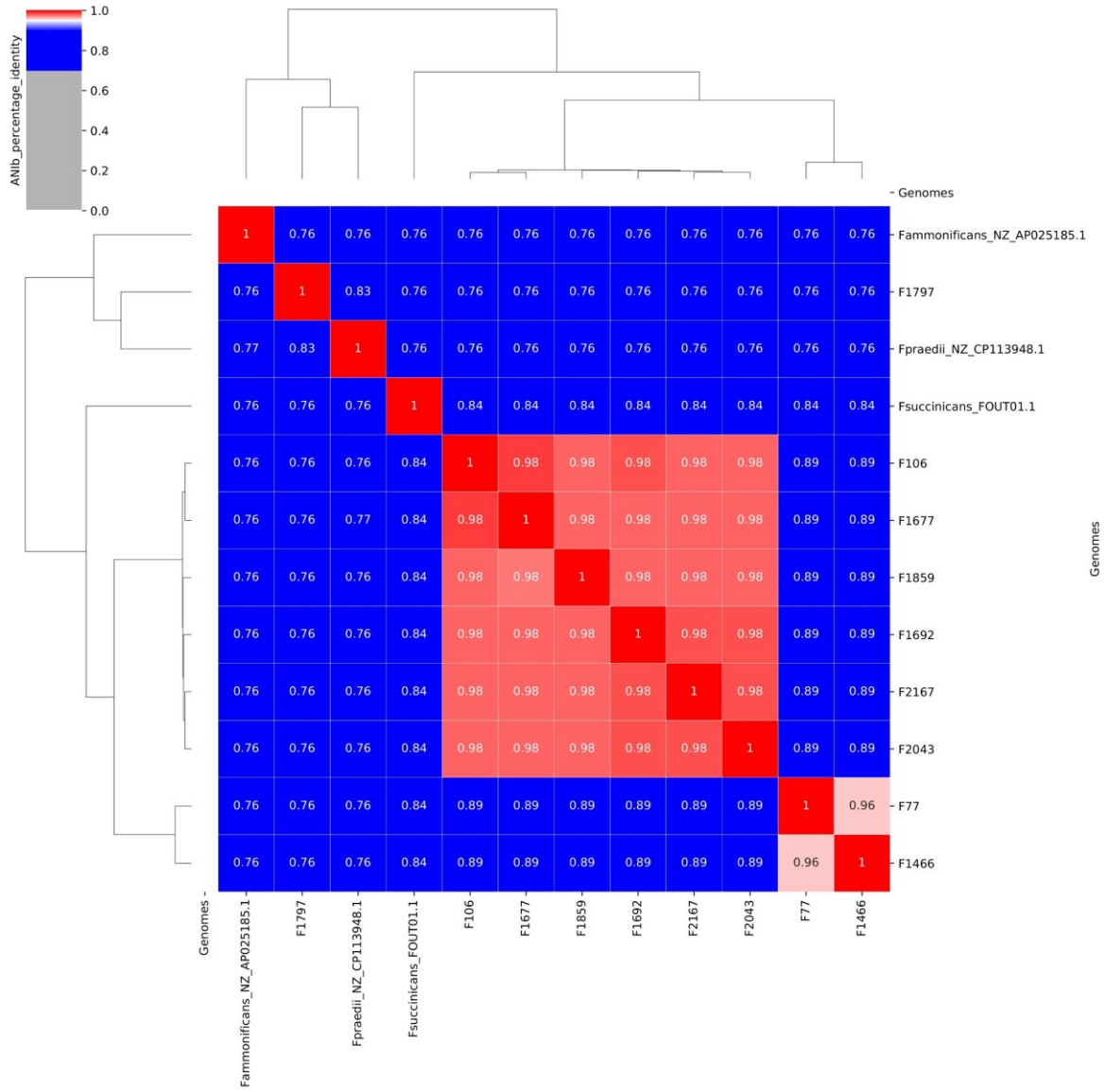
Appendix 4: Pairwise comparison of blast-based average nucleotide identity (ANIb) values between *Flavobacterium* species isolates and their closest phylogenetic relatives

