

Shrimp production in Europe and diversity of potential pathogenic bacteria in digestive tracts of shrimp

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UND MEERESFORSCHUNG

13th Annual Workshop of the National Reference Laboratories for Crustacean Diseases
National Institute of Aquatic Resources, Technical University of Denmark, 01.06.2022

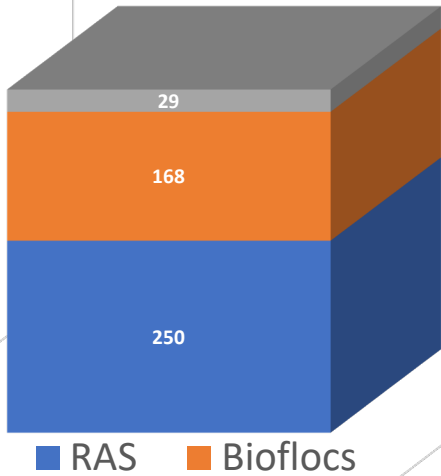
- Shrimp production in Europe
- Diversity of pathogenic bacteria in shrimp digestive: fresh water and marine species
- Utilization of *tlh* gene to detect pathogenic *Vibrio*

Shrimp production in Europe

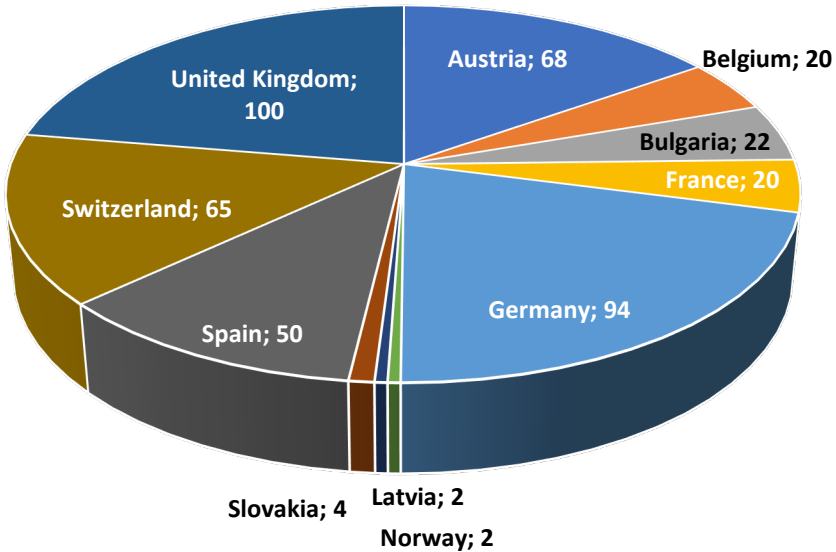
Cultured shrimps:
Pacific white-leg shrimp (*Penaeus vannamei*),
Blue shrimp (*Litopenaeus stylirostris*),
Kuruma shrimp (*Marsupenaeus japonicus*)



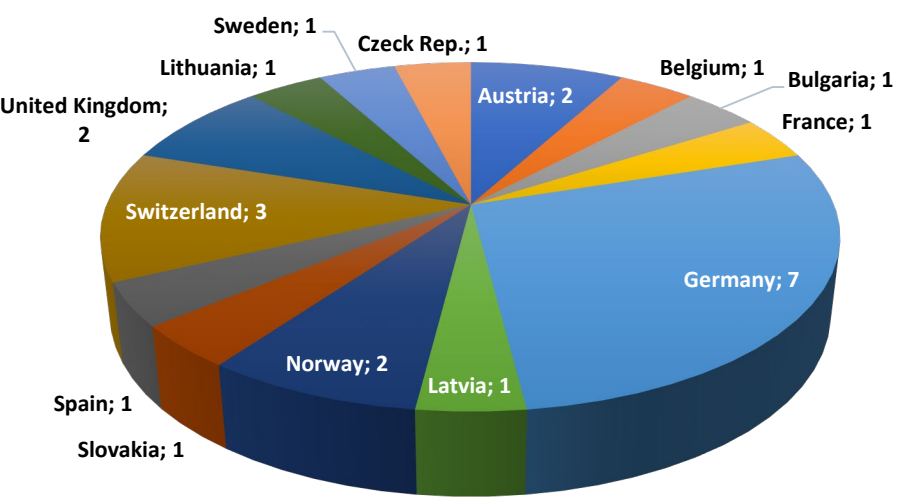
Culture systems and shrimp yield (in tonnes)



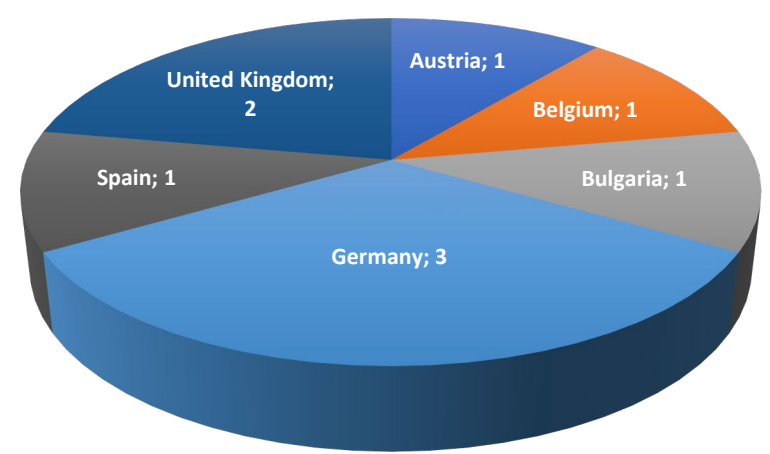
Shrimp production in 2020 (in tonnes)



Shrimp production in Europe



Farms



Hatcheries

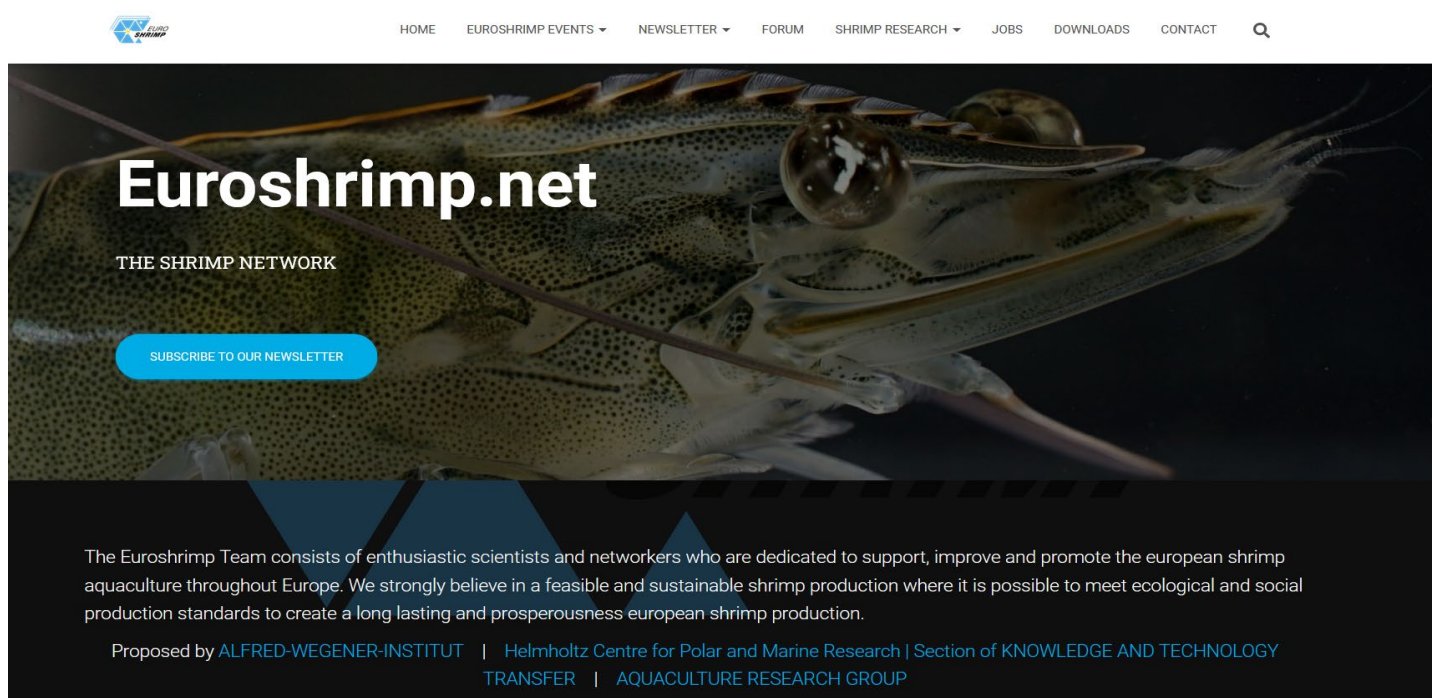
Shrimp farms = 25 companies

Shrimp hatcheries = 9 companies

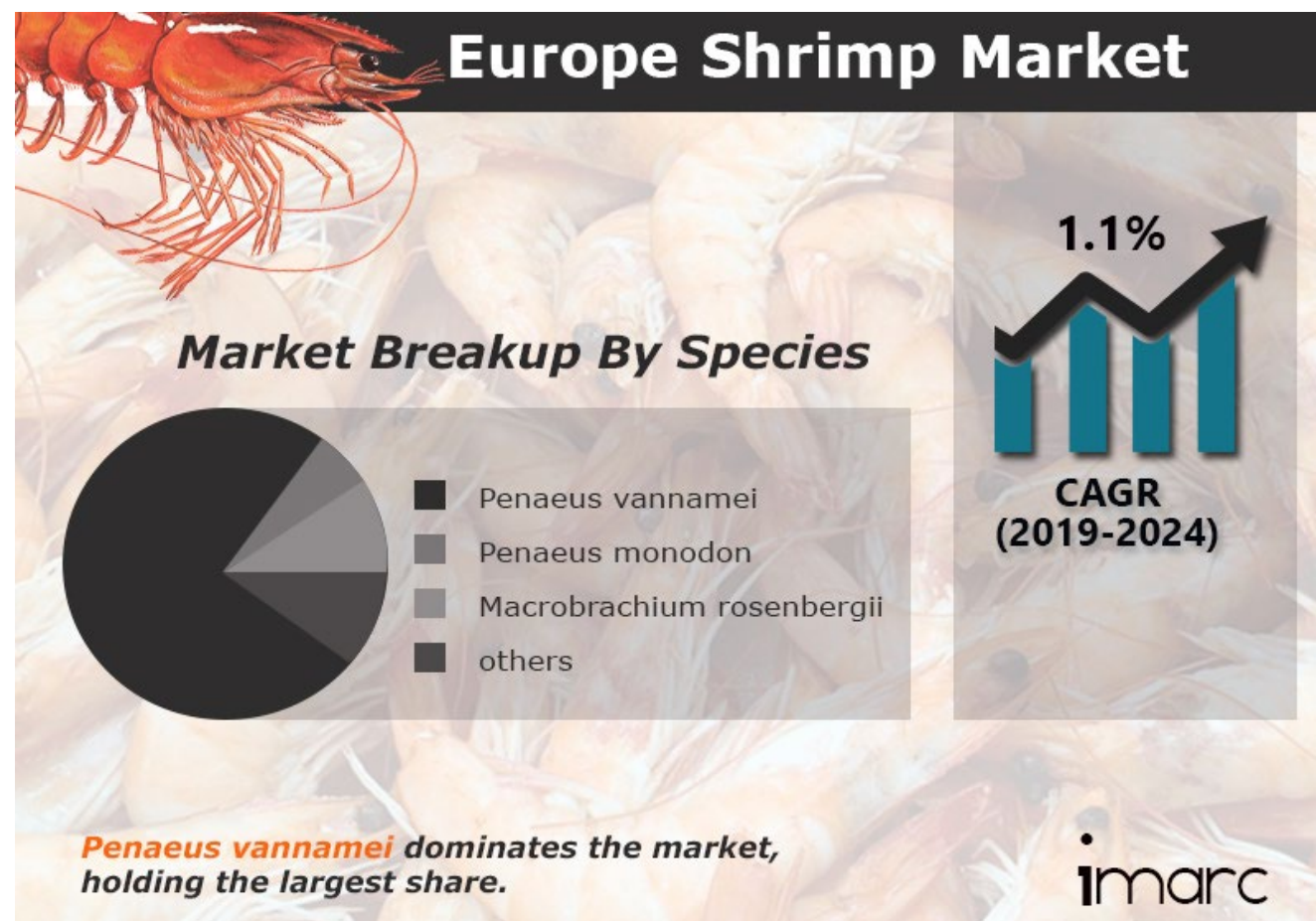
Feed producers/suppliers = 16 companies

Willing to contribute/submit data from your country?

