GHENT
UNIVERSITY

FACULTY OF BIOSCIENCE ENGINEERING

DEPARTMENT OF ANIMAL PRODUCTION

LABORATORY OF AQUACULTURE & ARTEMIA REFERENCE CENTER

Impact of prebiotics and probiotics on animal health and production

Peter Bossier and Collaborators

Ghent University

GHENT UNIVERSITY

Artemia Reference Center

XIX CONGRESO ECUATORIANO DE ACUICULTURA

Tecnologías para una producción sustentable

Starting point

Aquaculture target organisms, live in an environment conducive to the proliferation of micro-organisms

Strategies for microbial community management

Controlling microbial numbers:

- Non-selective: hygiene
    - Surface desinfection
    - Reduction of substrate input
    - Internal removal of organic matter

- selective
    - phages

Strategies for microbial community management

Selective enhancement of microbes

- Probiotics
- Prebiotics/probiotics
- Controlling microbial biomass substrate loading :
  - mg C/mg microbial biomass per hour

Strategies for microbial community management

Manipulation of microbial activity

- **Quorum sensing interference**
- **Stress hormones (?)**

Strategies for microbial community management

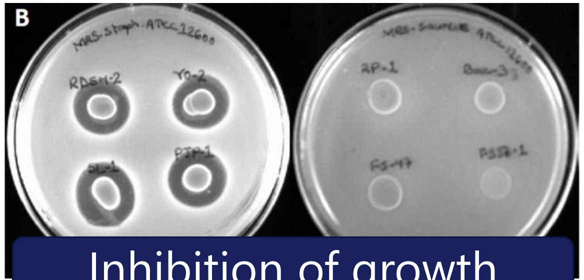
Enhancing host response

- **Immunostimulants**
- **Vaccine**
- **Broodstock conditioning (heat shock proteins)**

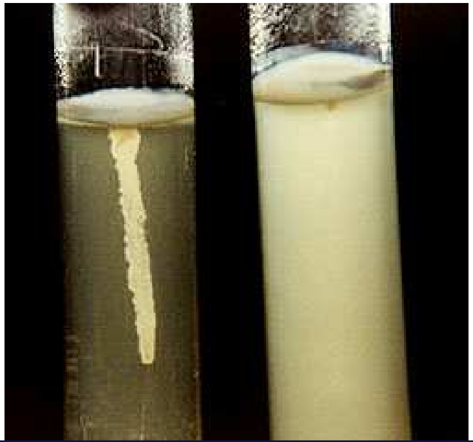
Strategies:

- Probiotics (i.e. Bacillus)
- Prebiotics
- Vaccines
- Immunostimulants
- Bacteriophages (lytic)
- Quorum sensing disruption
- microbial community composition management
- Poly-β-hydroxybutyrate
- Broodstock conditioning?

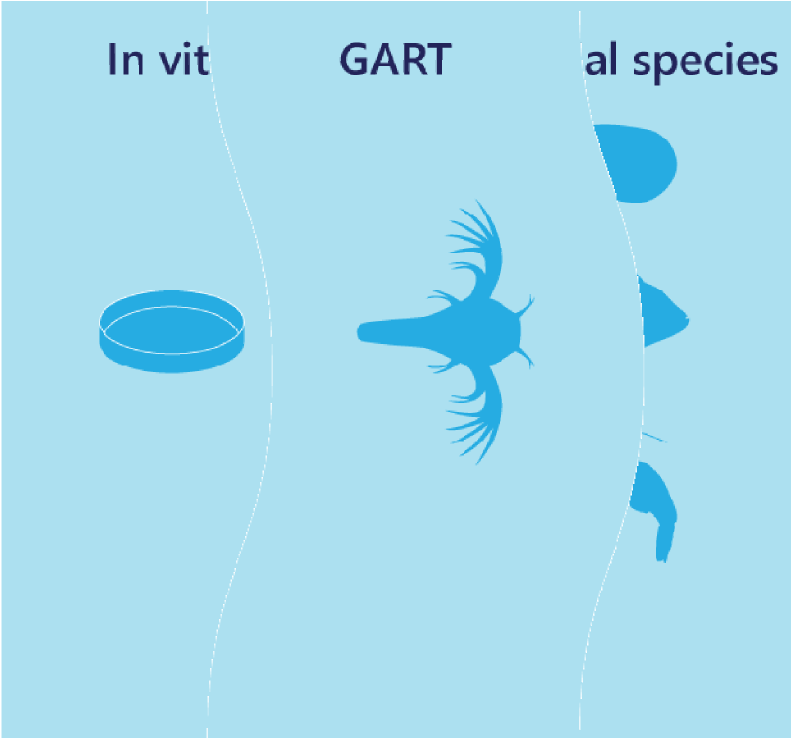
Development of MCM control strategies



Inhibition of growth



Virulence factor activity



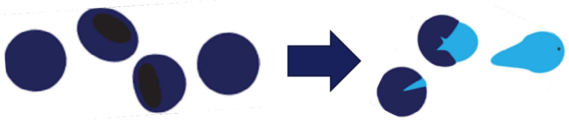
Intermediate step:
GART, the <u>gnotobiotic *Artemia*</u> screening platform

Under real production conditions: trial and error, uncontrolled, risk for production

Under laboratory conditions: no risk for production, more control, yet labour intensive

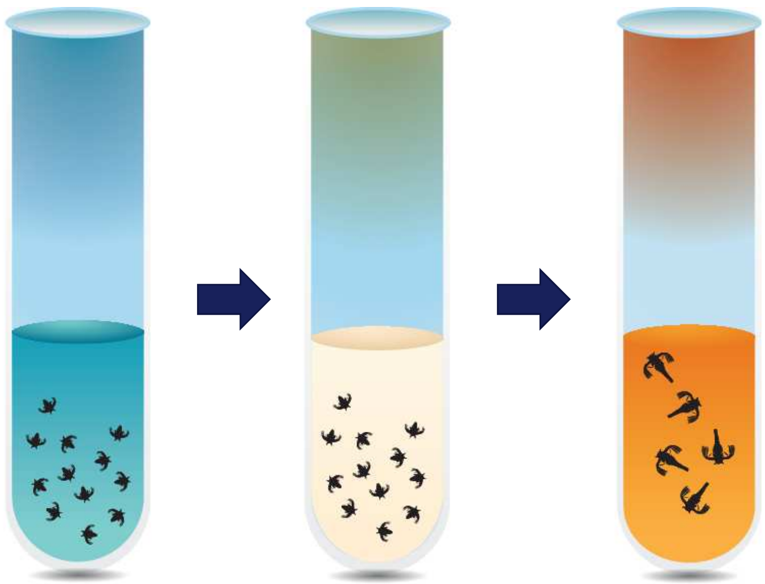
Gnotobiotic *Artemia* screening platform: pathogenic challenge

1 Decapsulation and disinfection of cysts to remove all bacteria



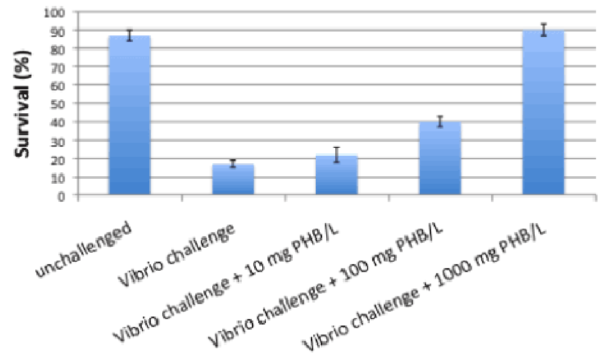
2 Hatching *Artemia* nauplii in sterile seawater

3 Stocking of sterile *Artemia* nauplii in sterile seawater



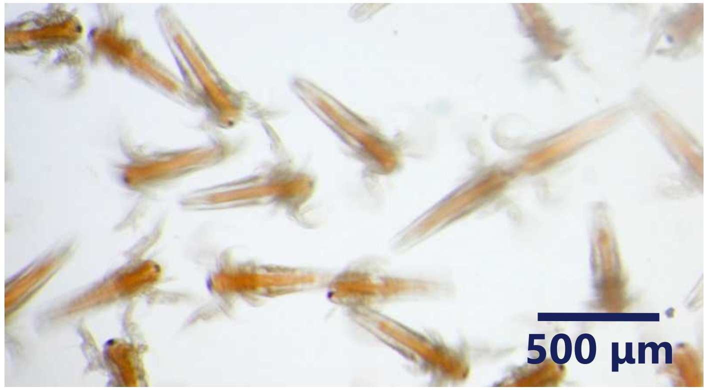
4 Treatment and/or pathogenic challenge (*V. harveyi;V. campbellii;...*)

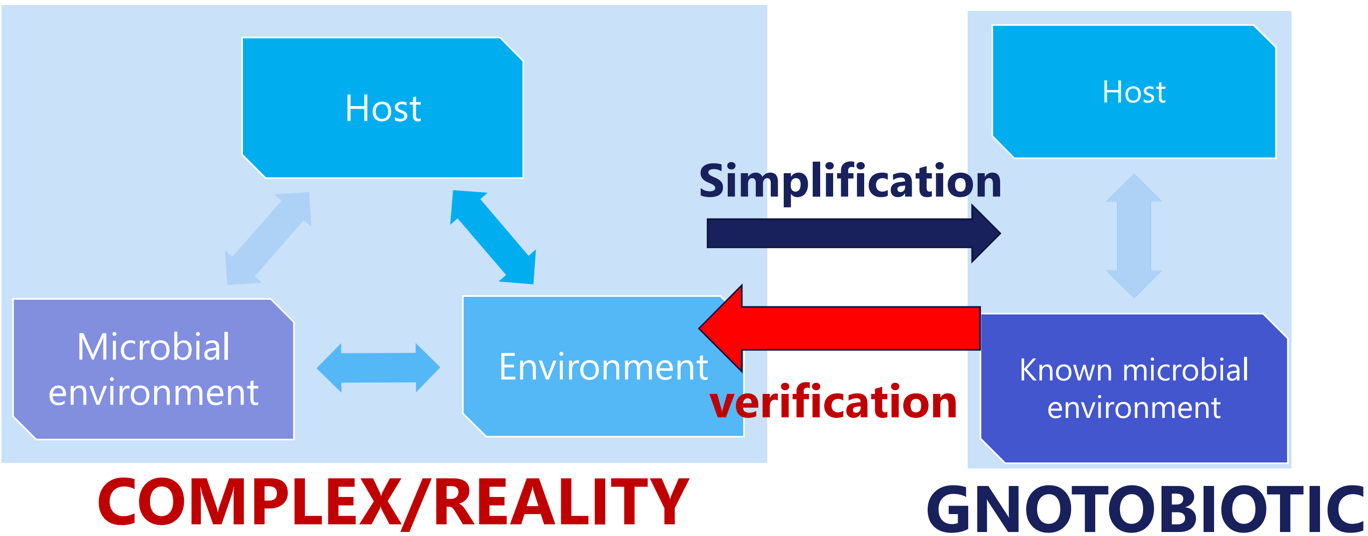
5 Count surviving nauplii; growth; activity;... after 24-48h

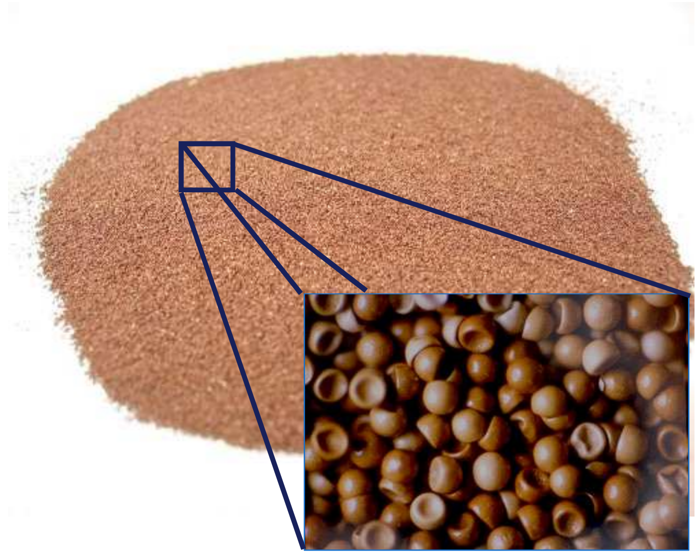


Gnotobiotic *Artemia* screening platform

- Small scale (ml)
- Continuous supply
- High throughput (48-72h)
- Highly controllable
- Realistic response