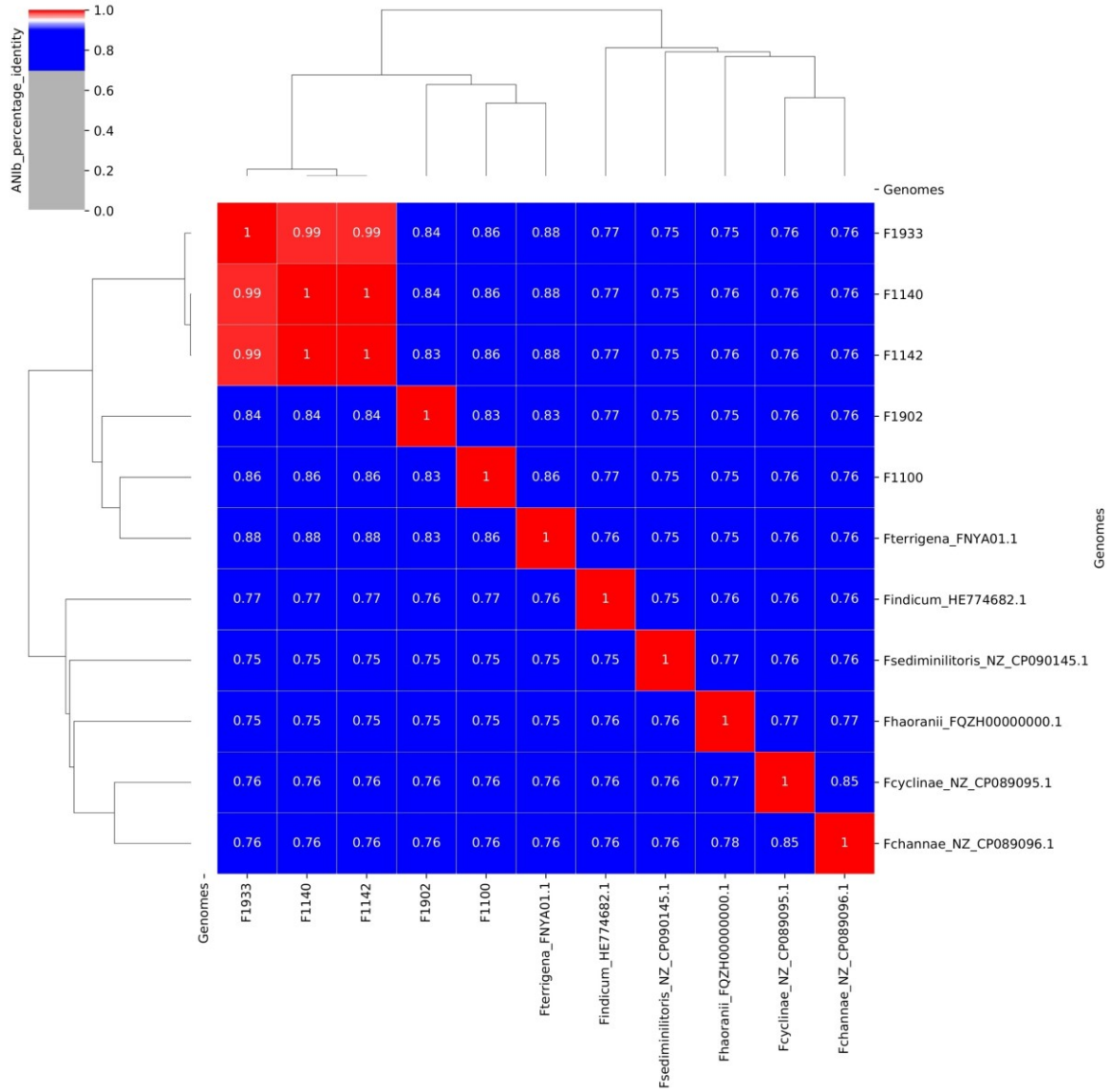


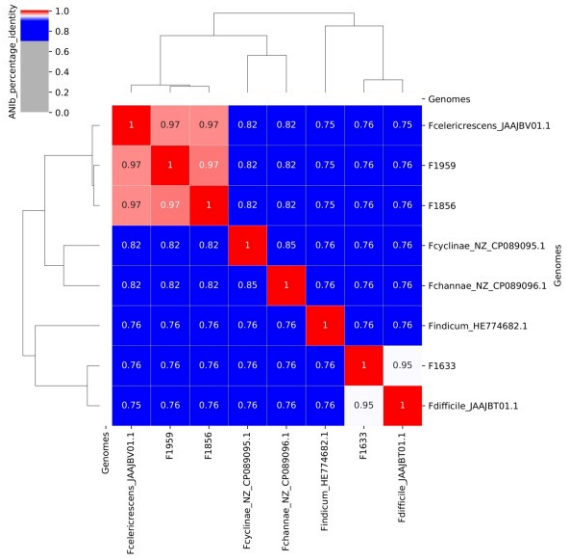


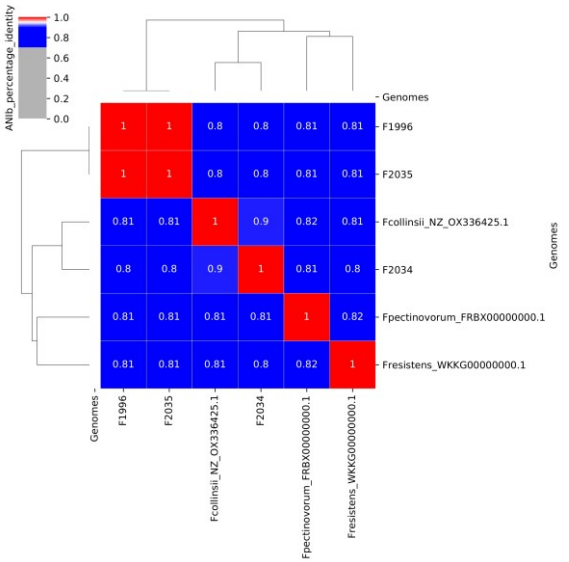

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F1678
F944
F1511
F2050
Fhercynium_MUGW00000000.1
F1259
F1213
Fflavipigmentatum_NZ_CP087133.1
Fbizetiae
Ffrigidimaris_MUGV01.1
Flaiguense_QCZH01.1
F1757
Fpraedii_NZ_CP113948.1
Fgawalongense_VJZP00000000.1
Facetivorans_NZ_CP087132.1

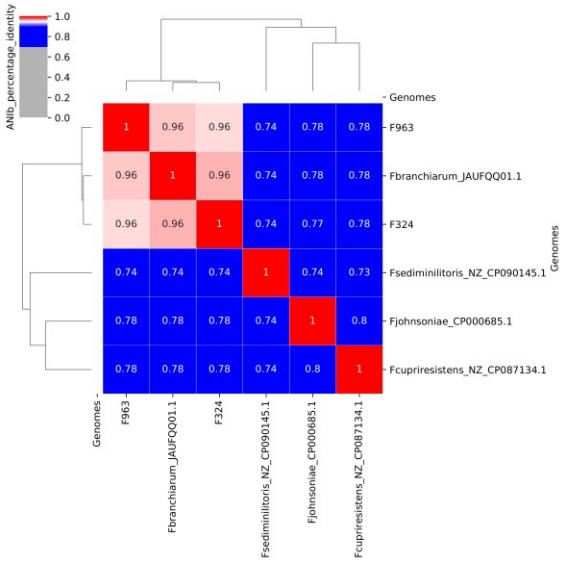

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F1527
F1593
F1996
F2035
Fflavipigmentatum_NZ_CP087133.1
Fbizetiae_NZ_CP091861.1
Fpiscis_LVEN01.1
F1228
Fresistens_WKKG00000000.1
Fpectinovorum_NZ_CP130042.1
Fsaccharophilum_FRBY00000000.1
F1486
Fpraedii_NZ_CP113948.1
F2020
F1715

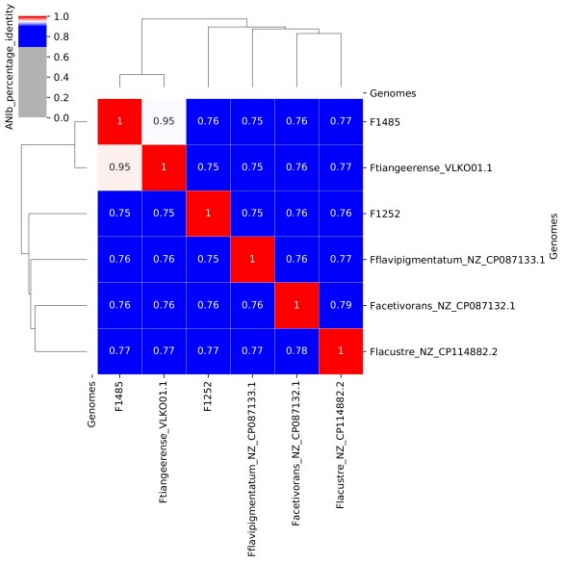

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fammonificans_NZ_AP025185.1
F1797
Fpraedii_NZ_CP113948.1
Fsuccinicans_FOUT01.1
F106
F1677
F1859
F1692
F2167
F2043
F77
F1466
Genomes