Please contact us at euroshrimp.net or send e-mail to euroshrimp@awi.de



Projection of shrimp demand in Europe



European shrimp market to reach 918,000 tonnes by 2024

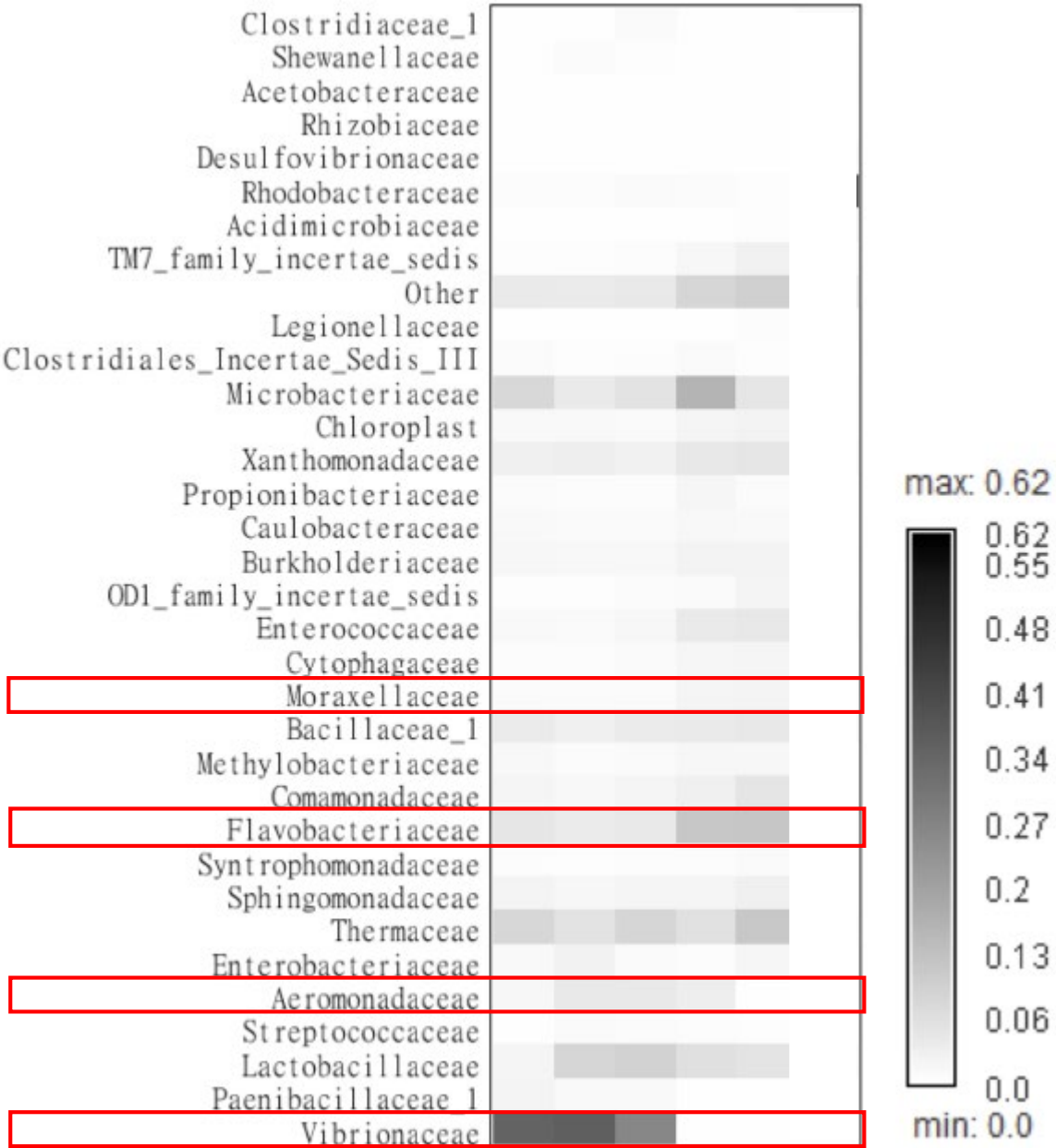
European consumers prefer:

- ready-to-eat and easy-to-prepare products,
- frozen and canned products as well as value-added variants such as marinated shrimp,
- sustainability certificated product

How do EU countries boost shrimp production?

1. the European Commission encourages investments in the aquaculture sector (*i.e.* the “EU Aquaculture Online”)
2. Production of good quality shrimp larvae and feed
3. Digitalization of culturing system
4. Thorough water quality as well as microbial assessment including pathogen surveillance

Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



Macrobrachium nipponense

- Intestine (N= 25 samples),
- platform: 454 Pyrosequencing,
- Target: 16S rRNA V1-V2 region



Potential pathogens:
Acinetobacter, Aeromonas, Flavobacterium, Vibrio

Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



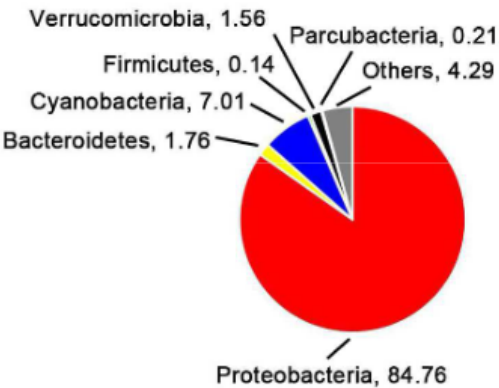
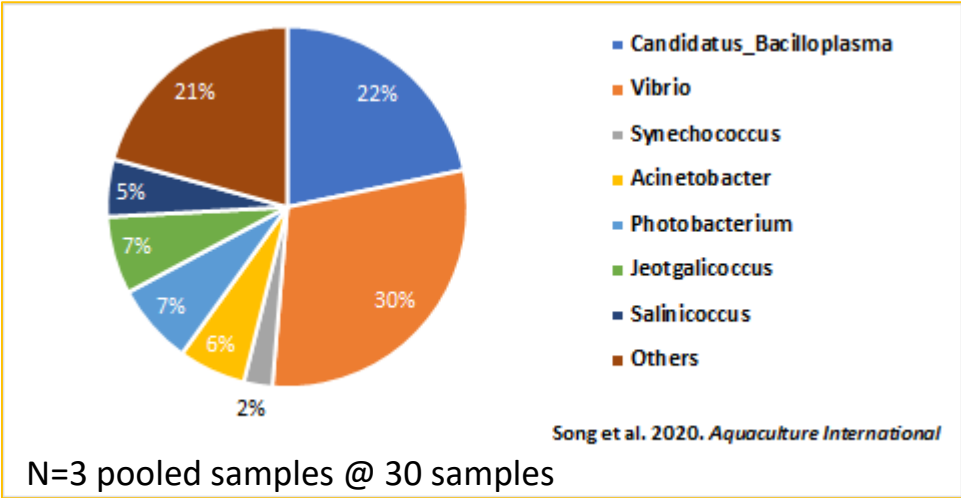
J. Mazurie (2005)

Marsupenaeus japonicus

- Gut and stomach content,
- Illumina HiSeq2500, Ion S5TM XL, Illumina Miseq,
- Target: 16S rRNA V4-V5 and V3-V4 region

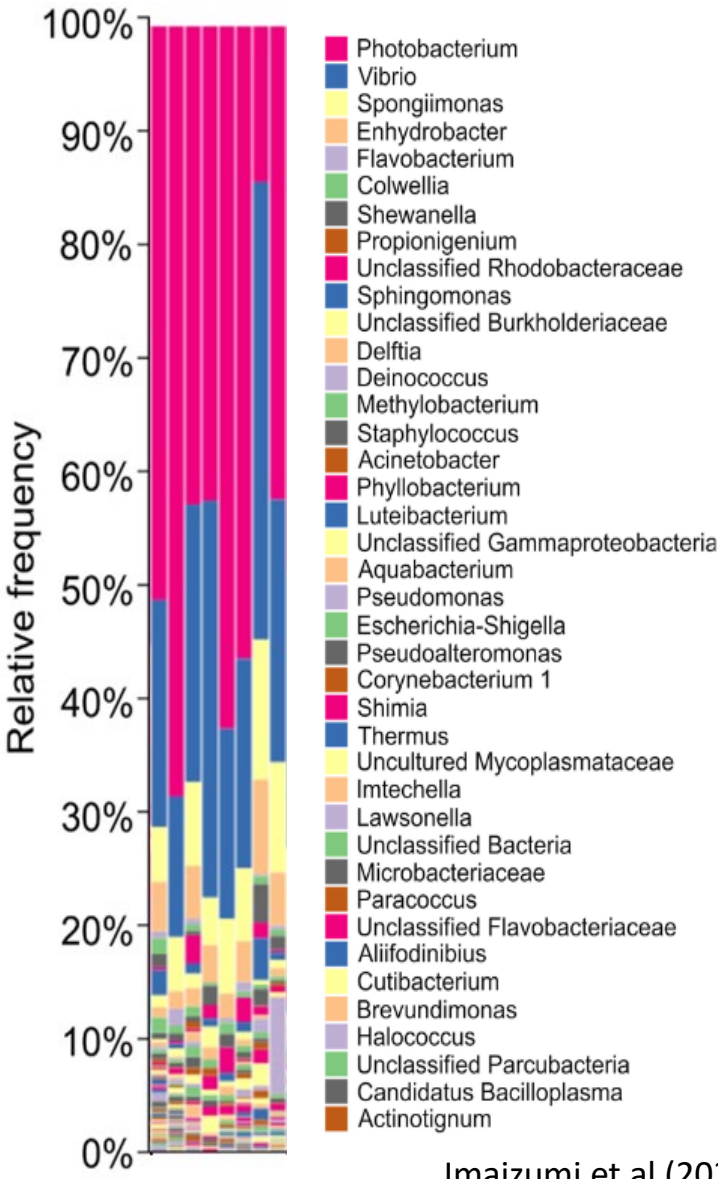
Potential pathogens:

- Photobacterium
- Vibrio
- Escherichia-Shigella
- Flavobacterium
- Pseudoalteromonas
- Staphylococcus