Ghent University: Artemia genome on the ORCAE platform

Gnotobiotic *Artemia* screening platform:

STUDYING INTRAKINGDOM SIGNALING: QUORUM SENSING

1. **What is quorum sensing?**
2. **Quorum sensing and aquaculture**

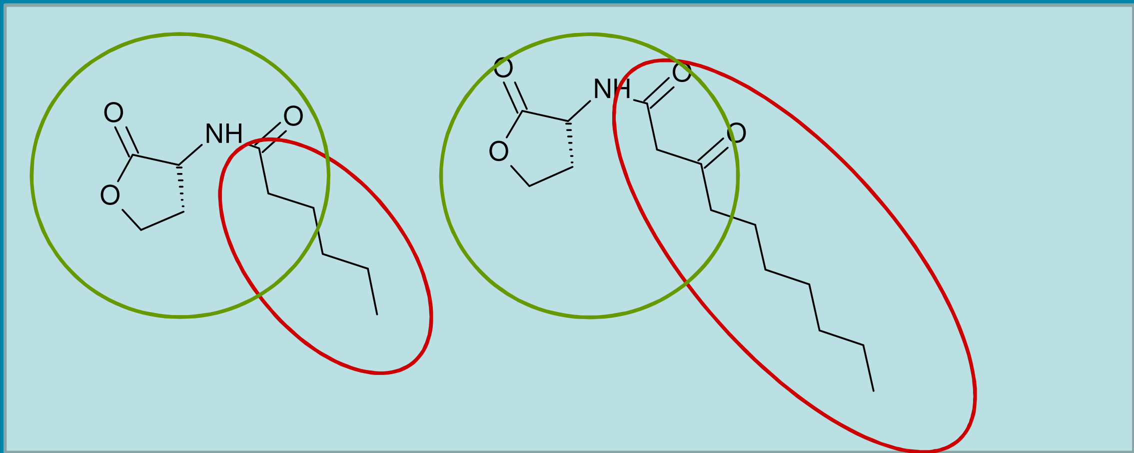
QUORUM SENSING

- Before: bacteria = separate entities

- Now: bacteria sense and respond to environment **and to each other**
  - Extracellular signal molecules
  - ≈ hormones in higher organisms

AHL QUORUM SENSING

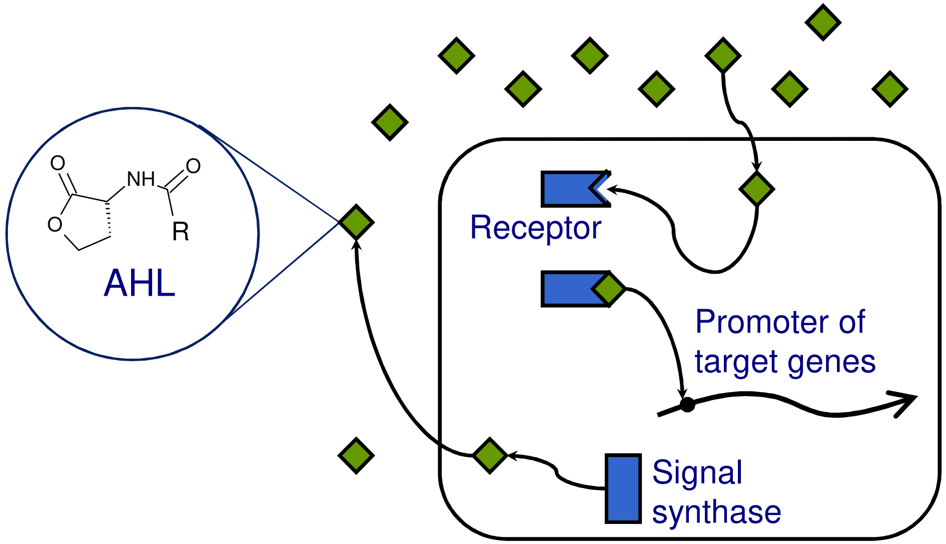
- Homoserine lactone is conserved
- AHLs of different species have <u>different acyl side chains</u>



- <u>Concentrations</u>: liquid culture: micromolar
  biofilm: millimolar ≈ 1 g/L !

QUORUM SENSING SYSTEMS

- Acylhomoserine lactone (AHL) QS:

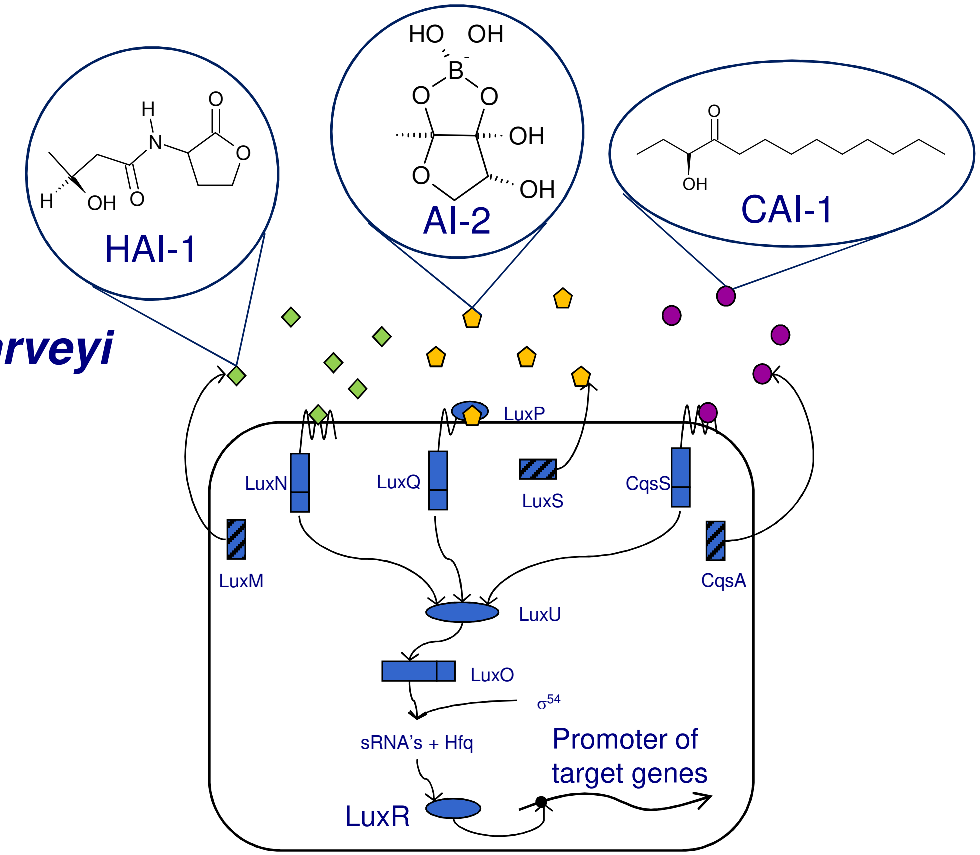


- Found in different aquaculture pathogens:
  - *Aeromonas hydrophila, A. salmonicida*
  - *Edwardsiella tarda*
  - *Yersinia ruckeri*
  - Some vibrios e.g. *V. anguillarum*

QUORUM SENSING SYSTEMS

- QS in vibrios: multi-channel systems:

- Documented in:
  - *V. alginolyticus*
  - *V. anguillarum*
  - ***V. campbellii / V. harveyi***
  - *V. ichthyoenteri*
  - *V. mimicus*
  - *V. parahaemolyticus*
  - *V. salmonicida*
  - *V. scophthalmi*
  - *V. vulnificus*



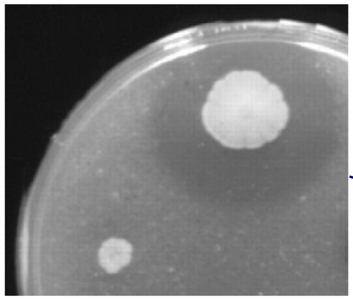
QS-REGULATED PHENOTYPES

- <u>Virulence factors</u>
  - *In vitro* activity assays
  - *In vitro* gene expression (RT realtime PCR)

Quorum sensing

+ Metalloprotease
  "Extracellular toxin"

- Type III secretion system
  Siderophore
  Phospholipase
  Chitinase

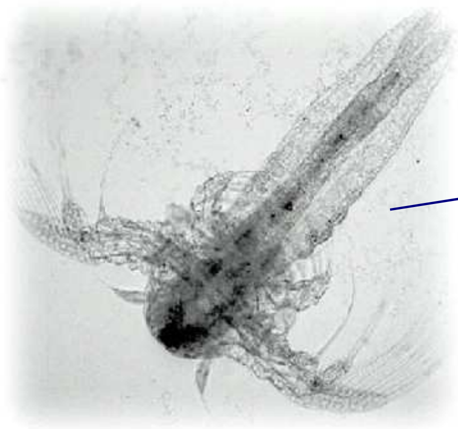


Chitinase assay

More info: Ruwandeepika et al. (2012) Reviews in Aquaculture 4: 59-74

INTERACTION: Vibrio - Artemia

- Brine shrimp (*Artemia*): model organism for shrimp

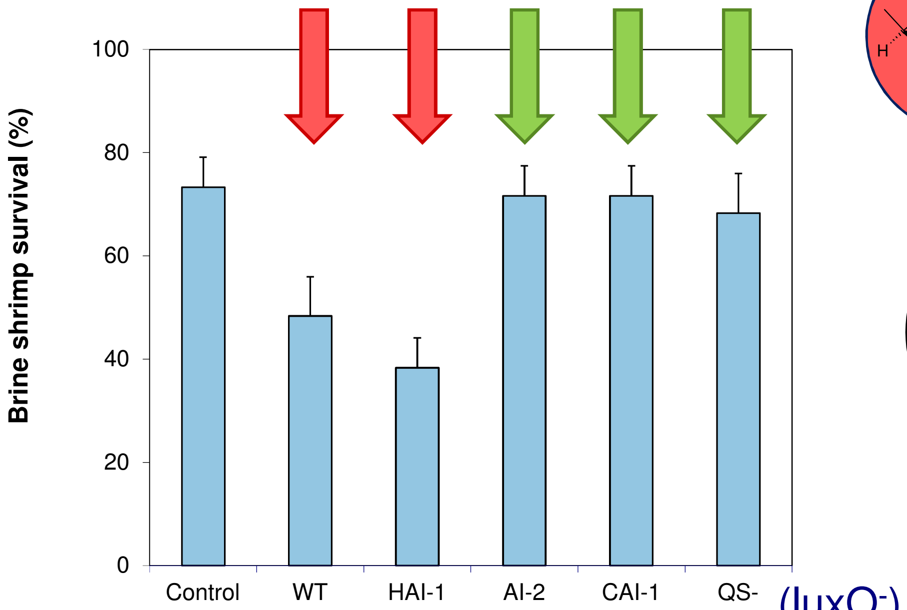


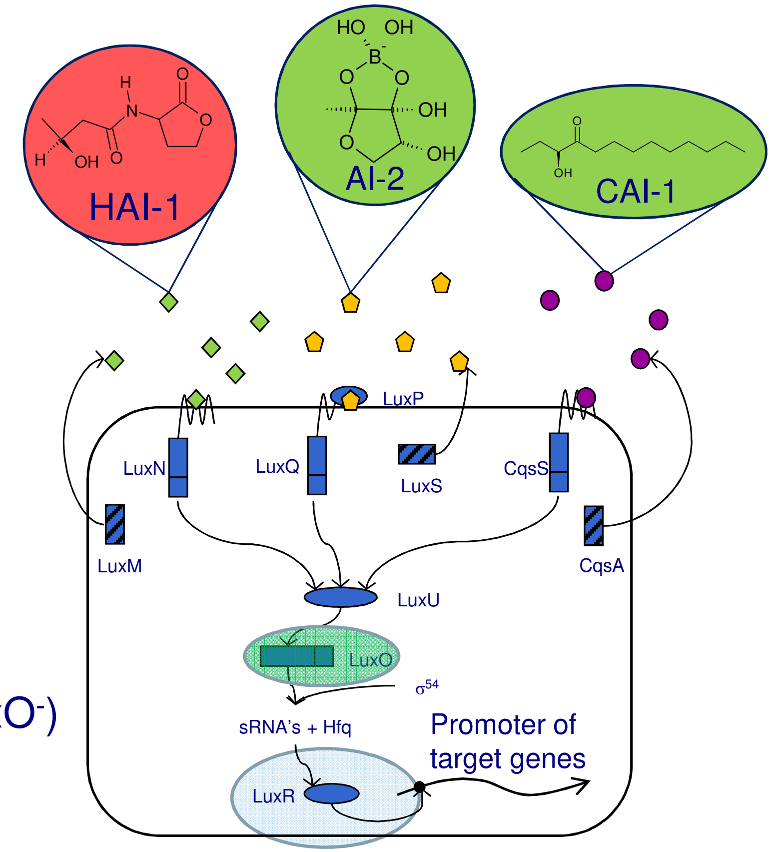
- Creating gnotobiotic cultures starting with axenic larvae (Instar II nauplii)

  → Only bacteria added to cultures are present!