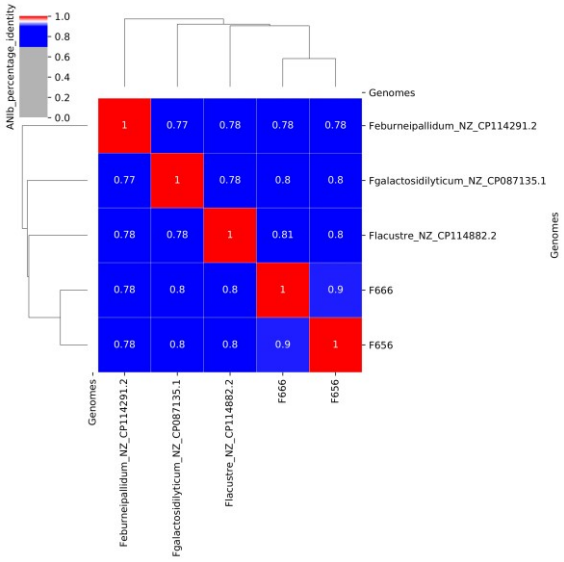

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F1933
F1140
F1142
F1902
F1100
Fterrigena_FNYA01.1
Findicum_HE774682.1
Fsediminilitoris_NZ_CP090145.1
Fhaoranii_FQZH00000000.1
Fcyclinae_NZ_CP089095.1
Fchannae_NZ_CP089096.1

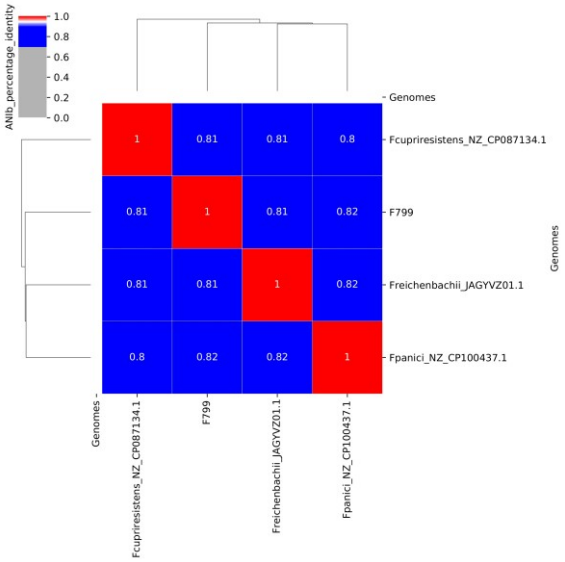

ANIb_percentage_identity
Genomes
Fcelericrescens_JAAJBV01.1
F1959
F1856
Fcyclinae_NZ_CP089095.1
Fchannae_NZ_CP089096.1
Findicum_HE774682.1
F1633
Fdifficile_JAAJBT01.1

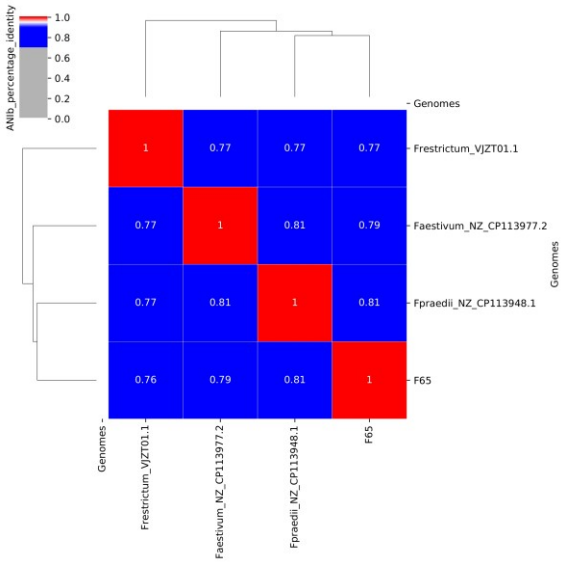

ANIb_percentage_identity
Genomes
F1996
F2035
Fcollinsii_NZ_OX336425.1
F2034
Fpectinovorum_FRBX00000000.1
Fresistens_WKKG00000000.1

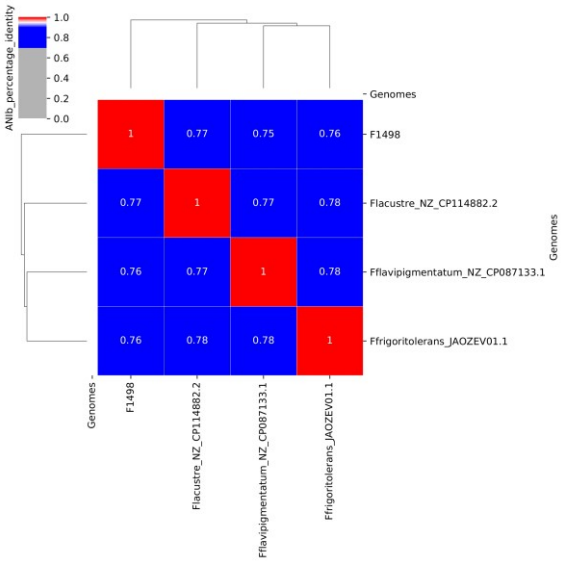

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F963
Fbranchiarum_JAUFQQ01.1
F324
Fsediminilitoris_NZ_CP090145.1
Fjohnsoniae_CP000685.1
Fcupriresistens_NZ_CP087134.1
1 0.96 0.96 0.74 0.78 0.78
0.96 1 0.96 0.74 0.78 0.78
0.96 0.96 1 0.74 0.77 0.78
0.74 0.74 0.74 1 0.74 0.73
0.78 0.78 0.78 0.74 1 0.8
0.78 0.78 0.78 0.74 0.8 1