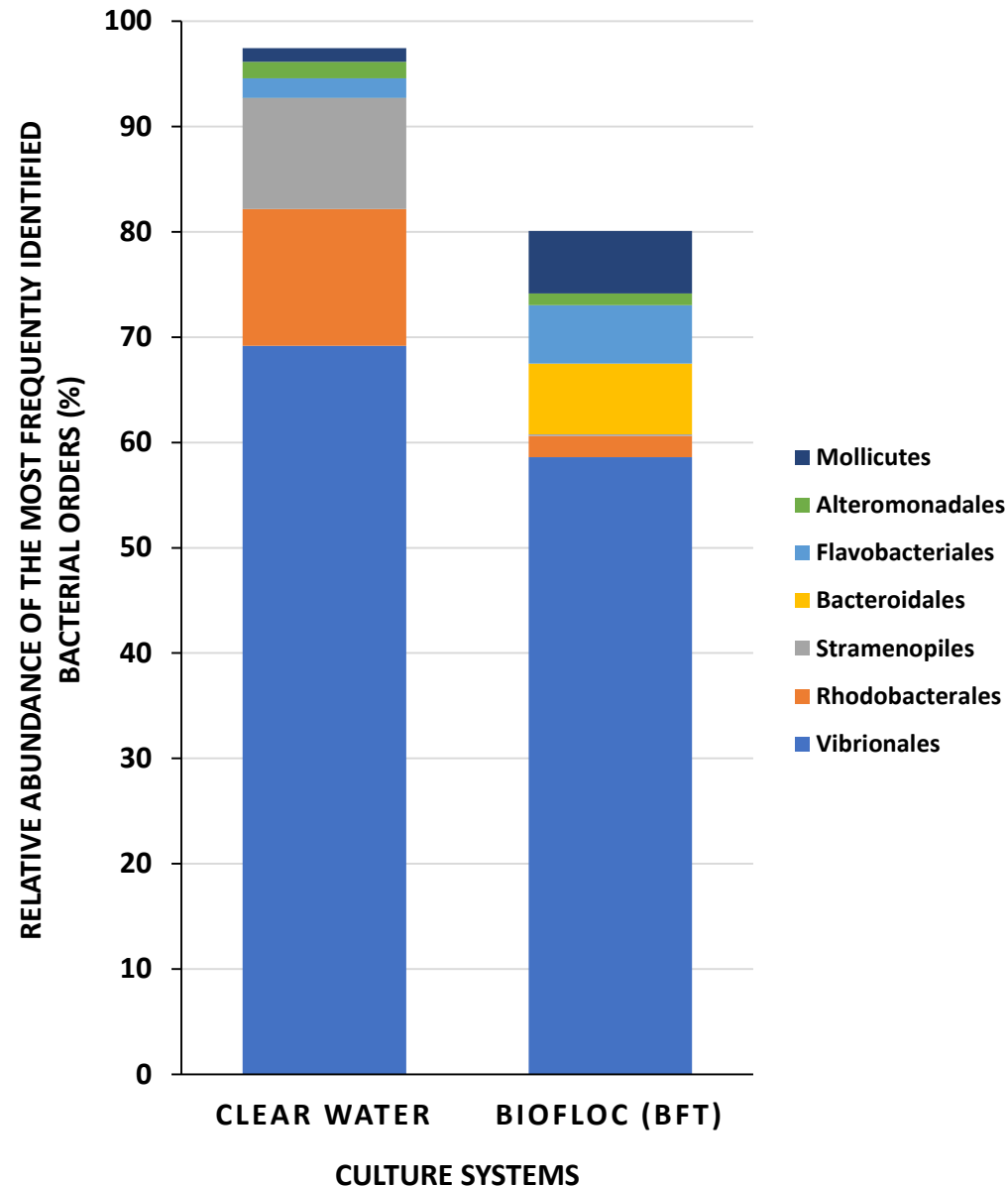


- Photobacterium,
- Ruegeria,
- Vibrio,
- Marivita

Zhou et al (2021). *Aquaculture*



Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



Litopenaeus stylirostris

- Intestine
- Clear water and biofloc systems
- Illumina Miseq,
- Target: 16S rRNA V3-V4 region



Potential pathogens:

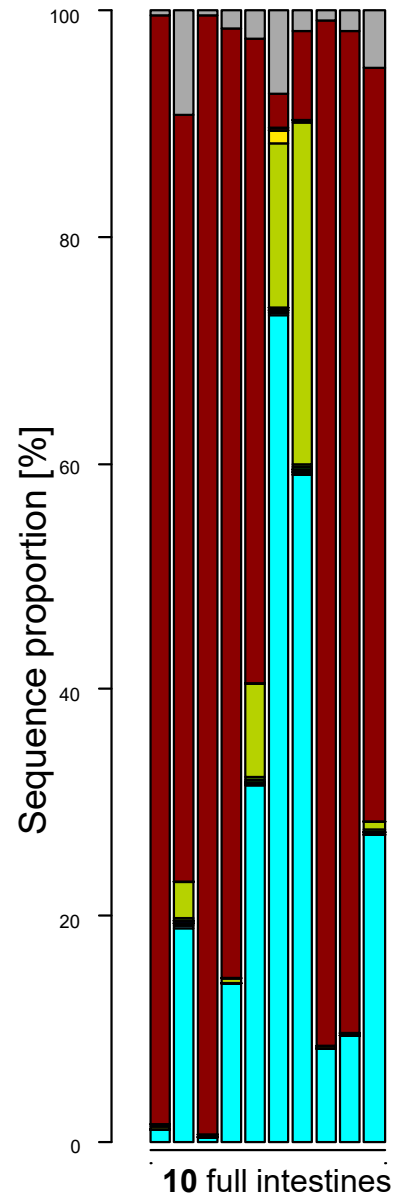
Alteromonas

Pseudoalteromonas

Flavobacterium

Vibrio

Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



Intestines of healthy shrimps (N=10)

■ Vibrio
■ Pseudomonas
■ Acinetobacter

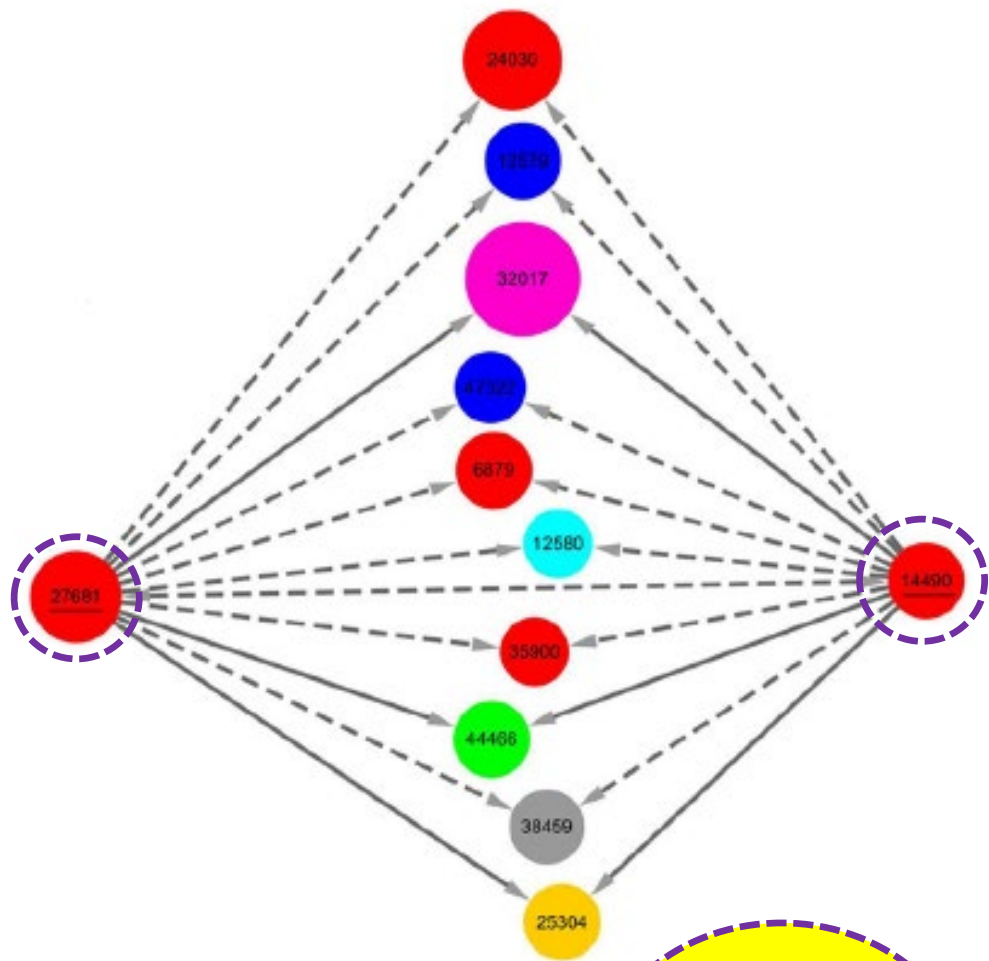
Penaeus vannamei

- Intestine (N= 10 samples),
- Illumina Miseq,
- Target: 16S rRNA V3-V4 region



Potential pathogen:
Vibrio

Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



P. vannamei

- Intestine (N= 30), shrimp ponds
- Sequencing platform: Illumina Miseq,
- Target: 16S rRNA V3-V4 region

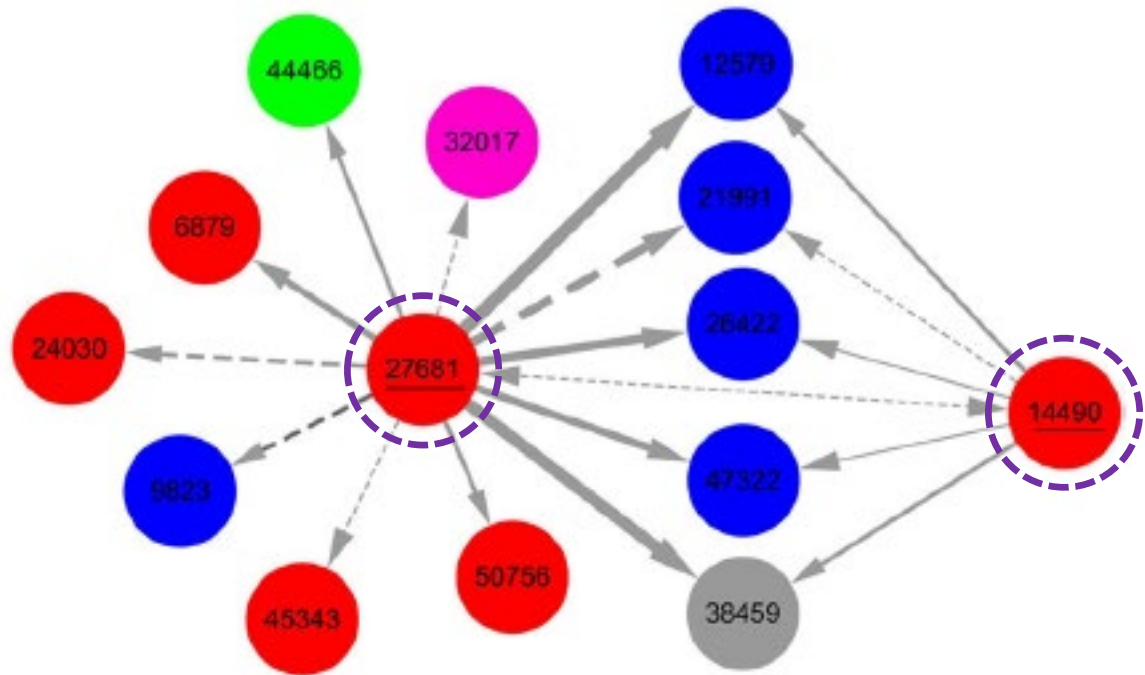


<i>γ-Proteobacteria</i>	<i>α-Proteobacteria</i>	<i>Actinobacteria</i>	Edge style → Positive - - - Negative
27681 g_ <i>Vibrio tubiashii</i>	12580 f_ <i>Rhodobacteraceae</i>	32017 o_ <i>Actinomycetales</i>	
14490 g_ <i>Vibrio harveyi</i>	Flavobacteriia	Bacilli	
50756 g_ <i>Shewanella</i>	12579 f_ <i>Flavobacteriaceae</i>	38459 s_ <i>garvieae</i>	
6879 g_ <i>Photobacterium</i>	47322 f_ <i>Flavobacteriaceae</i>	Planctomycetia	
24030 g_ <i>Salinivibrio</i>	9823 f_ <i>Flavobacteriaceae</i>	25304 g_ <i>Planctomyces</i>	
45343 g_ <i>Vibrio</i>	21991 f_ <i>Flavobacteriaceae</i>	Verrucomicrobiae	
35900 f_ <i>Marinicellaceae</i>	26422 g_ <i>Flavobacterium</i>	44466 g_ <i>Haloferula</i>	