INTERACTION: Vibrio – Artemia

- Effect of *V. harveyi* <u>QS mutants</u> on gnotobiotic brine shrimp survival

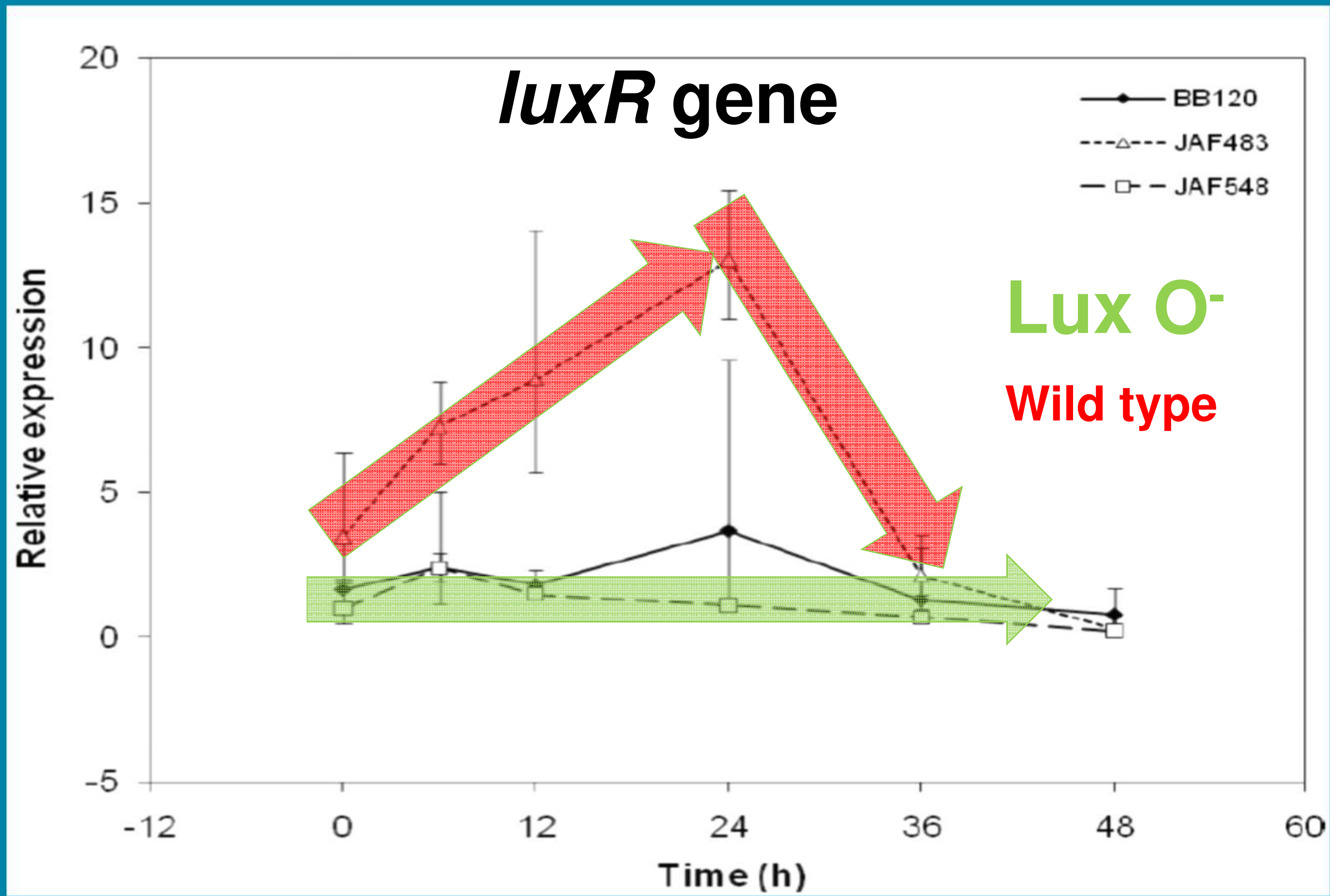




gnoto

Defoirdt et al. (2005) Environ. Microbiol. 7: 1239-1247

In vivo virulence gene expression by Vibrio in Artemia



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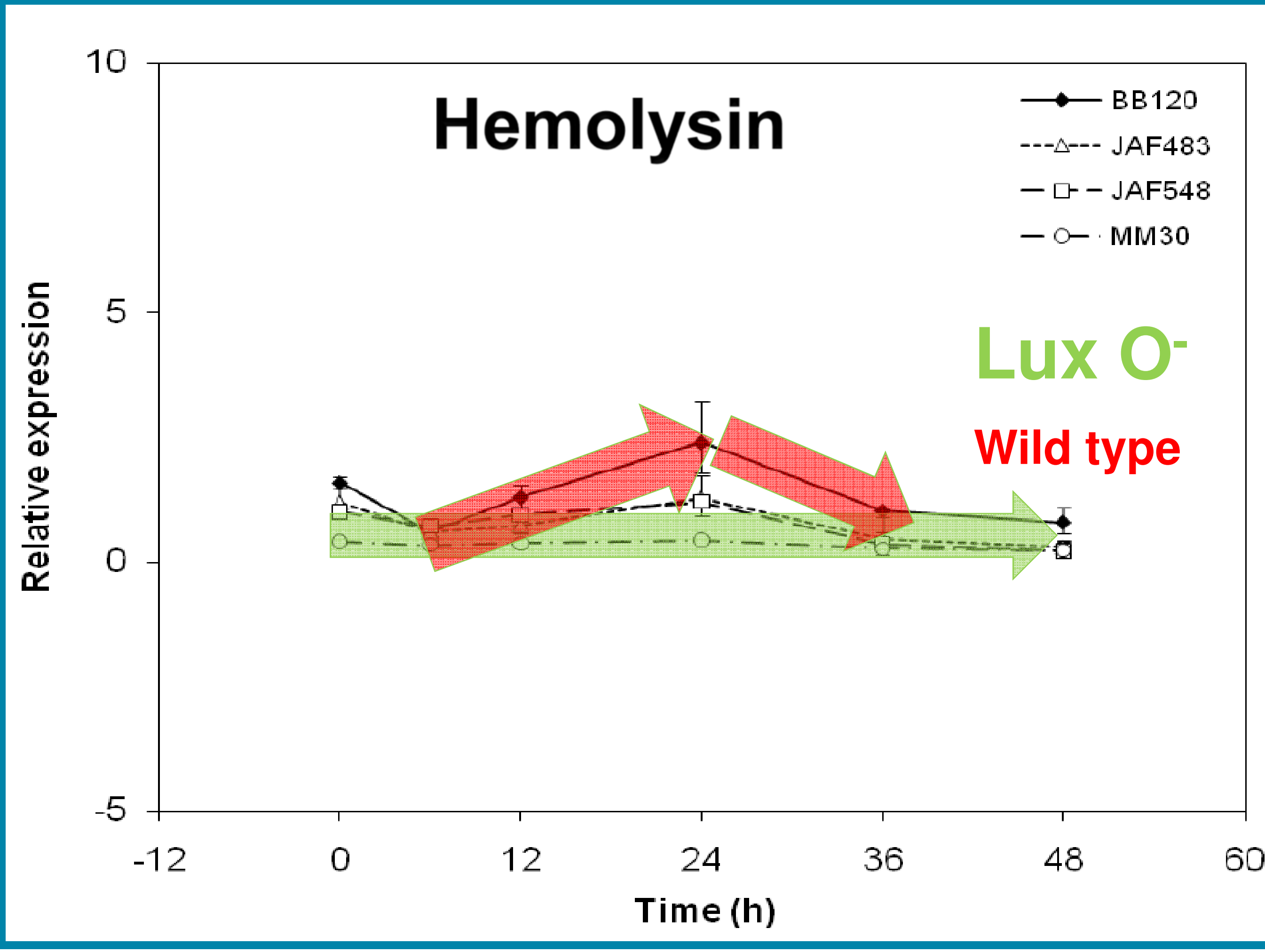
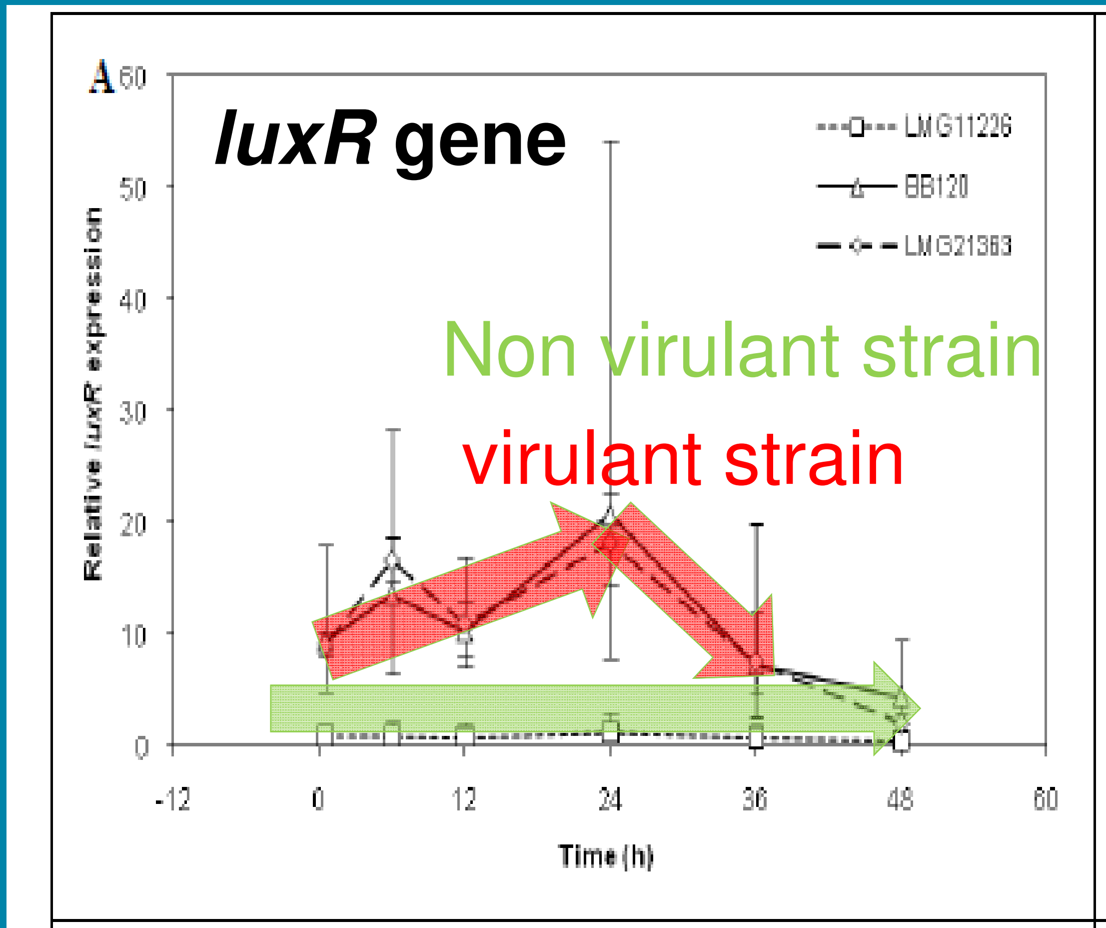
In vivo hemolysin gene expression by Vibrio in Artemia