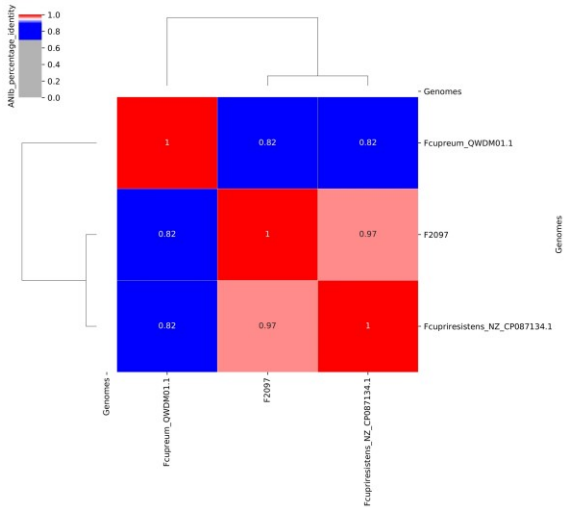

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F1485
Ftiangeerense_VLKO01.1
F1252
Fflavipigmentatum_NZ_CP087133.1
Facetivorans_NZ_CP087132.1
Flacustre_NZ_CP114882.2
1 0.95 0.76 0.75 0.76 0.77
0.95 1 0.75 0.75 0.76 0.77
0.75 0.75 1 0.75 0.76 0.76
0.76 0.76 0.75 1 0.76 0.77
0.76 0.76 0.76 0.76 1 0.79
0.77 0.77 0.77 0.77 0.78 1

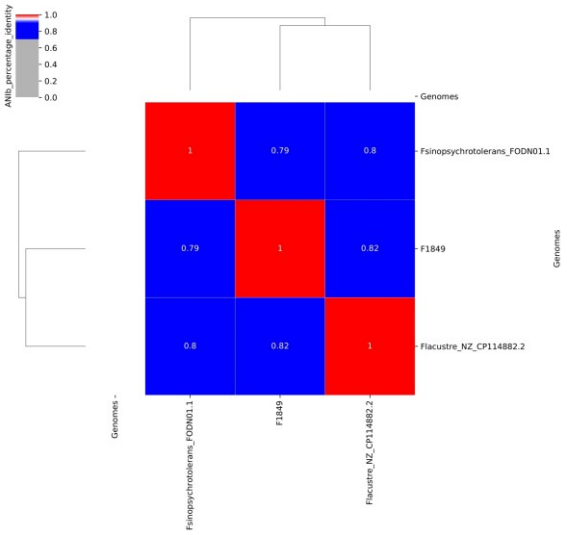

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Feburneipallidum_NZ_CP114291.2
Fgalactosidilyticum_NZ_CP087135.1
Flacustre_NZ_CP114882.2
F666
F656
1 0.77 0.78 0.78 0.78
0.77 1 0.78 0.8 0.8
0.78 0.78 1 0.81 0.8
0.78 0.8 0.8 1 0.9
0.78 0.8 0.8 0.9 1

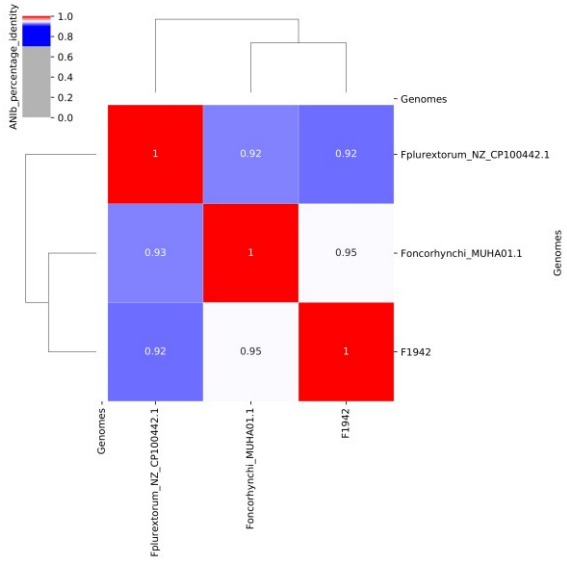

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fcupriresistens_NZ_CP087134.1
F799
Freichenbachii_JAGYVZ01.1
Fpanici_NZ_CP100437.1
1 0.81 0.81 0.8
0.81 1 0.81 0.82
0.81 0.81 1 0.82
0.8 0.82 0.82 1

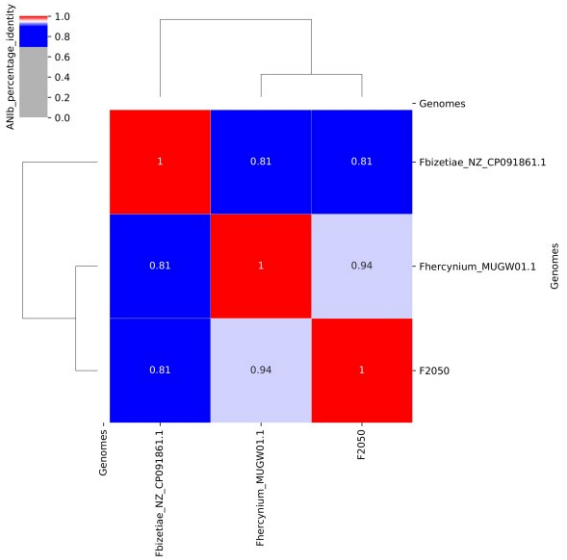

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Frestrictum_VJZT01.1
Faestivum_NZ_CP113977.2
Fpraedii_NZ_CP113948.1
F65
1 0.77 0.77 0.77
0.77 1 0.81 0.79
0.77 0.81 1 0.81
0.76 0.79 0.81 1

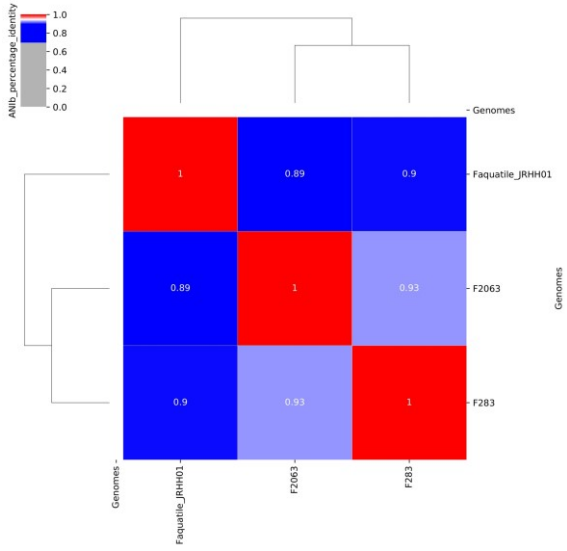

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F1498
Flacustre_NZ_CP114882.2
Fflavipigmentatum_NZ_CP087133.1
Ffrigoritolerans_JAOZEV01.1
1 0.77 0.75 0.76
0.77 1 0.77 0.78
0.76 0.77 1 0.78
0.76 0.78 0.78 1