Potential pathogens:

V. tubiashii
V. harveyi
=10.17%

Diversity of pathogenic bacteria in shrimp digestive - Diseased shrimps



White Feces Syndrom (WFS)

- Intestine (N= 30),
- Illumina Miseq,
- Target: 16S rRNA V3-V4 region

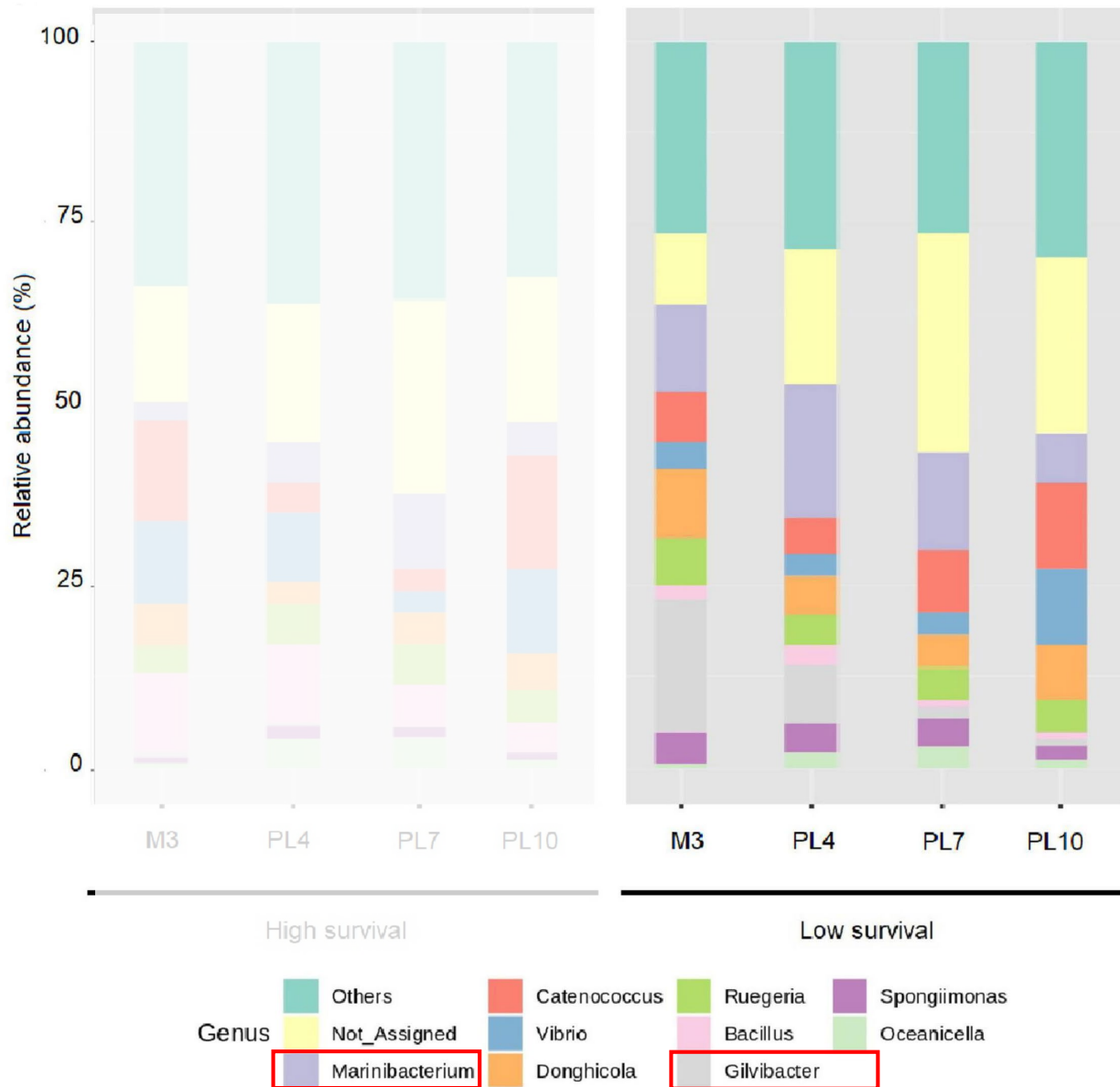


V. tubiashii
V. harveyi
= 64.86%

V. tubiashii
V. harveyi
= 10.17%

<i>γ-Proteobacteria</i>	<i>α-Proteobacteria</i>	<i>Actinobacteria</i>	Edge style → Positive - - - Negative
27681 g_ <i>Vibrio tubiashii</i>	12580 f_ <i>Rhodobacteraceae</i>	32017 o_ <i>Actinomycetales</i>	
14490 g_ <i>Vibrio harveyi</i>	<i>Flavobacteriia</i>	<i>Bacilli</i>	
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35900 f_ <i>Marinicellaceae</i>	26422 g_ <i>Flavobacterium</i>	44466 g_ <i>Haloferula</i>	

Diversity of pathogenic bacteria - Diseased shrimps



AHPND

Whole body

N= 233 samples;

Mysis 3 (M3, N = 64), Postlarvae 4 (PL4, N=64),
Postlarvae 7 (PL7, N= 56, Postlarvae 10 (PL10, N= 49)

Illumina NovaSeq 6000 P250

Target: 16S rRNA V3-V4 region

AHPND

Hepatopancreas

Bacterial cultivation → Challenge test → 16S rRNA

Pathogens:

- *V. owensii*
- *V. parahaemolyticus*
- *V. harveyi*
- *V. campbellii*
- *V. punensis*

Restrepo et al (2021). *Microbiome*, Reyes et al (2022). *Frontiers in Microbiology*

Diversity of pathogenic bacteria - Diseased shrimps

The main causative agent for mass mortality of Kuruma shrimps in Japan are **Vibriosis** and **Fusariosis**, caused by *V. penaeicida* and *Fusarium solani*, respectively (Yuasa et al., 2016)

Sakai et al. (2007)

魚病研究 Fish Pathology, 42 (3), 141–147, 2007. 9

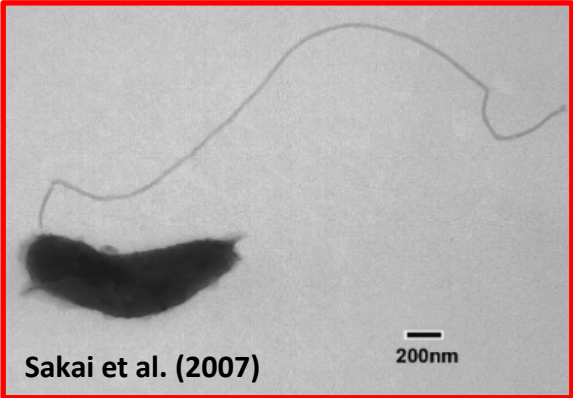
© 2007 The Japanese Society of Fish Pathology

Mass Mortality of Cultured Kuruma Prawn *Penaeus japonicus* Caused by *Vibrio nigripulchritudo*

Heart and muscles → bacterial cultivation → bacterial isolates
The isolates → 16S rDNA → species determination
N= 6 + 60 samples.

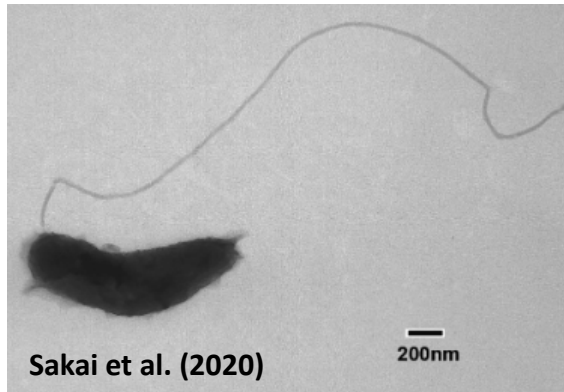
Inoculum doses (CFU/prawn)	No. of dead prawn/challenged
1.4×10^7	10/10
1.4×10^5	8/10
1.4×10^3	5/10
Control	1*/10

* Death at the molting. No bacterium was isolated.

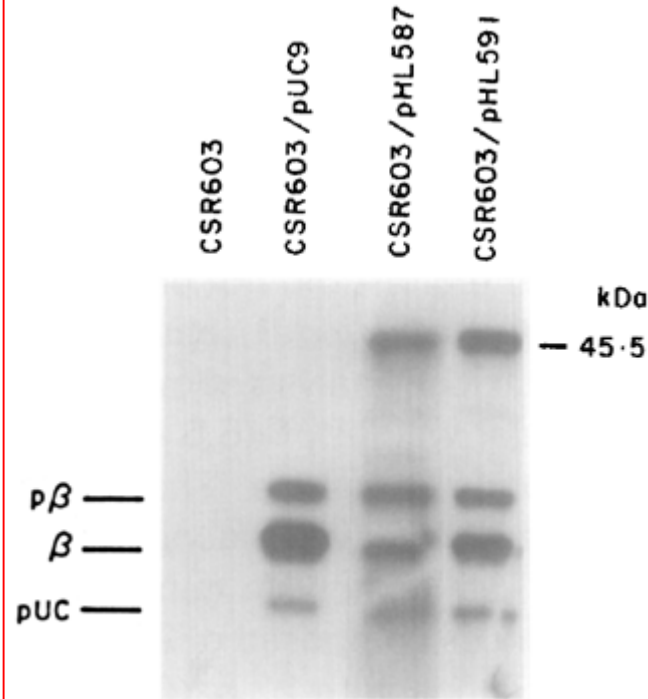


- Pathogens:
- *V. tapetis*
 - *V. nigripulchritudo*

Common pathogenic bacteria in shrimp and potential marker



- Thermolabile hemolysin/*tlh* gene



- *tlh* gene contains an open reading frame of 1254 bp
- encoding protein (45.5 - 47 kDa)
- type II toxins: bacterial toxins that enzymatically damage the membrane
- heat labile (60°C for 10 min)
- G + C content = 47.6%

Taniguchi et al (1985) Journal Bacteriology, Taniguchi et al (1986) Microbial Pathogenesis, Wang et al (2012) Applied and Environmental Microbiology