In vivo virulence gene expression by Vibrio in Artemia



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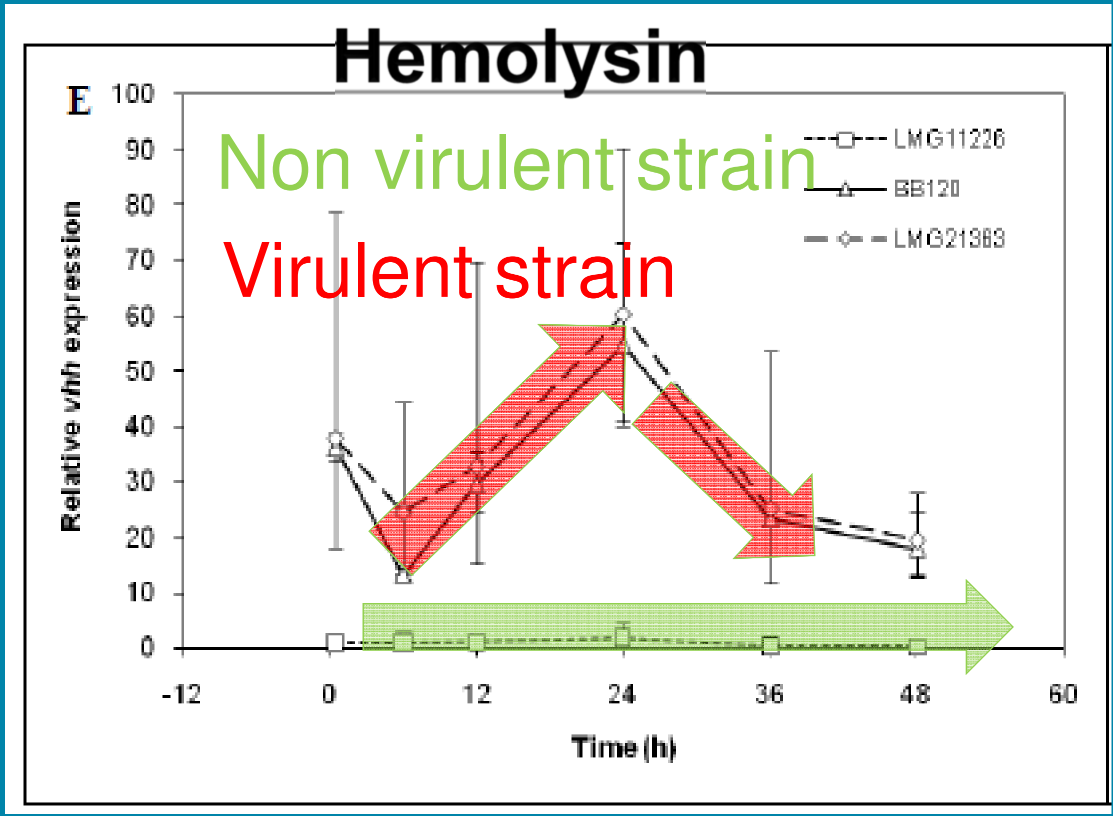
In vivo hemolysin expression by Vibrio in Artemia



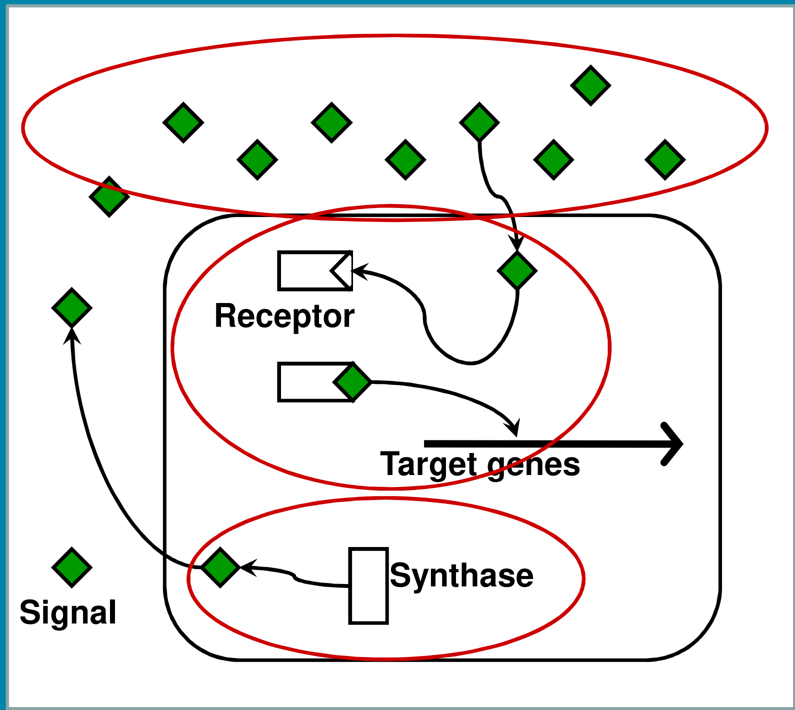
Steering microbial communities activity to the benefit of the host

Quorum quenching

QUORUM QUENCHING (QQ)

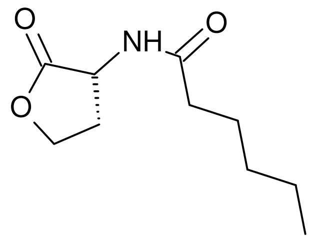
= disruption of quorum sensing

- Possible targets:
  - Signal production
  - Signal molecules
  - Signal detection

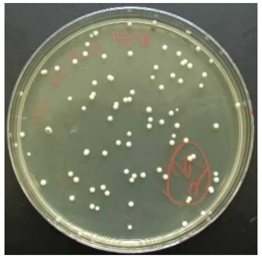


ENZYMATIC SIGNAL MOLECULE DEGRADATION

- Enrichment of AHL degrading bacteria in medium containing AHL as
  - Sole C source (e.g. add NH_4^+ as N source)
  - Sole N source (e.g. add glycerol as C source)
  - Sole C and N source

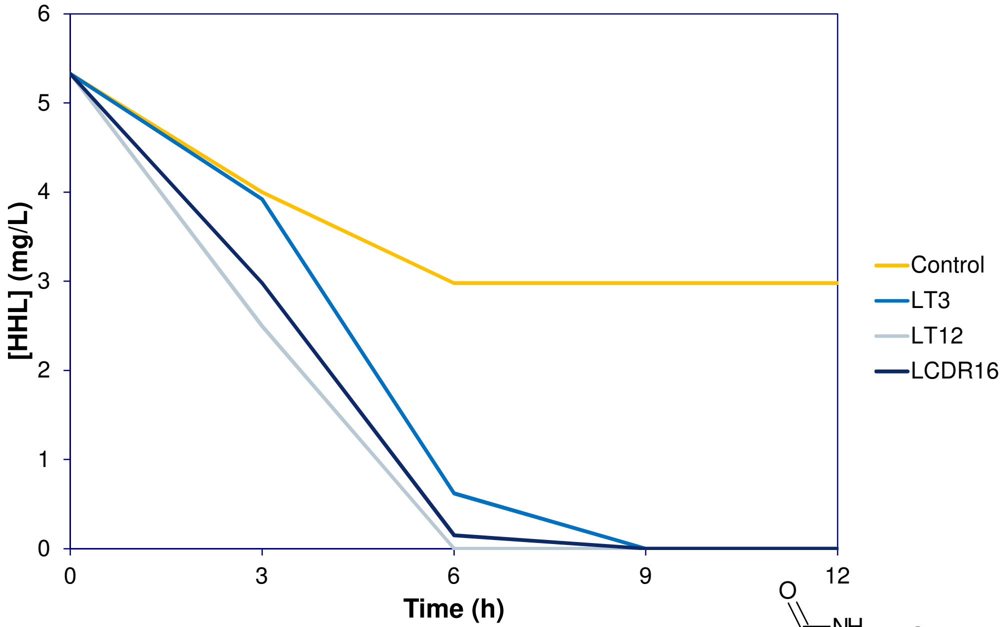


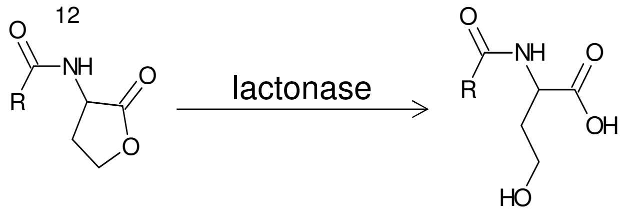
- Isolation of pure strains from the mixed culture by spread-plating and picking of single colonies



ENZYMATIC SIGNAL MOLECULE DEGRADATION

- AHL degradation by *Bacillus* strains LT3, LT12 and LCDR16



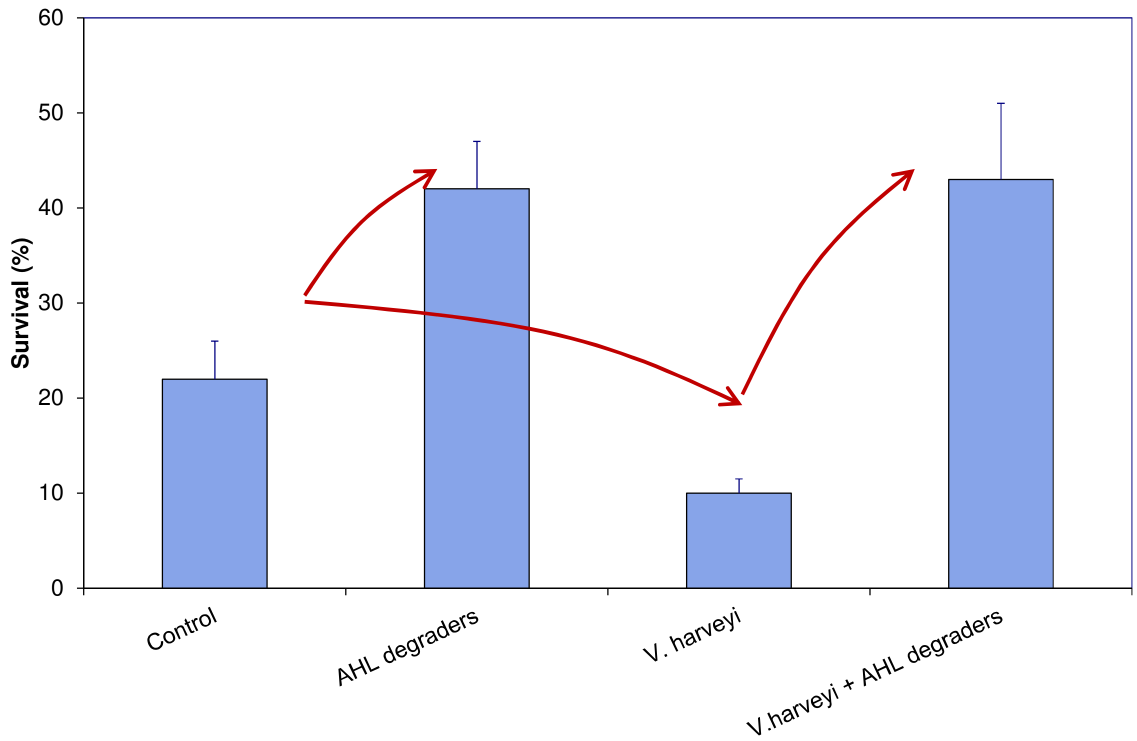


gnoto

Defoirdt et al. (2011) Aquaculture 311: 258-260

ENZYMATIC SIGNAL MOLECULE DEGRADATION

- Use of signal-degrading bacteria as probionts, e.g. in *Macrobrachium* larvae:

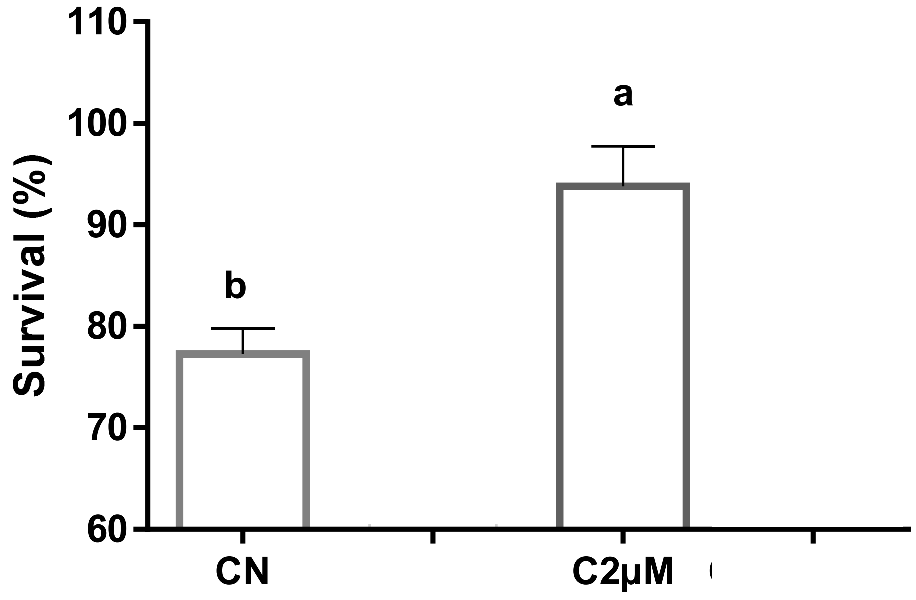


Nhan et al. (2011) J. Appl. Microbiol. 109: 1007-1016

- Effect of cinnamaldehyde on shrimp survival (PL 1 to PL 10)
- QUORUM QUENCHING BY RECEPTOR INTERFERENCE

Collaboration

- Ugent
- Universidad de Camagüey Ignacio Agramonte Loynaz (CUBA)
- Yaguacam shrimp hatchery (CUBA)



Application of cinnamaldehyde at 2 µM 6 h before preharvest

REALITY

CONCLUSIONS: Quorum sensing

- Quorum sensing regulates virulence in different aquatic host-pathogen systems

- Quorum sensing-disrupting agents can be recruited
  - Signal-degrading bacteria isolated from healthy fish, shrimp and algal cultures
  - Antagonists: natural or synthetic (cinnamaldehyde)

  and protect aquatic animals from infection

INTERKINGDOM SIGNALING:

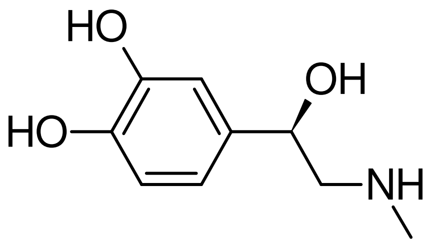
STRESS HORMONES INFLUENCE MICROBIAL ACTIVITY

Interkingdom signaling: stress hormones

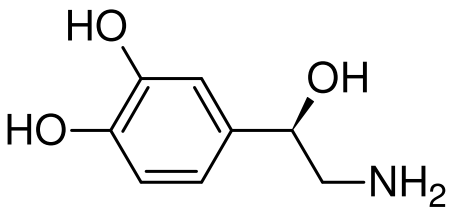
Host stress → increased susceptibility to infection

- Decreased activity of host defense
- Recently: increased virulence of pathogenic bacteria

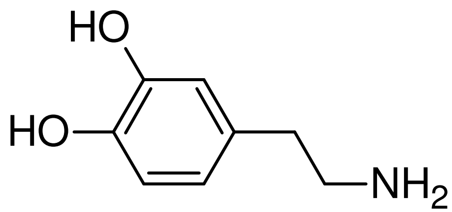
Catecholamines: “fight and flight” stress response



Epinephrine



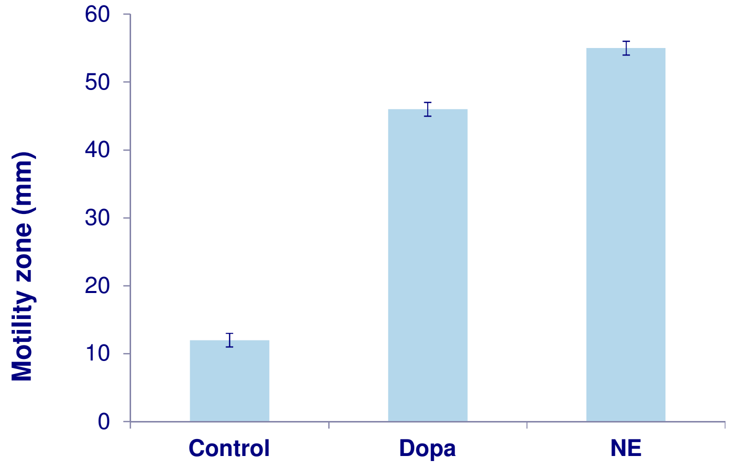
Norepinephrine



Dopamine

Catecholamines: effect on bacterial swimming motility

- **Motility:** important virulence factor
  Helps bacteria to attach to host
- **Flagellum:** rotating "motor"
- **Plate assay:** soft LB12 agar (0.3% agar)



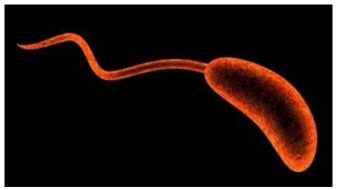
Swimming motility of
***V. harveyi* BB120 on soft agar**

In vitro

Catecholamines: effect on bacterial swimming motility

Strains	Control (mm)	Dopamine 100 µM (mm)
BB120 (V harveyi)	11.7 ± 0.6[a]	46.0 ± 1.0[b]
LMG21363 (V campbellii)	26.0 ± 1.0[a]	45.0 ± 2.6[b]
HI610 (V anguillarum)	22.3 ± 1.2[a]	42.0 ± 1.0[b]
NB10 (V anguillarum)	29.7 ± 0.6[a]	51.0 ± 1.0[b]

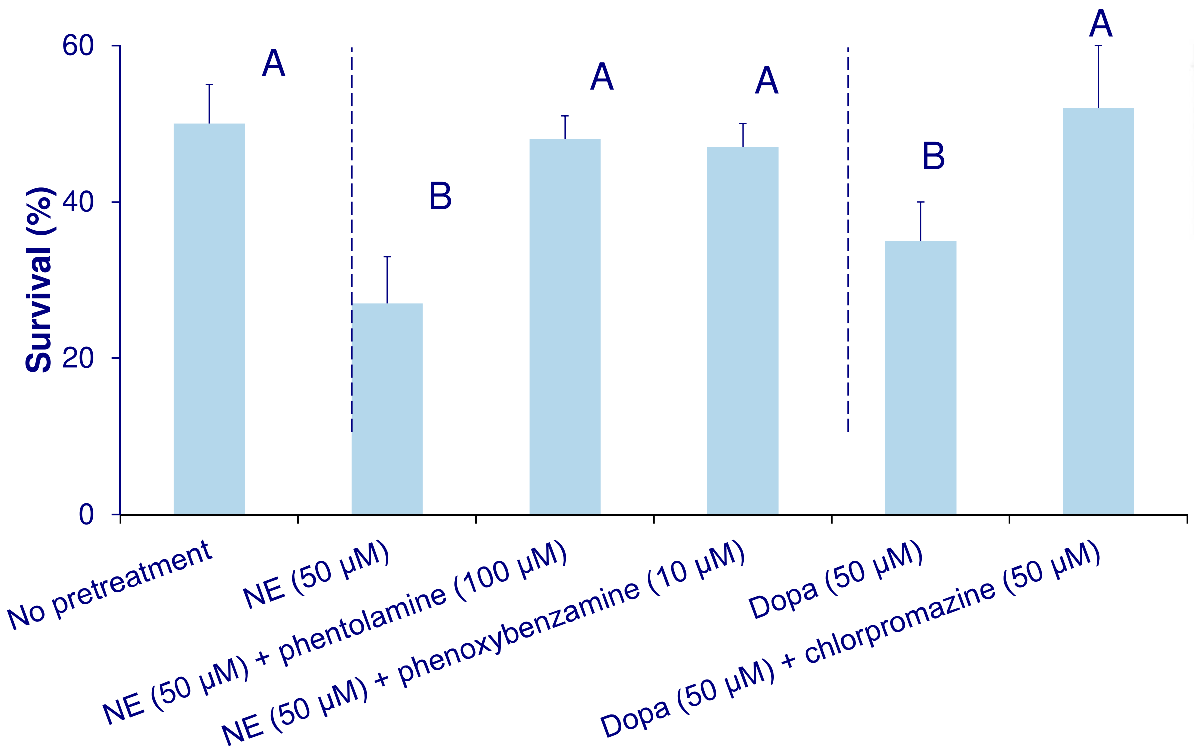
In vitro

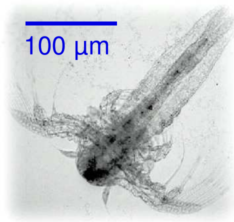


Impact on survival of brine shrimp

- Gnotobiotic brine shrimp: only *V. campbellii* present
- *V. campbellii* pretreated prior to inoculation

In vivo





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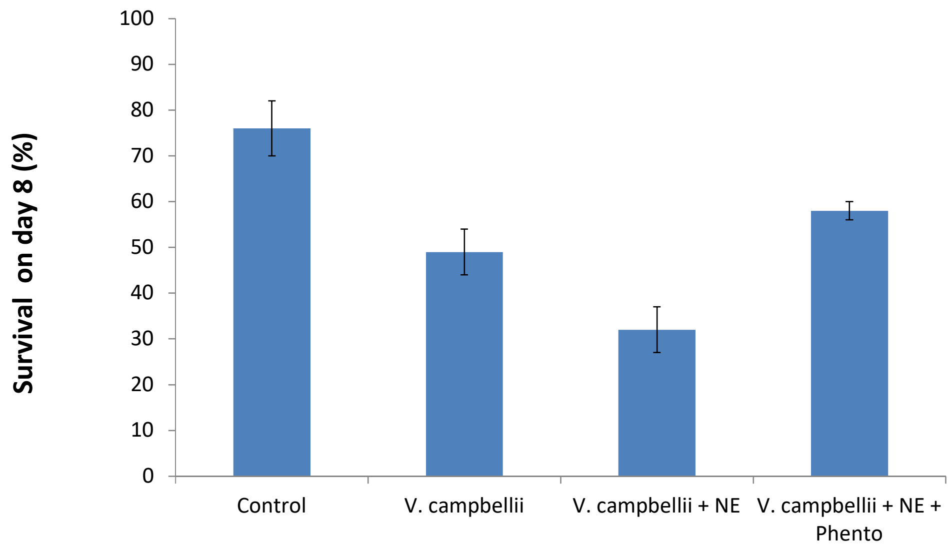
Catecholamines and infection

- Impact catecholamines on survival of prawn larvae
  - Pre-treatment of pathogen
  - Catecholamines washed away prior to challenge test



REALITY

CONCLUSIONS: stress hormones

- Aquatic pathogens such as Vibrio regulate phenotype in response to stress hormones: mobility *in vitro*

- Pathogen pretreatment with stress hormones has a negative influence on larval survival

OVERALL CONCLUSIONS

- Intrakingdom and interkingdom signaling is of importance in host microbial interactions for aquatic larvae
- Strategies influencing microbial community activity, specifically interfering with all these mechanisms, can be designed to the benefit of the host