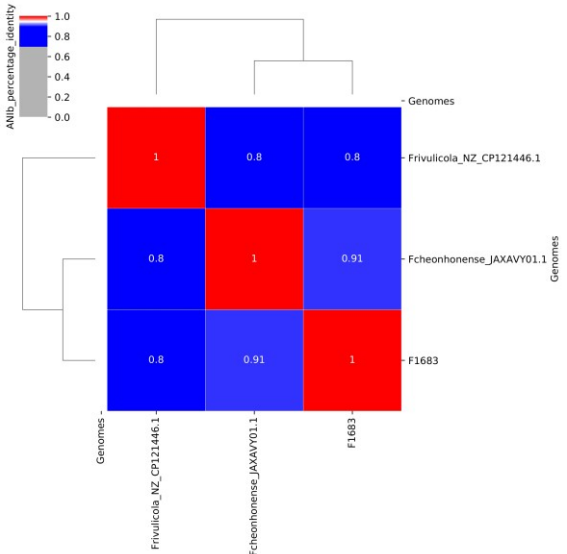

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fcupreum_QWDM01.1
F2097
Fcupriresistens_NZ_CP087134.1
1
0.82
0.82
0.82
1
0.97
0.82
0.97
1

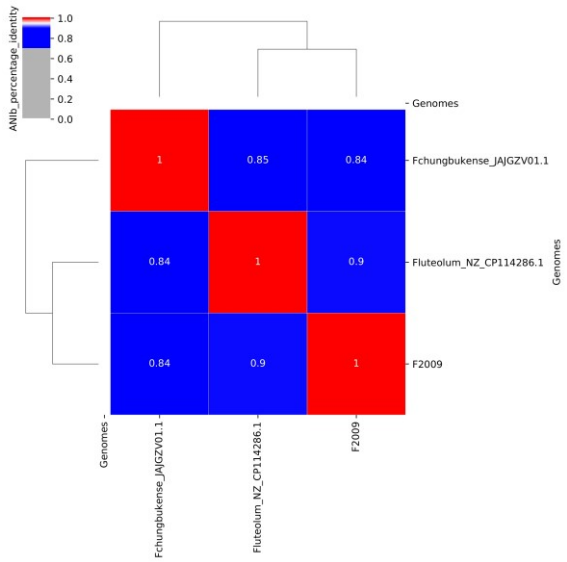

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fsinopsychrotolerans_FODN01.1
F1849
Flacustre_NZ_CP114882.2
1
0.79
0.8
0.79
1
0.82
0.8
0.82
1

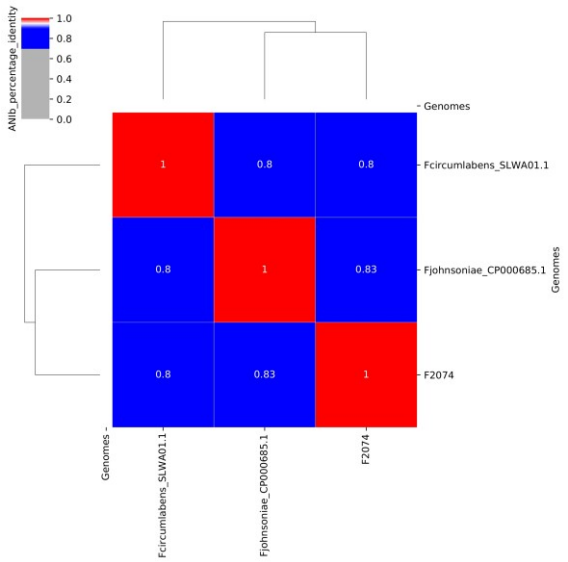

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fplurextorum_NZ_CP100442.1
Foncorhynchi_MUHA01.1
F1942
1
0.92
0.92
0.93
1
0.95
0.92
0.95
1

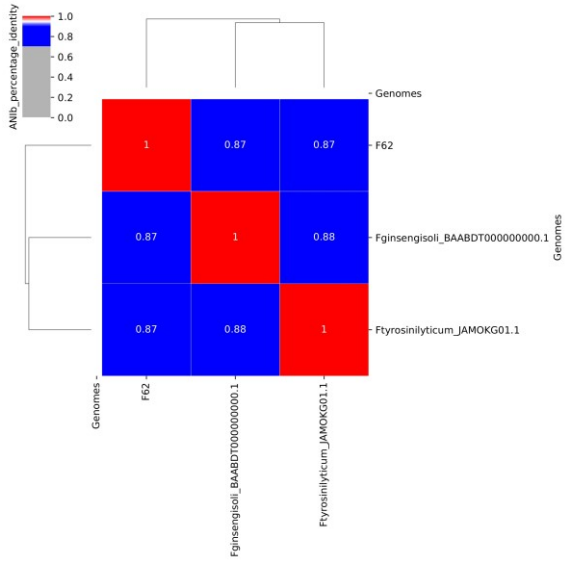

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fbizetiae_NZ_CP091861.1
Fhercynium_MUGW01.1
F2050
1
0.81
0.81
0.81
1
0.94
0.81
0.94
1

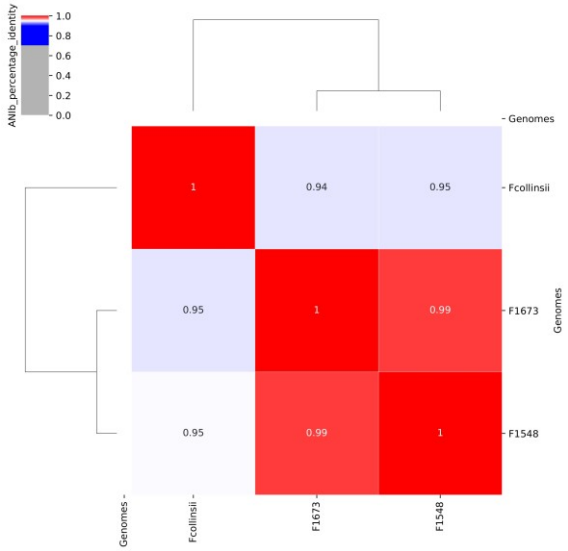

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Faquatile_JRHH01
F2063
F283
1
0.89
0.9
0.89
1
0.93
0.9
0.93
1