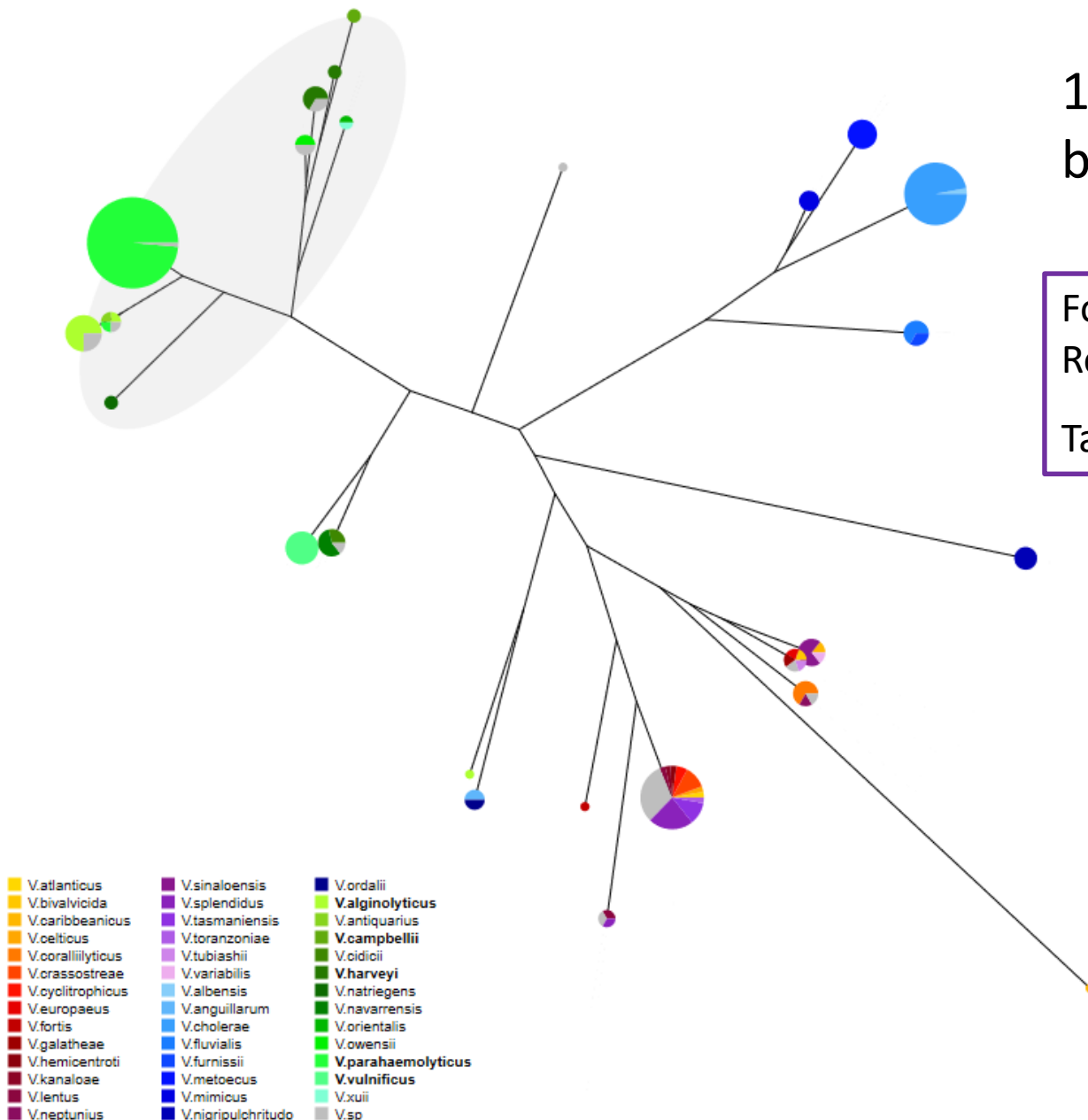
tlh gene to predict pathogenic *Vibrio* in shrimp

108 *tlh* sequences from 10 species (shaded branch) → primer design (5 primer pairs).

Forward: *tlh*-G-vibrio-0515-a-S-22: GCTGGTTCTTRGGDCAYTTCTC
Reverse: *tlh*-G-vibrio-0771-a-A-22: TGGAACGCYACGGTTRTAGTTC
Targeted amplicon: 150-400 bp

Validated target taxa:

- *V. alginolyticus* DSM 2171
- *V. campbellii* DSM 19270
- *V. harveyi* DSM 6940
- *V. parahaemolyticus* DSM 11058
- *V. vulnificus* DSM 10143
- *V. anguillarum* DSM 11323
- *Bacillus subtilis* DSM 347

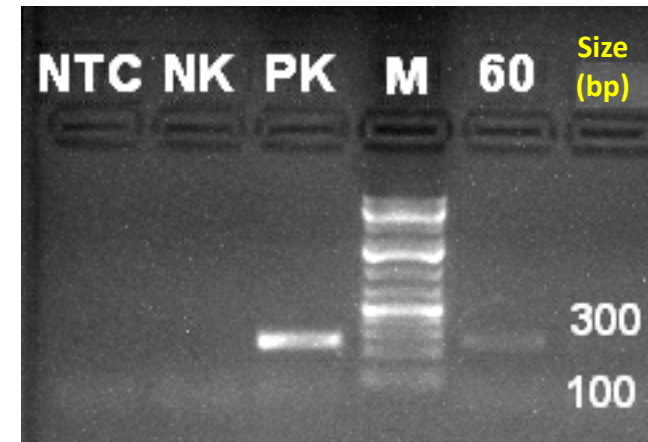
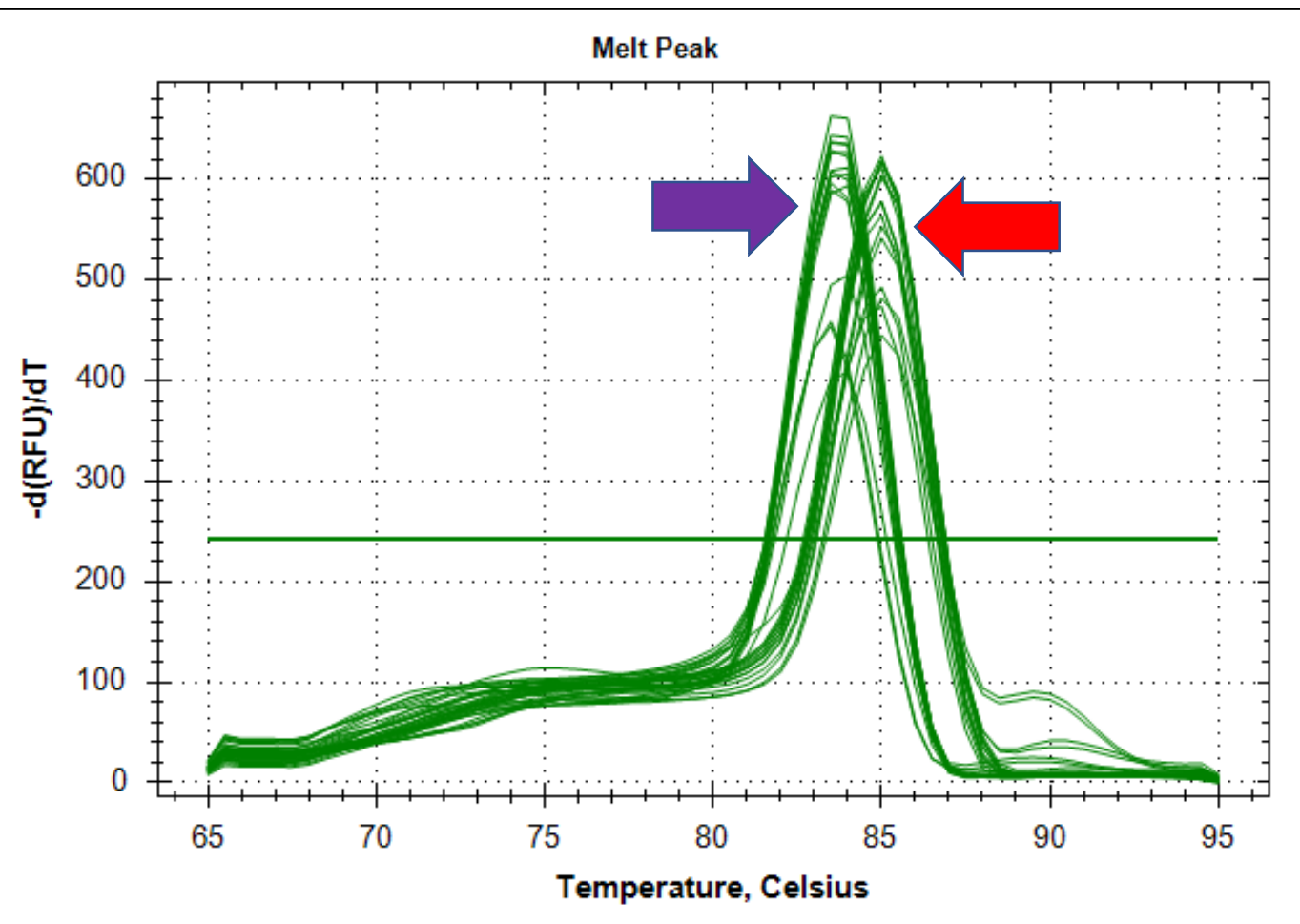


tlh gene to predict pathogenic *Vibrio* in shrimp

Forward: *tlh*-G-vibrio-0515-a-S-22: GCTGGTTCTTRGGDCAYTTCTC
Reverse: *tlh*-G-vibrio-0771-a-A-22: TGGAACGACYACGGTTRTAGTTC
Targeted amplicon: 150-400 bp, PCR & qPCR SYBRGreen

115 samples;

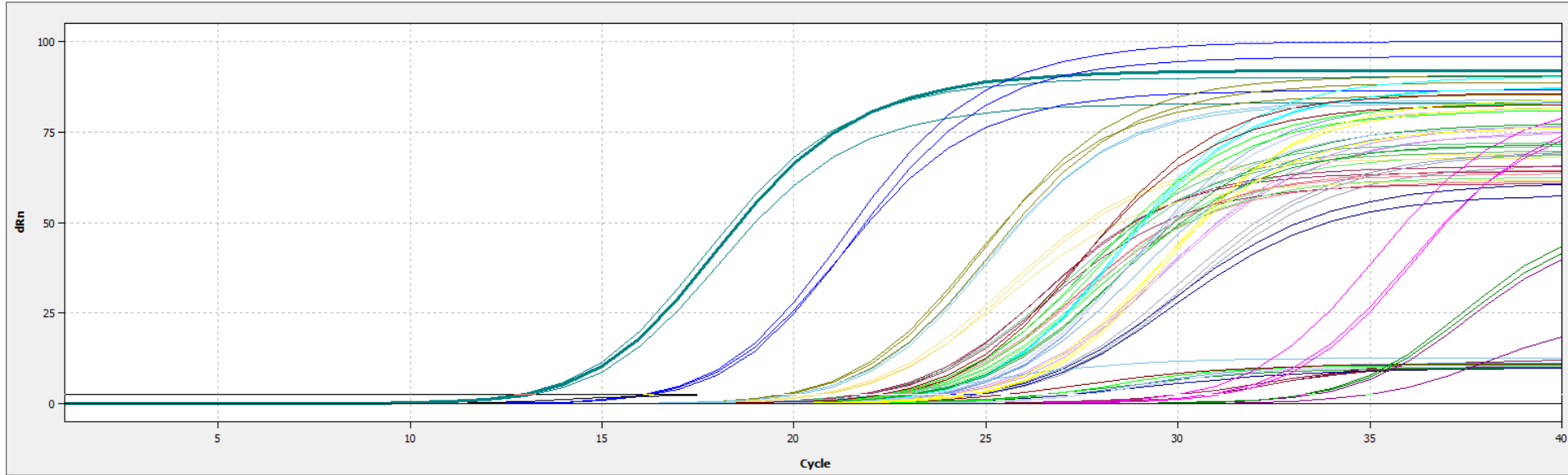
- Intestine of healthy shrimps: 85 samples
- Feces from diseased shrimps: 30 samples