MCM: selective enhancement

- probiotic
- prebiotics

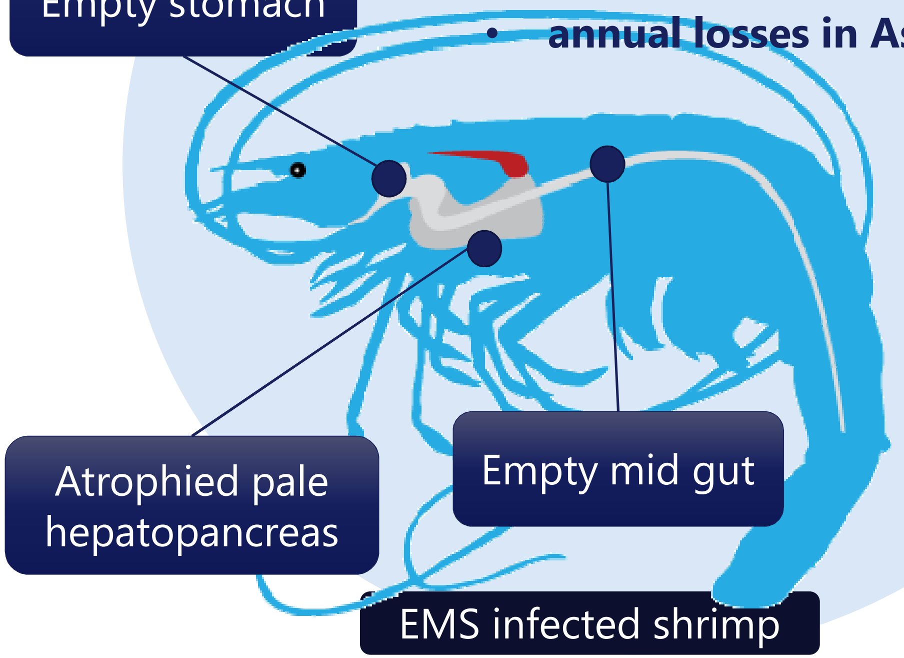
Use of gnotobiotic *Artemia* in Early Mortality Syndrome research

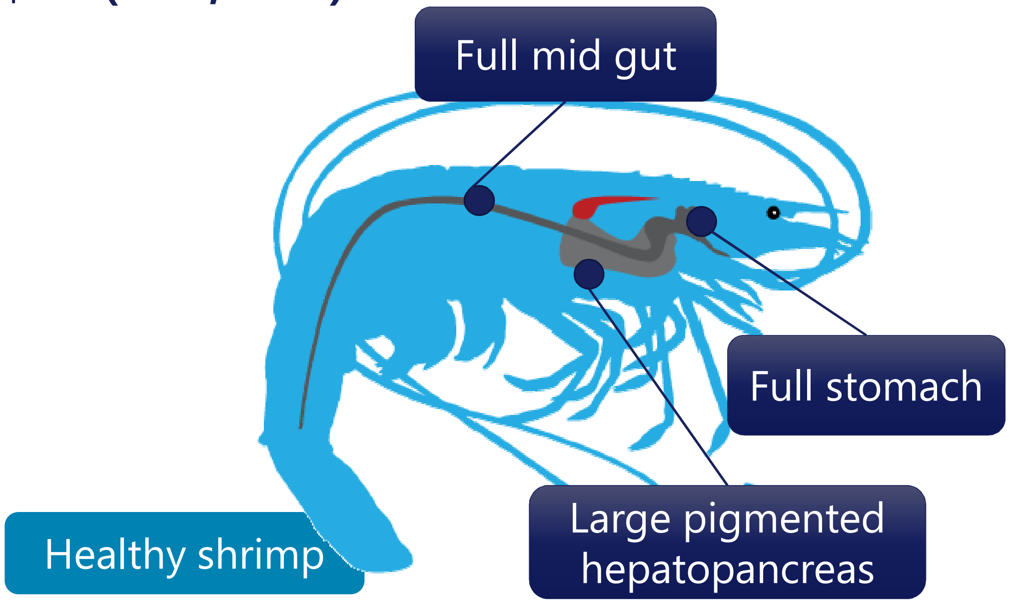
AHPND/EMS
on a global scale

Use of gnotobiotic *Artemia* in Early Mortality Syndrome research

Characteristics

- shrimp post-larvae
- 10 - 30 days after stocking in grow-out pond
- causes up to 100 % mortality
- annual losses in Asia: > 1 billion $ US (GAA, 2013)





Treatments against AHPND/EMS

*"AHPNS has a bacterial etiology and Koch's Postulates have been satisfied in laboratory challenge studies with the isolate, which has been identified as a member of the Vibrio harveyi clade, most closely related to **V. parahaemolyticus**"*

- *V. parahaemolyticus* is a normal member of the microbial community in aquaculture systems hence difficult to avoid.

- Application of "traditional" sustainable strategies may contribute in preventing the disease:
  - Probiotics/Prebiotics
  - Immunostimulation
  - Quorum sensing disruption
  - …

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DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org
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Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp

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[1]Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, Department of Veterinary Science and Microbiology, and [2]Department of Soil, Water and Environmental Science, University of Arizona, Tucson, Arizona 85721, USA

ABSTRACT: A new emerging disease in shrimp, first reported in 2009, was initially named early mortality syndrome (EMS). In 2011, a more descriptive name for the acute phase of the disease was proposed as acute hepatopancreatic necrosis syndrome (AHPNS). Affecting both Pacific white shrimp *Penaeus vannamei* and black tiger shrimp *P. monodon*, the disease has caused significant losses in Southeast Asian shrimp farms. AHPNS was first classified as idiopathic because no specific causative agent had been identified. However, in early 2013, the Aquaculture Pathology Laboratory at the University of Arizona was able to isolate the causative agent of AHPNS in pure culture. Immersion challenge tests were employed for infectivity studies, which induced 100% mortality with typical AHPNS pathology to experimental shrimp exposed to the pathogenic agent. Subsequent histological analyses showed that AHPNS lesions were experimentally induced in the laboratory and were identical to those found in AHPNS-infected shrimp samples collected from the endemic areas. Bacterial isolation from the experimentally infected shrimp enabled recovery of the same bacterial colony type found in field samples. In 3 separate immersion tests, using the recovered isolate from the AHPNS-positive shrimp, the same AHPNS pathology was reproduced in experimental shrimp with consistent results. Hence, AHPNS has a bacterial etiology and Koch's Postulates have been satisfied in laboratory challenge studies with the isolate, which has been identified as a member of the *Vibrio harveyi* clade, most closely related to *V. parahemolyticus*.

OPEN ACCESS Freely available online
PLOS | PATHOGENS
Opinion

Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming?

Peter De Schryver*, Tom Defoirdt, Patrick Sorgeloos

Laboratory of Aquaculture & *Artemia* Reference Center, Department of Animal Production, Ghent University, Gent, Belgium

A recent disease of farmed Penaeid shrimp, commonly referred to as "early mortality syndrome" (EMS) or more technically known as "acute hepatopancreatic necrosis disease" (AHPND), was first reported in southern China in 2010 and subsequently in Vietnam, Thailand, and Malaysia [1]. The EMS/AHPND disease typically affects shrimp postlarvae and impoverished microbial community (and a consequent lack of competition) favors fast-growing bacteria (such as many pathogenic *Vibrio* spp.) in recolonizing the environment [4]. Considering that EMS/AHPND most probably is caused by a *Vibrio*, this practice is thus more likely to stimulate proliferation of the EMS/AHPND-causing agent in the pond than related with a lowered incidence of EMS/AHPND in practice. Greenwater systems (in contrast to clear water systems) are characterized by a mature micro-algal and bacterial community and have been shown before to result in decreased *Vibrio* levels and decreased animal mortality [9,10]. Several mechanisms have been linked to the beneficial effect of green-

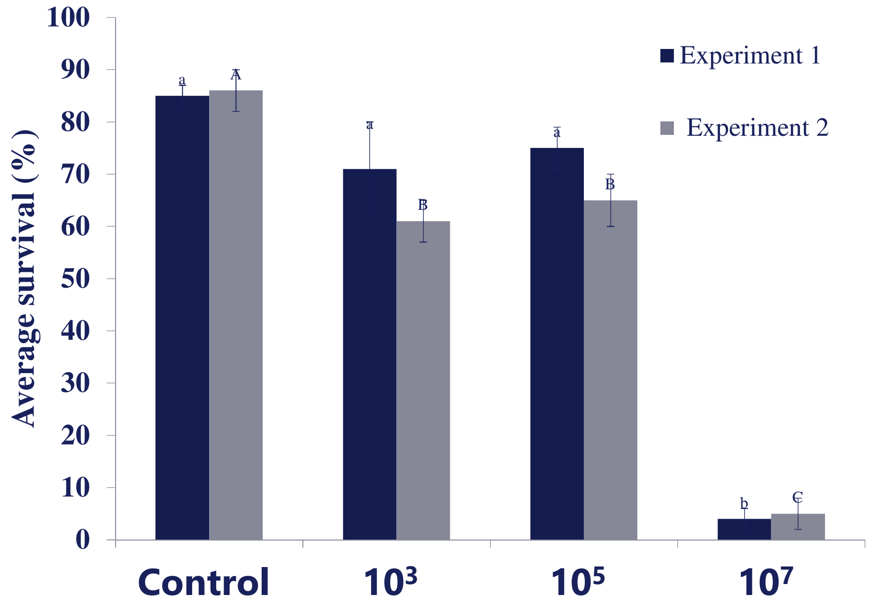
Using the gnotobiotic *Artemia* platform in AHPND/EMS research

1 Finding a representative pathogen: isolates obtained from diseased shrimp with AHPND/EMS symptoms:

Isolate	Origin (year)	Confirmed for Koch's postulates	AP2/AP3 positive	topA sequence analysis
PV1	China (2010)	✓	✓	*V. parahaemolyticus*
TW01	Thailand (2013)	✓	✓	*V. parahaemolyticus*
KM	Thailand (2013)	✓	✓	*V. parahaemolyticus*
M0605	Mexico (2013)	✓	✓	*V. parahaemolyticus*
M0904	Mexico (2013)	✓	✓	*V. parahaemolyticus*
M0903	Mexico (2013)	✗	✗	*V. parahaemolyticus*

Using the gnotobiotic Artemia platform in AHPND/EMS research

2 Challenge tests using the isolates as pathogens in the model:



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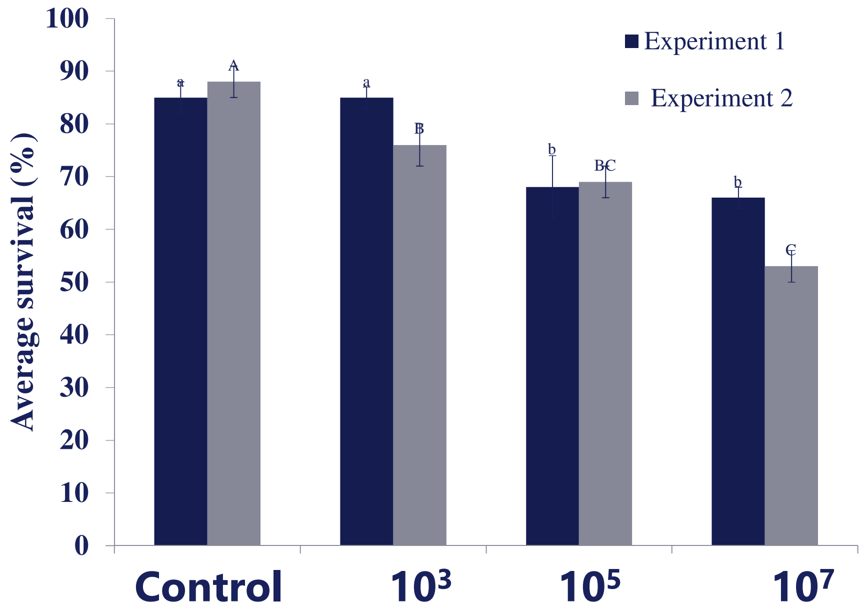