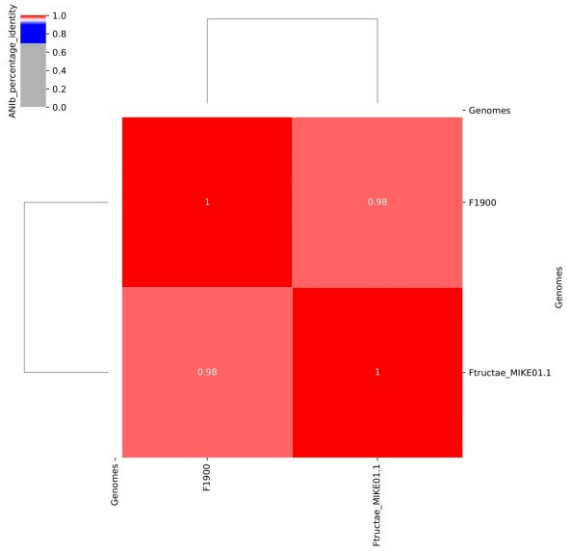

ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Frivulicola_NZ_CP121446.1
Fcheonhonense_JAXAVY01.1
F1683
1
0.8
0.8
0.8
1
0.91
0.8
0.91
1


ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fchungbukense_JAJGZV01.1
Fluteolum_NZ_CP114286.1
F2009
1
0.85
0.84
0.84
1
0.9
0.84
0.9
1


ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fcircumlabens_SLWA01.1
Fjohnsoniae_CP000685.1
F2074
1
0.8
0.8
0.8
1
0.83
0.8
0.83
1


ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F62
Fginsengisoli_BAABDT000000000.1
Ftyrosinilyticum_JAMOKG01.1
1
0.87
0.87
0.87
1
0.88
0.87
0.88
1


ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
Fcollinsii
F1673
F1548
1
0.94
0.95
0.95
1
0.99
0.95
0.99
1


ANIb_percentage_identity
1.0
0.8
0.6
0.4
0.2
0.0
Genomes
F1900
Ftructae_MIKE01.1
1
0.98
0.98
1

Appendix 5: Raw data on phenotypic antimicrobial susceptibility testing (minimum inhibitory concentrations in µg/ml) for the *Flavobacterium psychrophilum* ATCC 49418^T reference strain (Ref), the 54 Slovene *F. psychrophilum* isolates and other *Flavobacterium* spp., determined using the standardised method recommended for *F. psychrophilum* (CLSI, 2020a) for AMP, ampicillin; CTZ, ceftazidime; ENR, enrofloxacin; ERY, erythromycin; FLO, florfenicol; GEN, gentamicin; MER, meropenem; OXO, oxolinic acid; OXY, oxytetracycline; TRS, trimethoprim/sulfamethoxazole

Flavo.	AMP	CTZ	ENR	ERY	FLO	GEN	MER	OXO	OXY	TRS
F. psychrophilum Ref	0.12	2	0.015	1	0.5	0.25	≤ 0.008	0.12	0.06	> 1/19
F. psychrophilum F21	0.06	4	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	8	0.5/9.5
F. psychrophilum F23	0.06	4	0.12	1	0.5	0.12	≤ 0.008	> 1	> 8	0.25/4.75
F. psychrophilum F27	0.06	4	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	4	1/19
F. psychrophilum F29	0.06	4	0.25	1	0.5	0.12	≤ 0.008	1	> 8	1/19
F. psychrophilum F35	0.06	2	> 0.25	1	0.25	≤ 0.06	≤ 0.008	> 1	4	0.25/4.75
F. psychrophilum F36	0.06	1	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	4	> 1/19
F. psychrophilum F38	0.06	4	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	4	0.5/9.5
F. psychrophilum F82	0.06	2	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	8	> 1/19
F. psychrophilum F88*	0.06	4	0.015	1	0.25	≤ 0.06	≤ 0.008	0.12	0.06	> 1/19
F. psychrophilum F202	0.06	2	0.12	1	0.25	≤ 0.06	≤ 0.008	1	1	1/19
F. psychrophilum F206	0.06	2	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	8	> 1/19
F. psychrophilum F211	0.06	2	0.12	1	0.5	0.12	≤ 0.008	> 1	1	> 1/19
F. psychrophilum F328	0.06	4	> 0.25	1	0.5	≤ 0.06	≤ 0.008	1	4	0.5/9.5
F. psychrophilum F332	0.06	4	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	4	0.5/9.5
F. psychrophilum F296	0.06	4	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	4	0.25/4.75
F. psychrophilum F255	0.06	2	0.12	1	0.25	≤ 0.06	≤ 0.008	> 1	1	> 1/19
F. psychrophilum F341	0.06	2	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	8	> 1/19
F. psychrophilum F415	0.06	2	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	8	> 1/19
F. psychrophilum F418	0.03	4	0.06	1	0.25	≤ 0.06	≤ 0.008	1	4	> 1/19
F. psychrophilum F421	0.06	2	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	4	1/19
F. psychrophilum F444	0.03	2	> 0.25	1	0.25	≤ 0.06	≤ 0.008	> 1	0.03	0.12/2.38
F. psychrophilum F427	0.5	4	0.06	1	0.5	0.12	≤ 0.008	1	4	0.25/4.75
F. psychrophilum F1125	0.06	2	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	2	> 1/19
F. psychrophilum F1126	0.06	4	> 0.25	2	0.5	0.12	≤ 0.008	> 1	1	1/19
F. psychrophilum F937	0.06	1	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	0.03	0.5/9.5
F. psychrophilum F1127	0.03	4	0.06	1	0.5	≤ 0.06	≤ 0.008	1	2	0.25/4.75
F. psychrophilum F1070	0.06	1	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	> 8	> 1/19
F. psychrophilum F1146	0.06	2	0.06	0.5	0.5	≤ 0.06	≤ 0.008	1	2	0.5/9.5