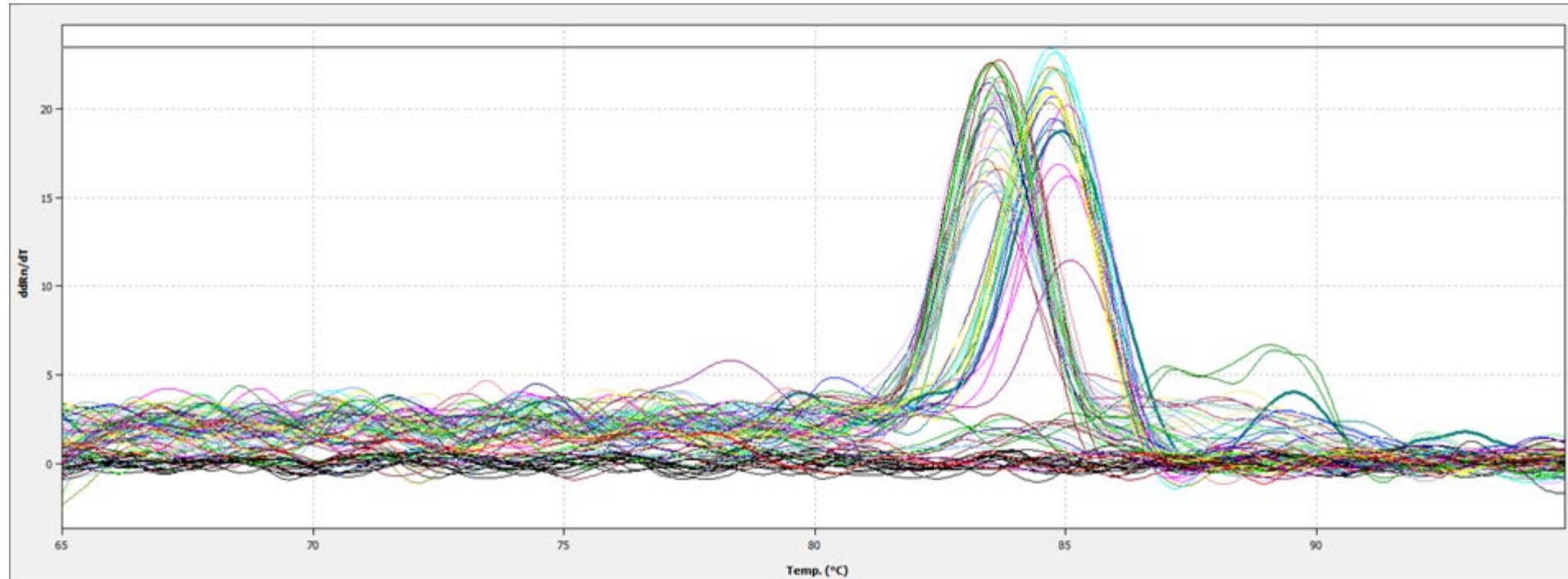
Melting curve for

- 83.5°C = *V. alginolyticus*
- 85°C = *V. parahaemolyticus*, *V. campbellii*,
V. harveyi and *V. vulnificus*

tlh gene to predict pathogenic *Vibrio* in shrimp



115 samples;
- Intestine: 85
- Feces: 30



tlh gene to predict pathogenic *Vibrio* in shrimp

115 samples → 52 samples contain *tlh* gene

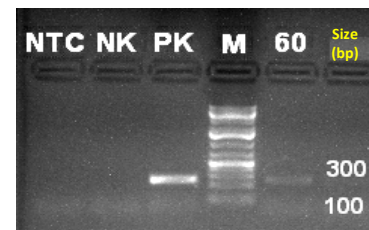
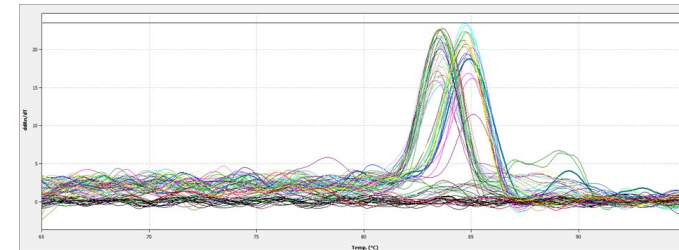
Species	Type of samples (N)	+ Results (%)	Average <i>tlh</i> concentrations (log10 gene copies/ng DNA)	Note
<i>P. vannamei</i>	Intestine (10 , 53)	8 (80%) , 12(23%)	3.9 ± 2.5 , 5.6 ± 0.41	Pond (healthy shrimps), Biofloc (healthy shrimps)
	Feces diseased shrimps (30)	30 (100%)	3.7 ± 1.4	Pond (diseased shrimps)
<i>L. stylirostris</i>	Intestine (22)	2 (9%)	5.8 ± 0.7	Biofloc (healthy shrimps)

Conclusions

1. European shrimp production tends to increase in upcoming years due to incentive, demand, business expansion as well as application of advance shrimp aquaculture technology.
2. Healthy and diseased shrimps may harbor potential pathogenic bacteria. Microbial community seems to influence disease status. When the pathogenic bacteria (i.e. *Vibrio*) reach certain threshold, they may cause disease. In addition, dysbiosis may also provoke a severe bacterial disease.
3. *tlh* gene belonging to *Vibrio* is a prominent candidate to predict the presence of pathogenic *Vibrio*.

Outlooks

1. Further investigation on species diversity possessing *tlh* gene.
2. Investigation of lethal concentration of pathogenic *Vibrio* based on the numbers of *tlh* gene.
3. Correlation among shrimp microbiome, water quality, and shrimp health status.

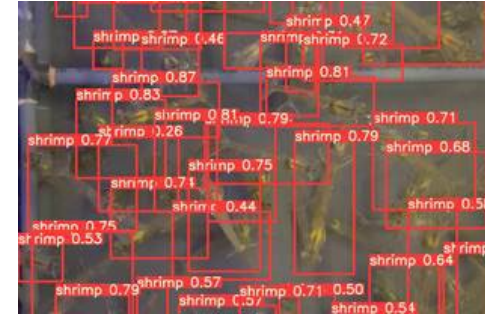




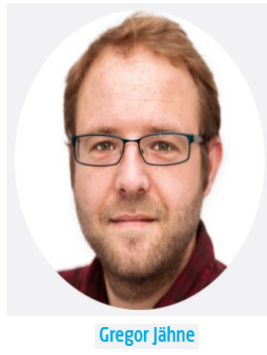
& WG Aquaculture Research, AWI Bremerhaven



RAS: *Grade Aid, MonitorShrimp*



Feed and Sustainable Resources: *TRUE, Sidestream, InEVal, Kraft*



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Head of the working group:

Prof. Dr. Astrid Gärdes (astrid.gaerdes@awi.de)

- Yustian Rovi Alfiansah (postdoc)
- Susanne Spahic (technician)
- Henrike Hohnholz (technician)

Collaborators:



Dr. Christiane Hassenrück
(christiane.hassenrueck@io-warnemuende.de)



Sonja Peters
(sonja.peters@leibniz-zmt.de)

Completed project:

- Mikrobiologische Untersuchungen des Tilapiawachstumsversuchs mit Futter Additiven
- Tropical Macroalgae Bio-based Products for Use in Natural Cosmetics
- Aquaculture practice in tropical coastal ecosystems- Understanding ecological and socio-economic consequences (ACUTE)
- Diversity of microorganisms in both natural and aquaculture tropical seaweed systems: biotechnology potential for sustainable development

Ongoing projects:

- **Microbiome in shrimp and sponges**
- **Bacteria-diatom interaction**

Thank you for your kind attention!

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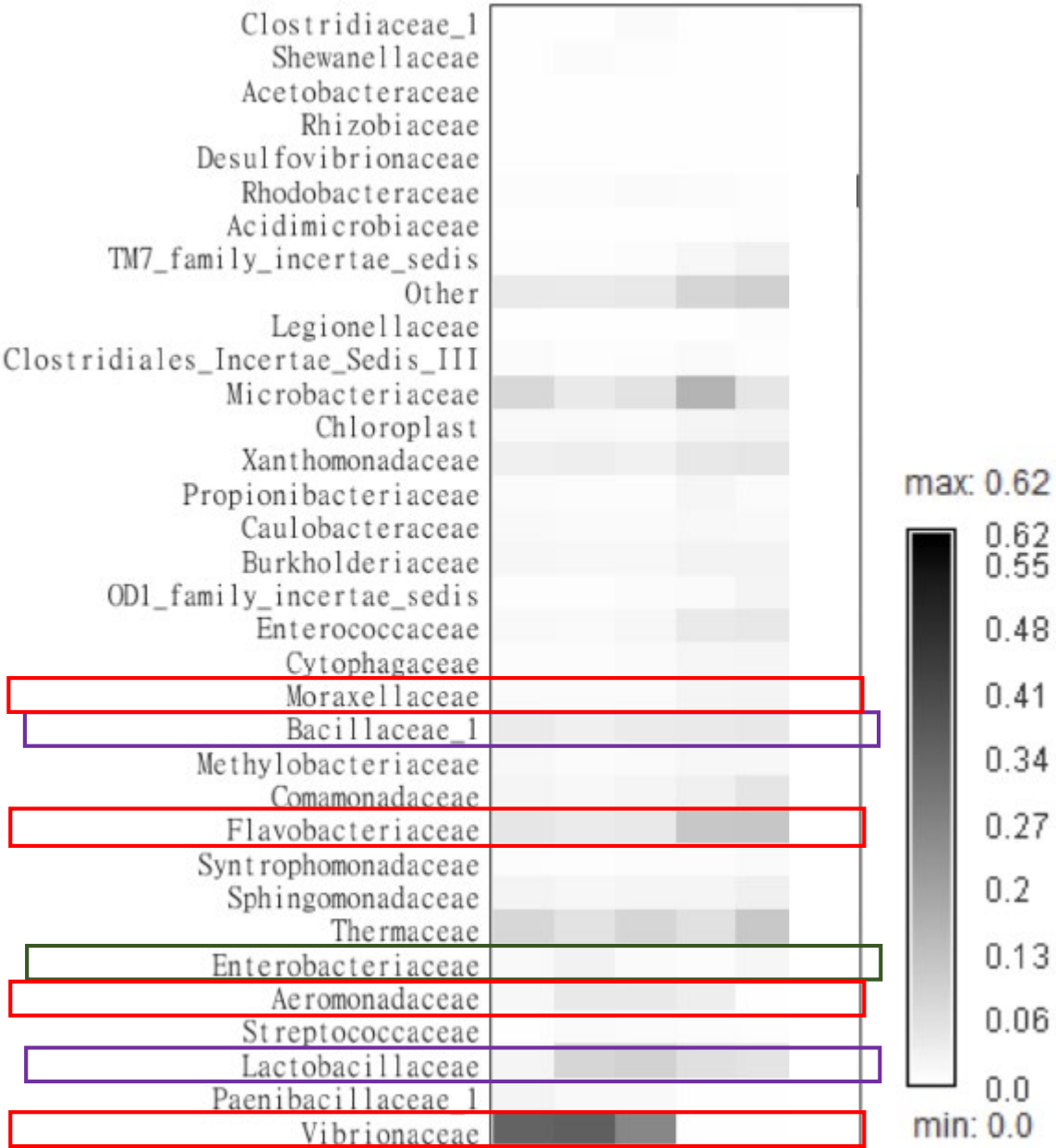
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UND MEERESFORSCHUNG



Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



Macrobrachium nipponense

- Intestine (N= 25 samples),
- platform: 454 Pyrosequencing,
- Target: 16S rRNA V1-V2 region