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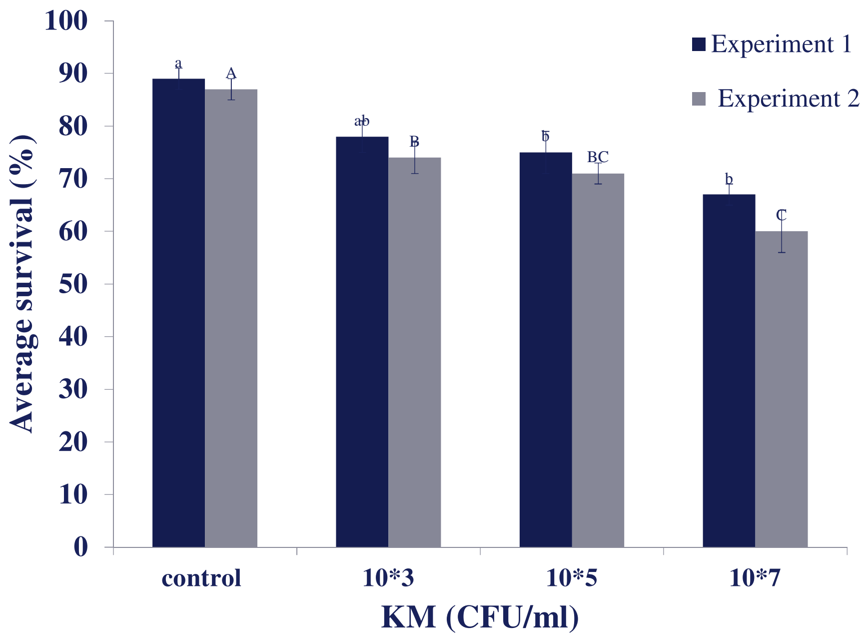
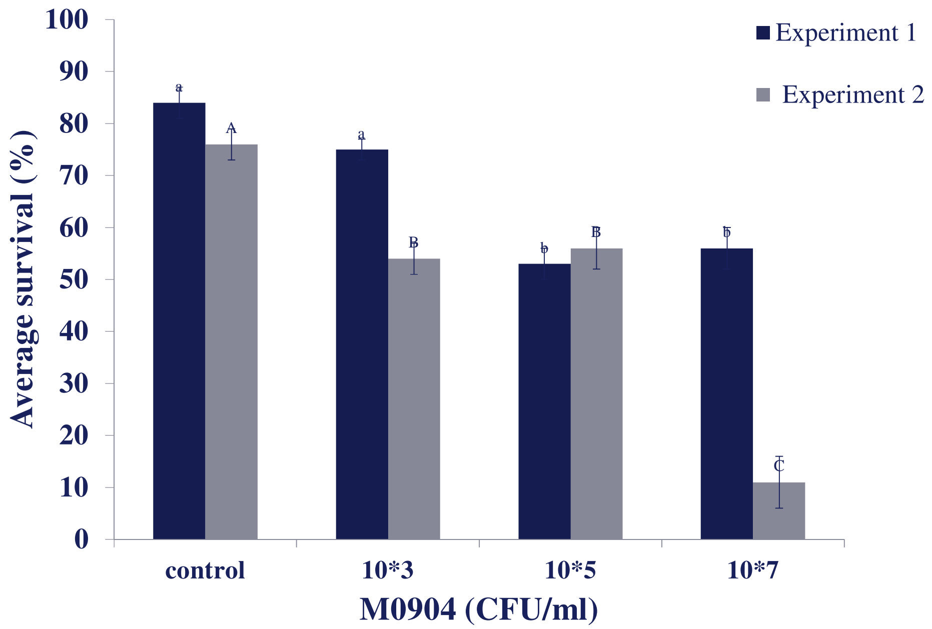
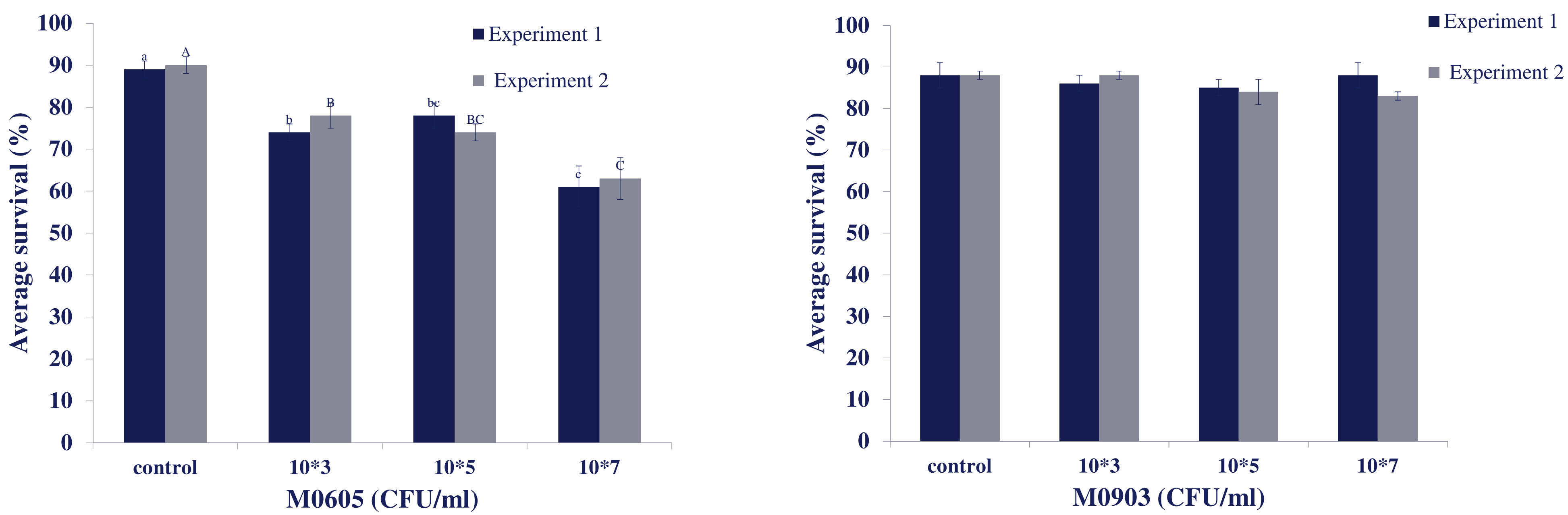




Control **10^3** **10^5** **10^7** **Control** **10^3** **10^5** **10^7**

Using the gnotobiotic Artemia platform in AHPND/EMS research

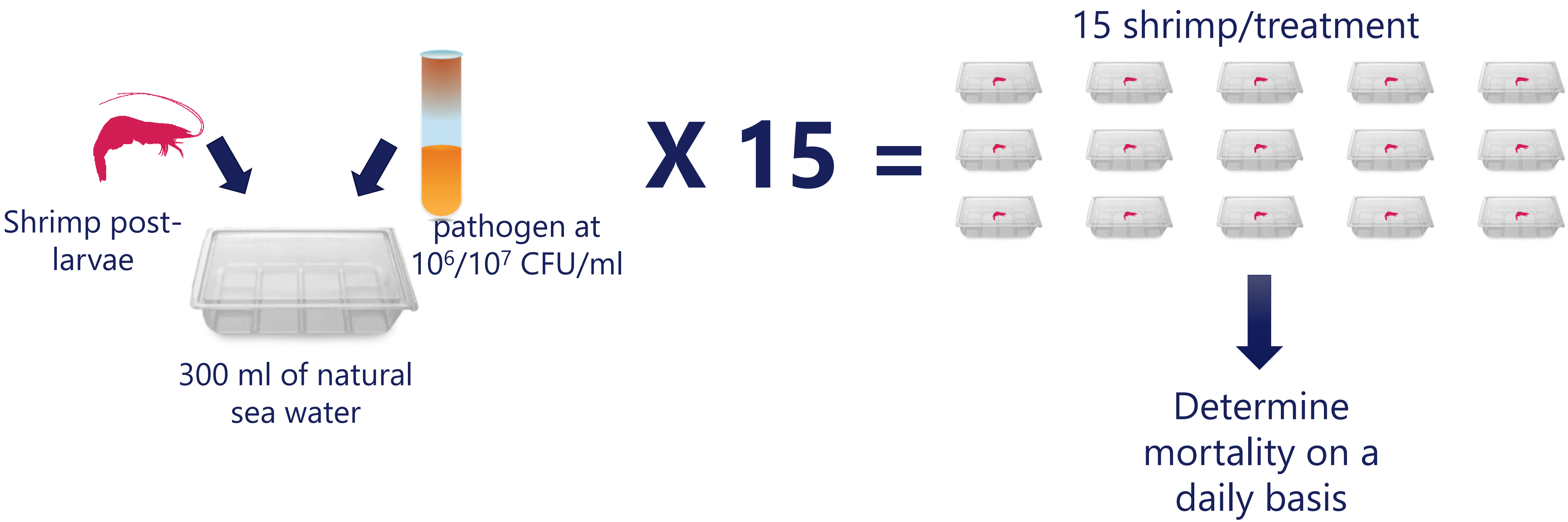
2 Challenge tests using the isolates as pathogens in the model:



Control **10^3** **10^5** **10^7** **Control** **10^3** **10^5** **10^7**

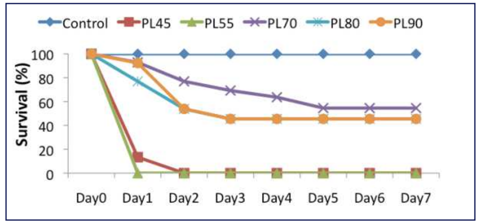
Using the gnotobiotic Artemia platform in AHPND/EMS research

3 Verification of the results in a shrimp model:



Using the gnotobiotic Artemia platform in AHPND/EMS research

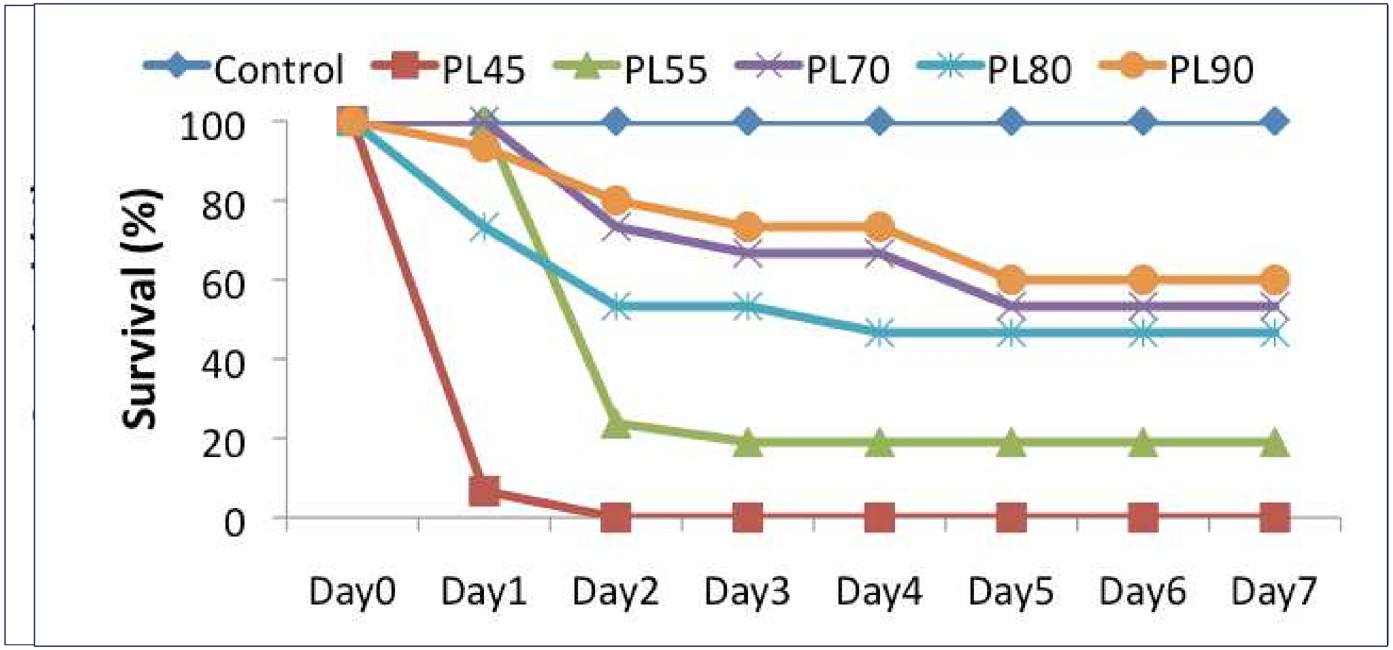
3 Applying the isolates as pathogens in a shrimp model system:



Challenge with EMS-pathogen PV1 at 10^6 CFU/mL

Challenge with EMS-pathogen TW01 at 10^6 CFU/mL

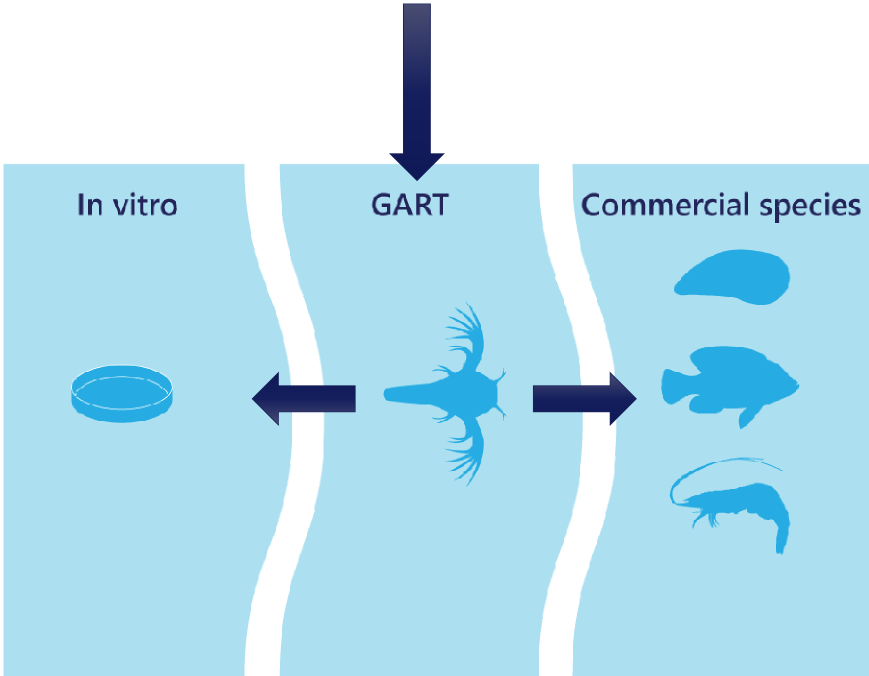
REALITY



Example: screening for probiotics to control AHPHND/EMS

A library of bacilli from culture collections and environmental samples.
Which ones to test on shrimp post-larvae?