F. psychrophilum F1147	0.06	> 4	> 0.25	2	0.5	0.12	≤ 0.008	> 1	1	1/19
F. psychrophilum F1184	0.06	2	> 0.25	2	0.5	0.12	≤ 0.008	> 1	1	> 1/19
F. psychrophilum F1186*	4	1	> 0.25	1	0.5	≤ 0.06	≤ 0.008	**> 1**	1	0.5/9.5
F. psychrophilum F1187*	0.06	2	0.12	1	0.5	≤ 0.06	≤ 0.008	**> 1**	0.06	> 1/19
F. psychrophilum F1188	0.06	2	0.12	2	0.5	0.12	≤ 0.008	> 1	0.03	> 1/19
F. psychrophilum F1266	0.06	2	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	8	> 1/19
F. psychrophilum F1267	0.06	1	> 0.25	1	0.25	≤ 0.06	≤ 0.008	> 1	8	> 1/19
F. psychrophilum F1268	0.03	2	0.004	1	0.5	≤ 0.06	≤ 0.008	0.06	0.25	0.03/0.6
F. psychrophilum F1560	0.06	2	0.008	0.5	0.25	0.12	≤ 0.008	0.06	0.5	0.03/0.6
F. psychrophilum F1516*	0.03	2	0.03	0.5	0.25	≤ 0.06	≤ 0.008	**0.5**	1	0.015/0.3
F. psychrophilum F1518	0.06	4	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	4	0.25/4.75
F. psychrophilum F1562	0.06	4	> 0.25	2	0.5	0.12	≤ 0.008	> 1	1	1/19
F. psychrophilum F1519*	0.06	1	> 0.25	1	0.5	≤ 0.06	≤ 0.008	**> 1**	8	> 1/19
F. psychrophilum F1595	0.12	> 4	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	4	0.5/9.5
F. psychrophilum F1719*	0.03	1	0.008	1	0.5	≤ 0.06	≤ 0.008	0.06	0.5	0.015/0.3
F. psychrophilum F1723	0.12	> 4	0.015	2	0.5	≤ 0.06	≤ 0.008	0.12	4	> 1/19
F. psychrophilum F1828	0.06	1	> 0.25	2	0.5	0.12	≤ 0.008	> 1	8	> 1/19
F. psychrophilum F1819	0.06	2	> 0.25	2	0.25	≤ 0.06	≤ 0.008	> 1	1	> 1/19
F. psychrophilum F1824	0.06	2	> 0.25	1	0.5	≤ 0.06	≤ 0.008	> 1	8	1/19
F. psychrophilum F1823	0.06	2	> 0.25	2	0.5	0.12	≤ 0.008	> 1	0.06	> 1/19
F. psychrophilum F551	0.06	2	0.12	2	0.25	0.12	≤ 0.008	> 1	1	> 1/19
F. psychrophilum F621*	≤ 0.015	1	0.015	1	0.25	≤ 0.06	≤ 0.008	**0.5**	0.25	0.06/1.19
F. psychrophilum F896	0.12	4	> 0.25	2	0.5	≤ 0.06	≤ 0.008	> 1	0.06	> 1/19
F. psychrophilum F2125	0.06	2	> 0.25	2	0.5	0.12	≤ 0.008	> 1	0.03	> 1/19
F. psychrophilum F2130	0.06	4	> 0.25	1	0.5	0.25	≤ 0.008	> 1	0.03	> 1/19
F. psychrophilum F2123	0.06	2	> 0.25	1	0.5	0.12	≤ 0.008	> 1	0.03	> 1/19
F. branchiarum F324	> 16	> 4	> 0.25	16	16	8	0.06	> 1	2	> 1/19
F. branchiarum F963	> 16	> 4	> 0.25	16	> 16	8	0.06	1	4	> 1/19
F. collinsii F1673	> 16	> 4	0.25	> 32	8	4	> 0.5	0.5	4	> 1/19
F. collinsii F1548	> 16	> 4	> 0.25	> 32	> 16	> 8	> 0.5	1	> 8	> 1/19
F. erciyesense F562	0.25	> 4	0.06	1	1	2	≤ 0.008	0.25	0.06	0.5/9.5
F. hercynium F2050	16	> 4	> 0.25	> 32	4	4	0.25	> 1	2	> 1/19
F. oncorhynchi F1942	16	> 4	0.25	> 32	8	8	0.5	0.5	2	> 1/19
F. piscis F1228	16	> 4	0.25	> 32	8	4	0.5	1	4	> 1/19
F. tructae F1900	> 16	> 4	0.12	32	> 16	4	0.5	0.25	8	> 1/19
FX9 F1616	0.12	> 4	0.06	0.5	0.5	1	≤ 0.008	0.5	0.03	1/19
FX9 F945	0.25	> 4	0.12	0.5	1	0.5	≤ 0.008	0.5	0.03	> 1/19
FX10 F944	0.25	> 4	0.06	1	1	0.5	≤ 0.008	0.12	0.06	1/19

FX10 F1511	0.25	> 4	0.06	1	1	0.5	≤ 0.008	0.12	8	1/19
FX15 F2167	1	> 4	0.12	4	1	1	0.015	0.5	0.12	0.25/4.75
FX15 F2043	1	> 4	0.25	8	2	1	0.015	0.5	0.12	> 1/19
FX15 F1859	1	> 4	0.12	8	1	1	0.015	0.25	0.12	0.5/9.5
FX15 F1692	0.5	> 4	0.12	8	1	0.5	0.015	0.25	0.12	0.25/4.75
FX15 F106	1	> 4	0.12	8	2	2	0.03	0.25	0.12	1/19
FX15 F1677	0.12	> 4	0.06	8	1	0.5	0.015	0.12	0.12	0.5/9.5
FX23 F1259	8	> 4	0.25	> 32	4	8	0.25	1	4	> 1/19
FX23 F1213	16	> 4	0.25	> 32	16	8	0.5	1	4	> 1/19
FX27 F1593	1	> 4	0.03	0.5	1	0.5	0.06	0.12	0.06	> 1/19
FX27 F1527	2	> 4	0.03	4	1	1	0.12	0.12	0.06	> 1/19
FX32 F1715	0.25	> 4	0.03	0.25	0.5	0.5	≤ 0.008	0.12	0.03	0.25/4.75
FX32 F2020	0.25	> 4	0.06	0.5	0.5	0.5	≤ 0.008	0.12	0.03	0.5/9.5
FX36 F1996	4	> 4	0.12	32	2	4	0.12	> 1	0.5	> 1/19
FX36 F2035	4	> 4	0.12	16	2	2	0.12	1	1	> 1/19

Seven *Flavobacterium psychrophilum* isolates that were further characterised using whole-genome sequencing are marked with an asterisk (*). Five of the seven *F. psychrophilum* isolates had MICs for OXO above the epidemiological cut-off value (ECV) and also had a corresponding T83A or T83V substitution in GyrA; these MICs are highlighted in bold and underlined. The remaining two isolates had no such substitution and had MICs for OXO below the ECV. For antimicrobials for which the ECV for *F. psychrophilum* is defined in the CLSI guideline VET04 (CLSI 2020) or for which a provisional epidemiological cut-off value for wild type (CO_{WT}) could be calculated, the MICs above the ECV or CO_{WT} are marked in red, and the MICs below are marked in green. Lighter colours are used for other *Flavobacterium* species. *Flavobacterium* isolates that grew in the upper end of the tested concentration range for antimicrobials without ECV or CO_{WT} for *F. psychrophilum* are marked grey.