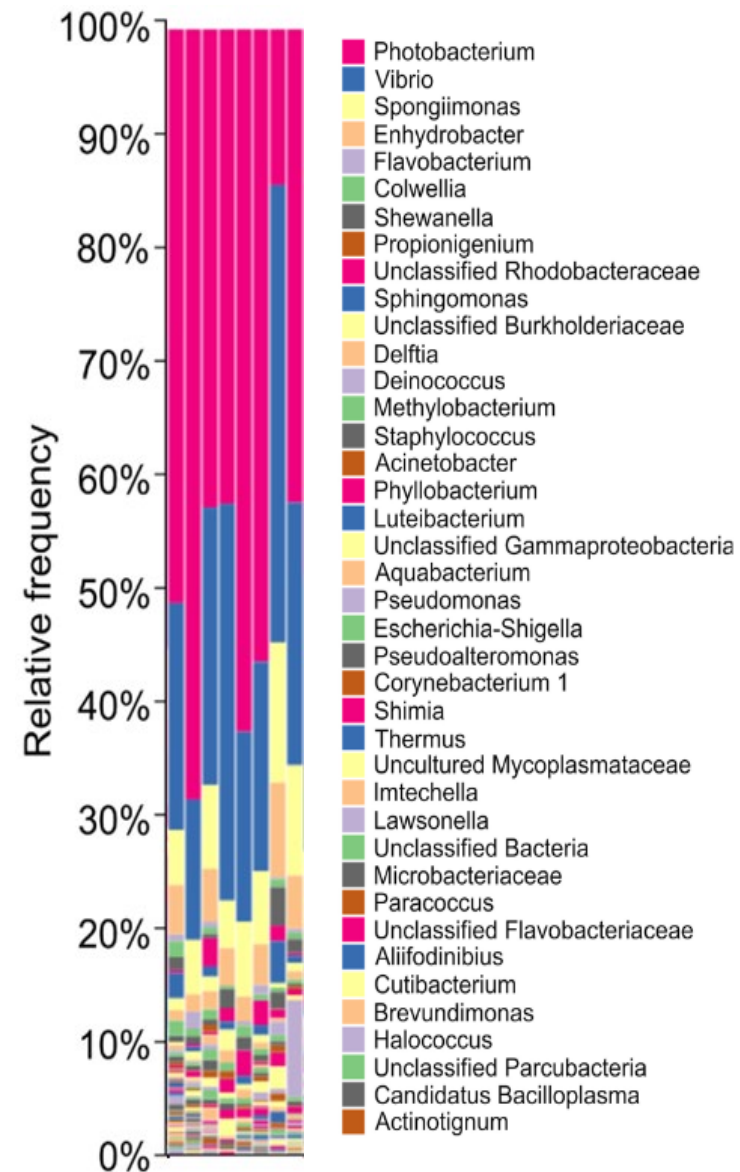


Probiotic bacteria:
Bacillus, Lactobacillus

Cellulose-degrading bacteria:
Citrobacter, Clostridium

Potential pathogens:
Acinetobacter, Aeromonas, Flavobacterium, Vibrio

Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



Marsupenaeus japonicus

- Stomach content (N= 8 samples),
- Illumina Miseq,
- Target: 16S rRNA V3-V4 region

Potential pathogens:

- Escherichia-Shigella
- Flavobacterium
- **Photobacterium**
- Pseudoalteromonas
- Staphylococcus
- **Vibrio**

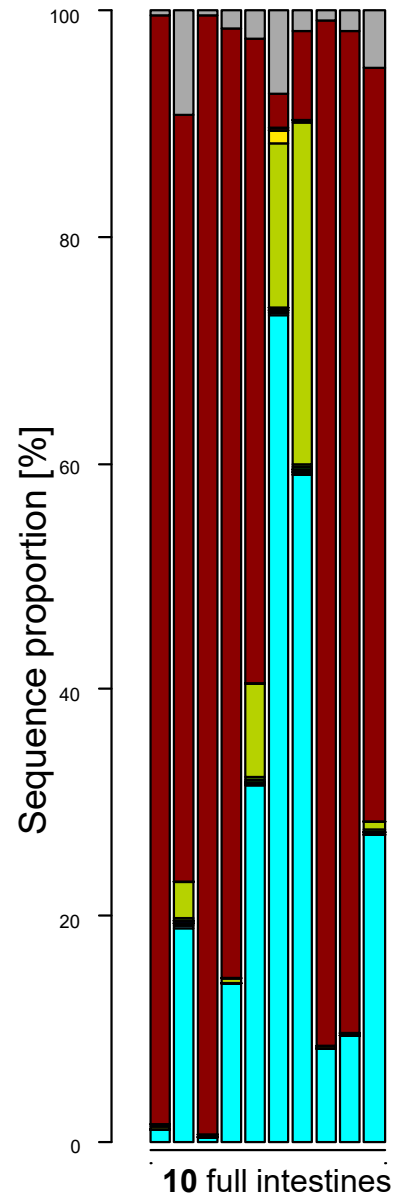
Probiotic bacteria:

- Lactobacillus plantarum,
- Lactococcus lactis,
- Vagococcus fluvialis),
- Lactococcus garvieae



J. Mazurie (2005)

Diversity of pathogenic bacteria in shrimp digestive - Healthy shrimps



Intestines of healthy shrimps (N=10)

■ Vibrio
■ Pseudomonas
■ Acinetobacter

Acinetobacter, *Acidovorax*, *Bacillus*, *Pseudomonas* are able to inactivate AHL, a type of quorum sensing molecule which regulates the virulence of pathogenic bacteria (Liu et al., 2010)

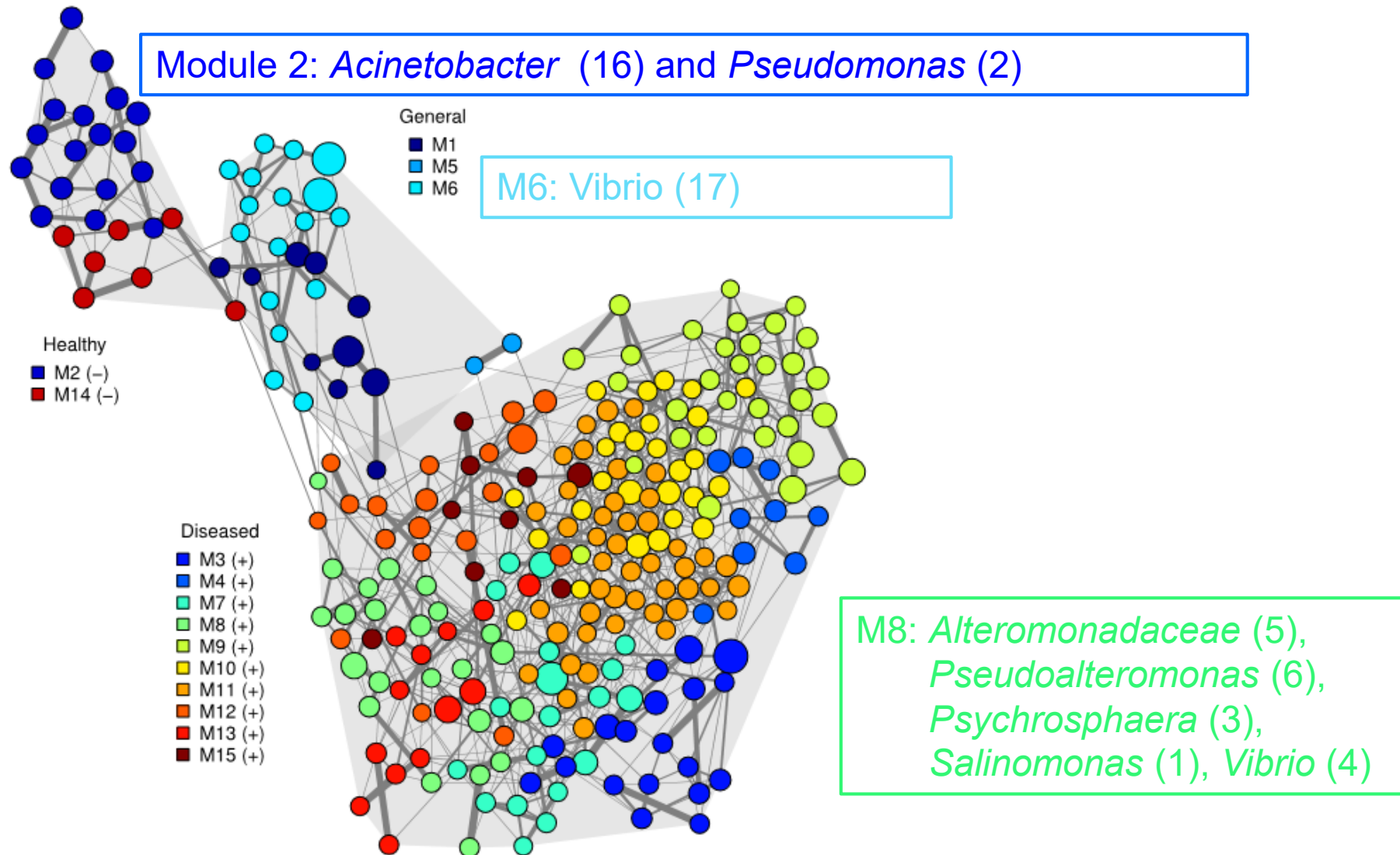
Penaeus vannamei

- Intestine (N= 10 samples),
- Illumina Miseq,
- Target: 16S rRNA V3-V4 region

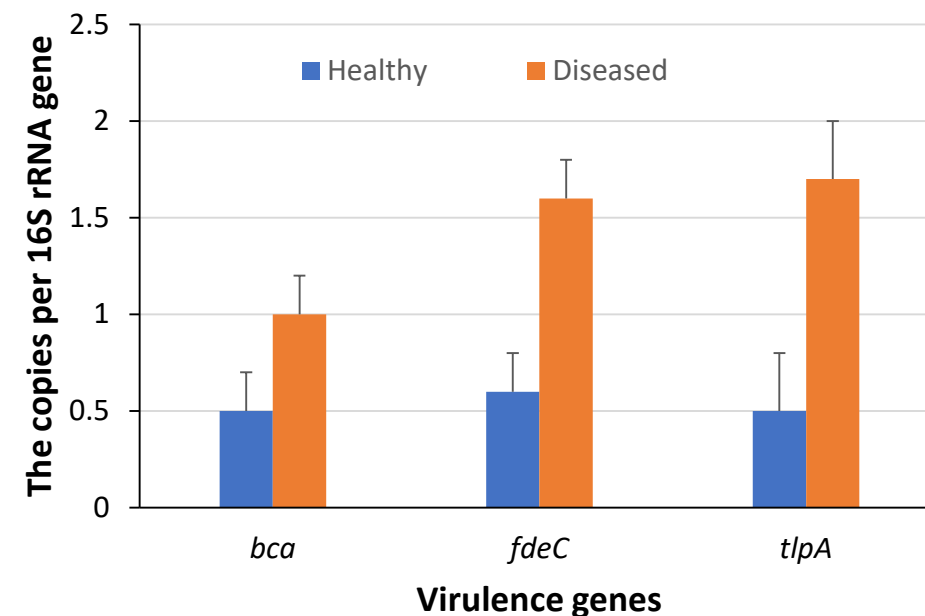
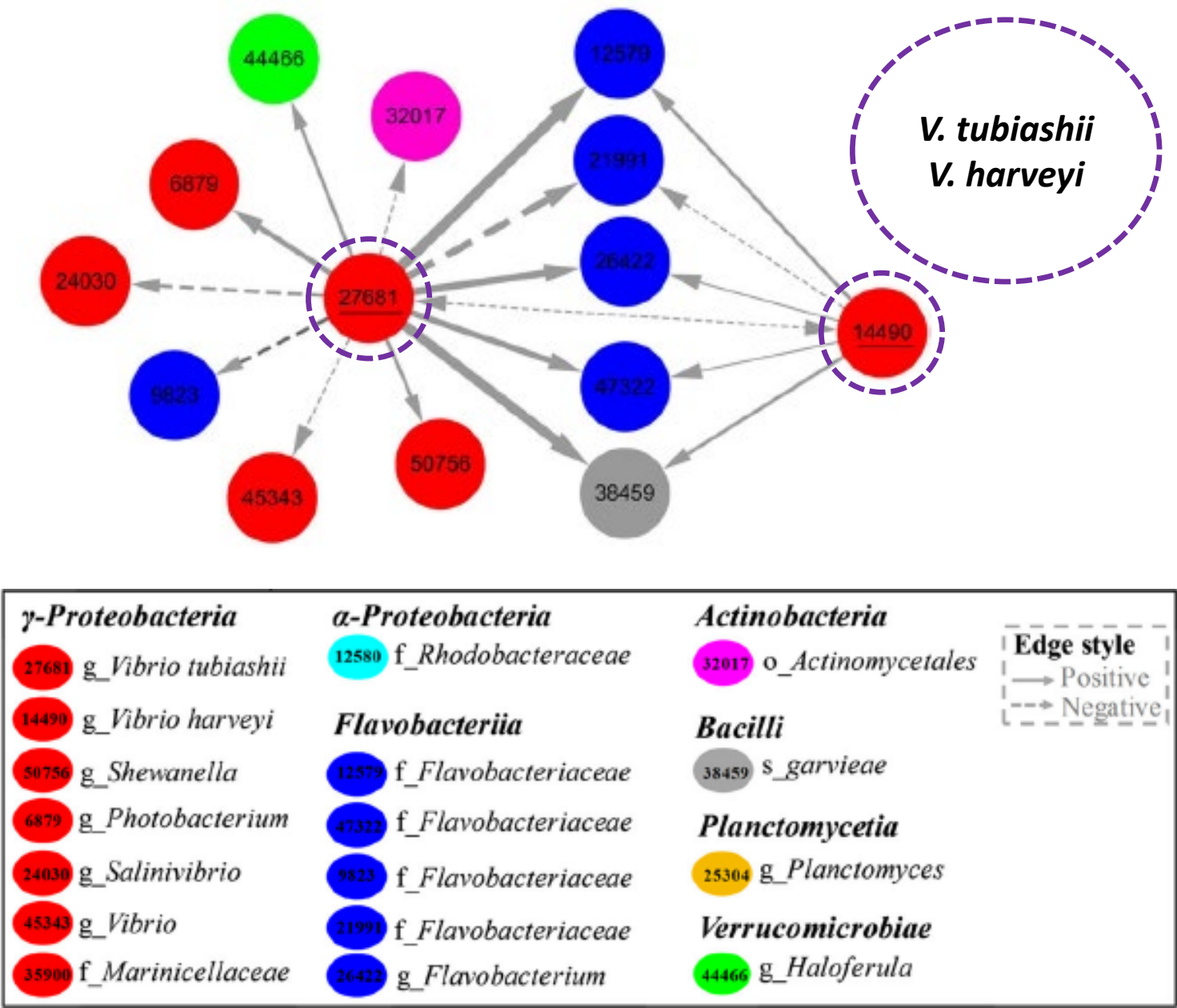
Potential pathogen:
Vibrio