Appendix 6: Raw data on phenotypic antimicrobial susceptibility testing (minimum inhibitory concentrations in µg/ml) for the eight Slovene *Flavobacterium branchiophilum* isolates and other *Flavobacterium* spp., determined using the adjusted method recommended for *F. psychrophilum* (CLSI, 2020a) for AMP, ampicillin; CTZ, ceftazidime; ENR, enrofloxacin; ERY, erythromycin; FLO, florfenicol; GEN, gentamicin; MER, meropenem; OXO, oxolinic acid; OXY, oxytetracycline; TRS, trimethoprim/sulfamethoxazole

	AMP	**CTZ**	**ENR**	**ERY**	**FLO**	**GEN**	**MER**	**OXO**	**OXY**	**TRS**
Isolate/growth medium	M	M	M	M	M	M	M	M	M	M
	A	A	A	A	A	A	A	A	A	A
F. branchiophilum F1517	0.12	> 4	0.06	1	0.5	≤ 0.06	≤ 0.008	0.25	0.25	0.03/0.6
	0.12	> 4	0.03	2	0.5	0.12	≤ 0.008	0.12	0.12	0.03/0.6
F. branchiophilum F1628*	0.12	> 4	0.015	1	0.5	0.12	≤ 0.008	0.12	0.25	0.03/0.6
	0.12	> 4	0.03	2	0.5	≤ 0.06	≤ 0.008	0.12	0.12	0.03/0.6
F. branchiophilum F1629	0.12	> 4	0.03	2	0.5	≤ 0.06	≤ 0.008	0.25	0.25	0.03/0.6
	0.12	> 4	0.03	2	0.5	≤ 0.06	≤ 0.008	0.12	0.12	0.03/0.6
F. branchiophilum F1727*	0.06	> 4	0.03	1	0.5	≤ 0.06	≤ 0.008	0.25	0.25	0.03/0.6
	0.12	> 4	0.03	2	0.5	0.12	≤ 0.008	0.12	0.12	0.03/0.6
F. branchiophilum F1728	0.12	> 4	0.03	2	0.5	≤ 0.06	≤ 0.008	0.25	0.25	0.03/0.6
	0.12	> 4	0.03	1	0.25	≤ 0.06	≤ 0.008	0.12	0.12	0.03/0.6
F. branchiophilum F1862	0.12	> 4	0.03	2	1	0.25	≤ 0.008	0.25	0.25	0.03/0.6
	0.12	> 4	0.03	2	0.5	0.12	≤ 0.008	0.12	0.12	0.03/0.6
F. branchiophilum F1822	0.12	> 4	0.03	2	0.5	≤ 0.06	≤ 0.008	0.12	0.25	0.015/0.3
	0.12	> 4	0.03	2	0.25	≤ 0.06	≤ 0.008	0.12	0.12	0.03/0.6
F. branchiophilum F1730	0.12	> 4	0.03	1	0.5	≤ 0.06	≤ 0.008	0.25	0.25	0.03/0.6
	0.12	> 4	0.03	2	0.5	0.12	≤ 0.008	0.12	0.12	0.03/0.6
Isolate/growth medium	M	M	M	M	M	M	M	M	M	M
F. bernardetii F1040	0.5	> 4	0.015	0.25	1	0.12	0.015	0.12	0.12	1/19
F. bernardetii F609	0.25	> 4	0.008	0.25	1	0.12	≤ 0.008	0.12	0.12	> 1/19
F. celericrescens F1856	0.25	> 4	0.06	4	2	0.12	≤ 0.008	0.5	0.12	0.5/9.5
F. celericrescens F1959	0.25	> 4	0.06	0.12	2	0.12	≤ 0.008	0.5	0.12	0.5/9.5
F. difficile F1633	0.12	> 4	0.06	0.12	0.5	≤ 0.06	≤ 0.008	0.25	0.12	> 1/19
F. tiangeerense F1485	2	4	0.03	0.5	1	2	≤ 0.008	0.25	0.12	> 1/19
FX2 F334	1	> 4	0.015	0.25	0.5	0.25	≤ 0.008	0.12	4	0.5/9.5
FX2 F1038	0.5	> 4	0.015	0.12	0.5	0.25	≤ 0.008	0.12	4	0.5/9.5
FX2 F317	1	> 4	0.03	0.5	2	1	0.03	0.25	> 8	> 1/19
FX3 F1048	0.5	> 4	0.008	0.25	1	≤ 0.06	0.015	0.12	0.06	1/19
FX3 F1821	0.5	> 4	0.008	0.25	1	0.12	0.015	0.12	0.06	1/19
FX3 F1782	1	> 4	0.015	0.25	1	0.12	0.03	0.25	0.12	0.5/9.5
FX5 F1933	1	> 4	0.03	1	1	0.25	0.015	0.25	0.25	> 1/19
FX5 F1140	0.25	> 4	0.015	0.25	1	0.12	0.015	0.25	0.12	> 1/19
FX5 F1142	0.12	> 4	0.03	0.5	2	0.25	≤ 0.008	0.25	0.12	> 1/19
FX18 F1773	0.5	> 4	0.004	0.25	1	0.12	0.015	0.06	0.12	0.5/9.5
FX18 F626	0.06	> 4	0.008	0.12	1	0.25	≤ 0.008	0.12	0.12	0.5/9.5

Two isolates that were further characterised using whole-genome sequencing are marked with an asterisk (*). None of the isolates had a T83V or T83A substitution in GyrA. The growth media used for each antimicrobial are specified in the second row with the abbreviations A for Anacker and Ordal (AO) broth and M for diluted cation-adjusted Mueller-Hinton broth supplemented with additional cations (DCAMHB+C). For antimicrobials for which the ECV for *F. psychrophilum* is defined in the CLSI guideline VET04 (CLSI 2020) or for which a provisional epidemiological cut-off value for wild type (CO_{WT}) could be calculated, the MICs above the ECV or CO_{WT} are marked in light red, and the MICs below are marked in light green. *Flavobacterium* isolates that grew in the upper end of the tested concentration range for antimicrobials without ECV or CO_{WT} for *F. psychrophilum* are marked grey.