Co-occurrence patterns



Diversity of pathogenic bacteria in shrimp digestive - Diseased shrimps



Diversity of pathogenic bacteria - Diseased shrimps

river prawn *Macrobrachium nipponense*

- Gills (N= 5 + 20 samples),
- Bacterial isolation followed by 16S rRNA characterization

Potential Pathogens:

- *Aeromonas hydrophila*
- *A. veronii*
- *Spiroplasma mirum*



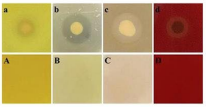
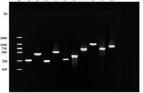
Bacterial isolation



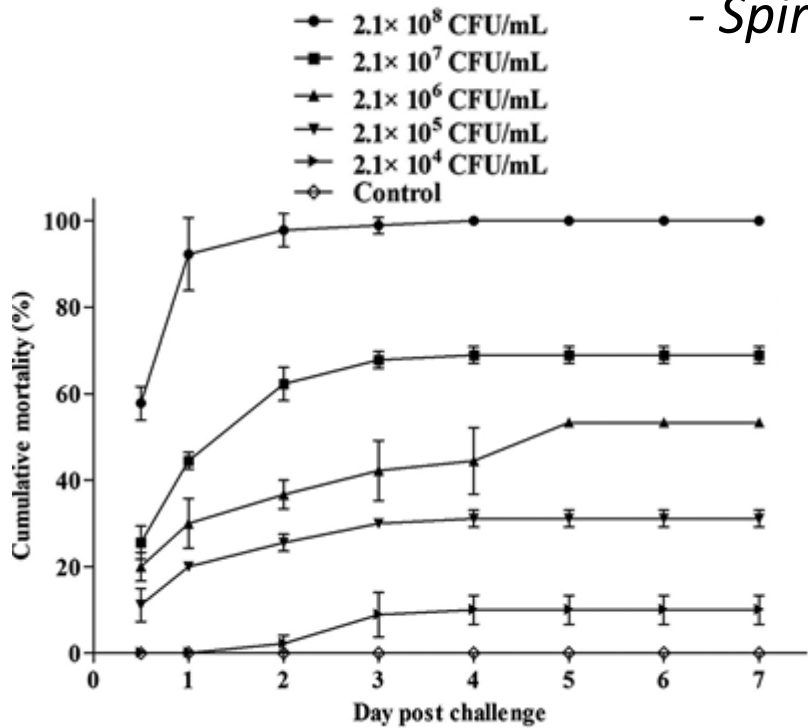
Aeromonas hydrophila

Artificial challenge showed *A. hydrophila* was pathogenic to *M. nipponense*, and the LD50 was 9.58×10^4 CFU/ml.

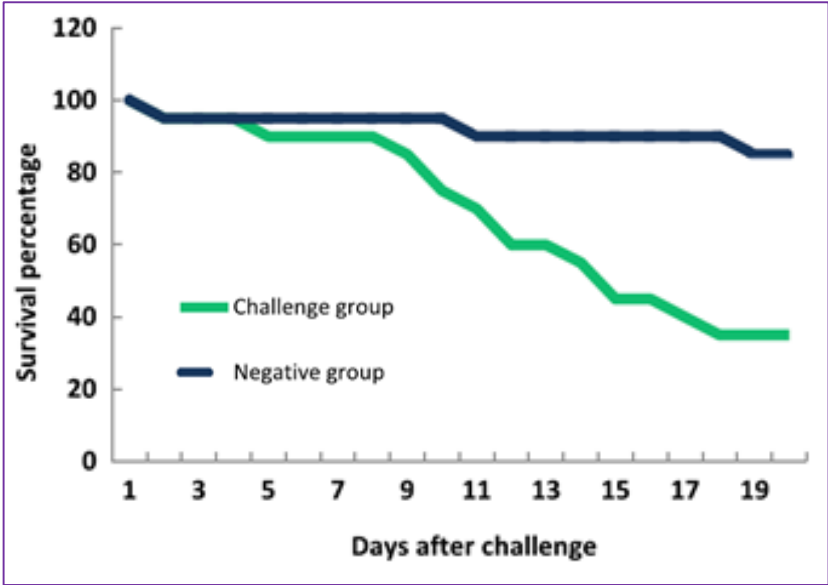
Carrying many virulence factors



A. hydrophila infection induced a wide defensive response of various immune related genes in *M. nipponense*.

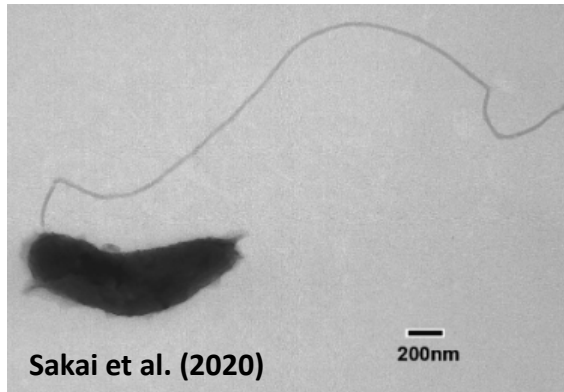


Challenge test against *A. veronii*



Challenge test against *S. mirum*

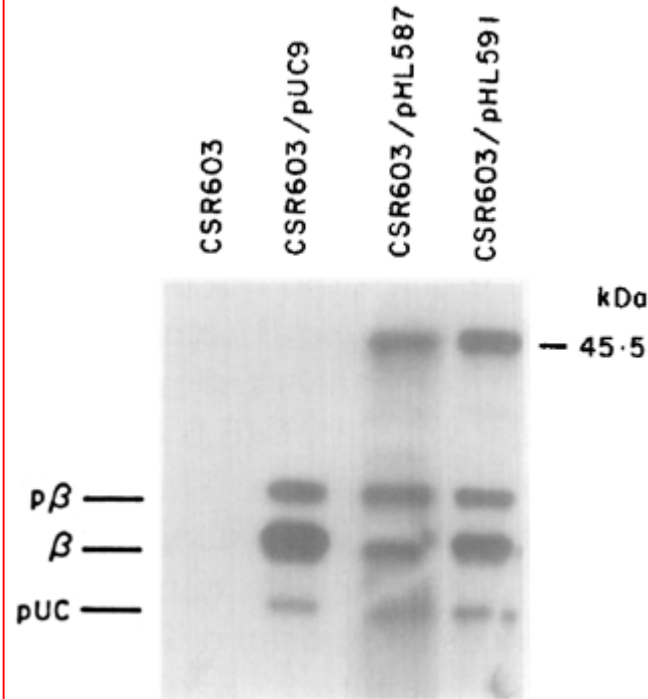
Common pathogenic bacteria in shrimp and potential marker



3125 publications

- *M. nipponense* : 34
- *M. japonicus* : 163
- *P. stylirostris* : 56
- *P. vanamei* : 1611

- Thermolabile hemolysin/*tlh* gene



- *tlh* gene contains an open reading frame of 1254 bp
- encoding protein (45.5 - 47 kDa)
- type II toxins: bacterial toxins that enzymatically damage the membrane
- heat labile (60°C for 10 min)
- G + C content = 47.6%

Taniguchi et al (1985) Journal Bacteriology, Taniguchi et al (1986) Microbial Pathogenesis, Wang et al (2012) Applied and Environmental Microbiology

TEST

Species	N	Gene accession numbers
<i>V. alginolyticus</i>	10	AGV19473, KIP72091, KLE26685, KLI71763, KOE03516, KOF24009, KPM93651, KPM96737, KPN04843, KTL56021
<i>V. antiquarius</i>	1	ACY53462
<i>V. campbellii</i>	2	AGU98041, EDL71063
<i>V. harveyi</i>	6	KIP65897, KIP77547, KNY43153, KNY48418, KTL39521, ODM56754
<i>V. natrie gens</i>	2	ANQ28223, EPM40804
<i>V. orientalis</i>	1	EEX93954
<i>V. owensii</i>	2	KIF44798, KIF51167
<i>V. parahaemolyticus</i>	71	AGQ93004, AGQ95930, AHJ01340, EFO35936, EFO46792, EFO52510, EQL99881, EQM15417, ETJ88037, EXJ30818, EXJ36541, EXJ45360, EXJ48645, KED26846, KED36275, KED40997, KED44804, KED49911, KED57653, KED66349, KED71956, KED95115, KEE21608, KEE45839, KEE60884, KEE64101, KFE93453, KHF08150, KHF09374, KHF12589, KHF18643, KIT21856, KIT26104, KIT36398, KIT40627, KIT46850, KIT56016, KJR19456, KJR20738, KJR31817, KJR39447, KKC83205, KKC87402, KKI11446, KKK60399, KKK73970, KKZ00601, KKZ08323, KLI86855, KOE01700, KOE75107, KOE84596, KOE86497, KOE95525, KOF13170, KOF25244, KOF37375, KOF48234, KOF51823, KOH09623, KON50554, KON52705, KOY22167, KOY32956, KOY37432, KOY37899, KOY45746, OAR48863, OAR64409, OAR69957, OEB23578
<i>Vibrio</i> sp	9	EKM19589, EKM27302, EKM30036, GAJ70087, GAJ74761, GAK15559, GAK22353, KFJ86910, KJQ90198
<i>V. xuii</i>	1	KOO15794