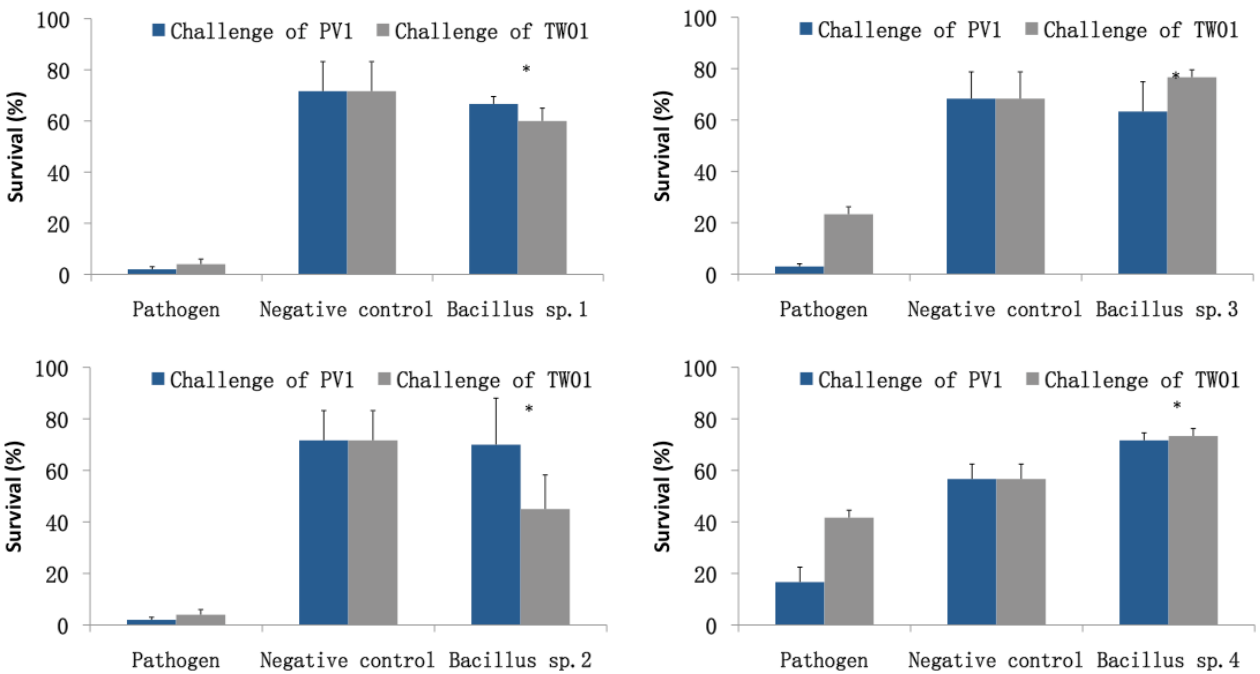
47 Probiotic candidates

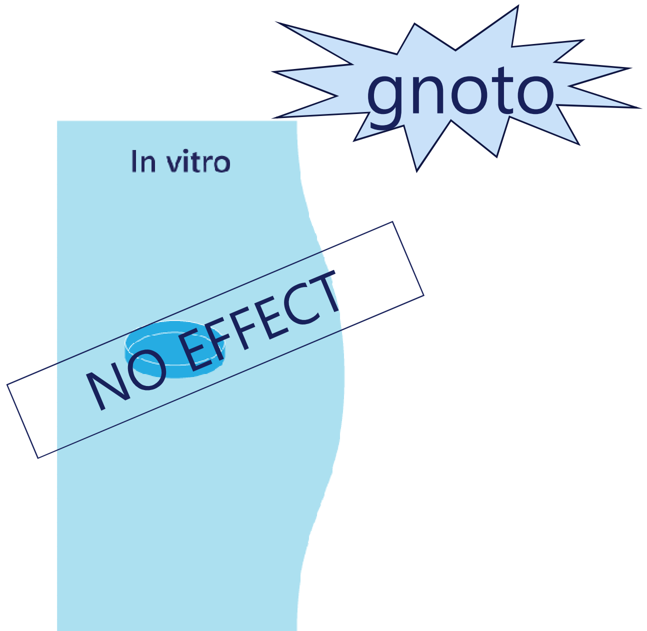


Example: screening for probiotics to control AHPHND/EMS

47 Probiotic candidates

4 most efficient ones against both PV1 and TW01 in the *Artemia* model selected for validation





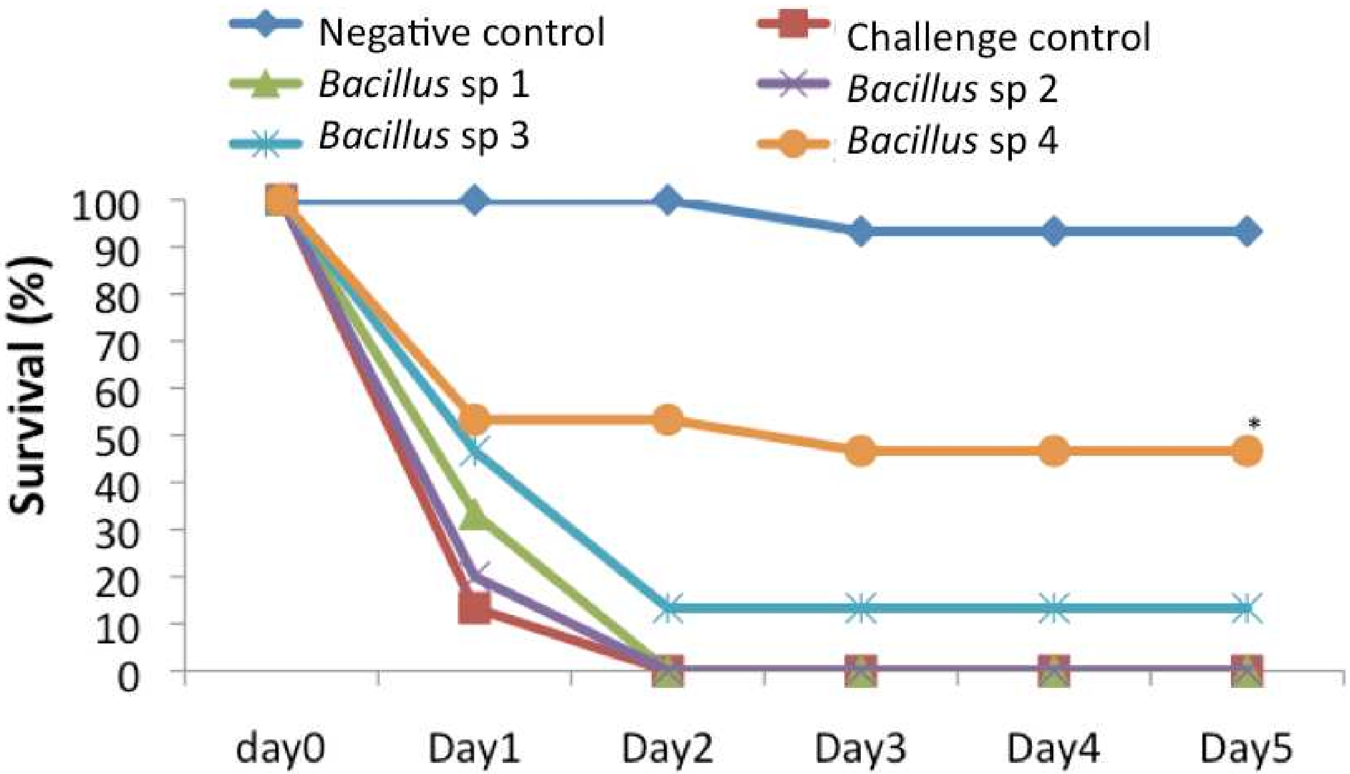
Example: screening for probiotics to control AHPHND/EMS

Validation of the 4 probiotic strains in the shrimp challenge model

Shrimp post-larvae PL44:

1) Treatment with the bacilli at 10^7 CFU/mL

2) Challenge with EMS-pathogen PV1 at 10^6 CFU/mL



Examples of GART based findings

- Identification of probiotics against *V. harveyi* for giant freshwater prawn larviculture

(Thai et al., Applied Microbiology and Biotechnology 2014, 98, 5205-5215)

- Identification of prebiotics against *V. campbellii* for giant tiger prawn larviculture

(Laranja et al. Veterinary Microbiology 2014, 170, 310-317)

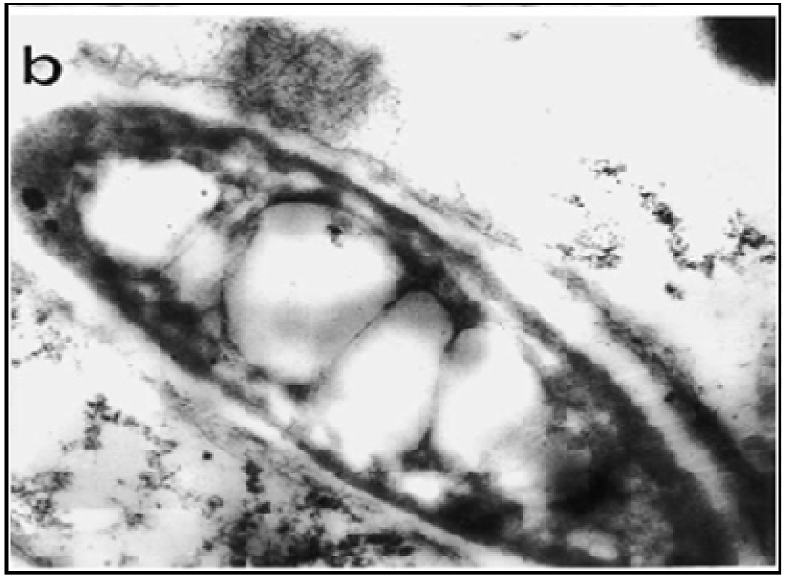
- Identification of prebiotics to be applied in European sea bass culture, Nile tilapia culture, blue mussel culture,…

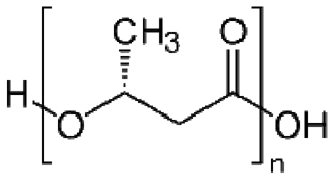
(De Schryver et al. Applied Microbiology and Biotechnology 2010, 86, 1535-1541; Hung et al. Aquaculture 2015, 446, 318-324; Situmorang et al. unpublished)

- Identification of Quorum Sensing disrupting compounds of *V. harveyi* for giant freshwater prawn larviculture

(Pande et al. Veterinary 2013, 406, 121-124)

PHB (Poly-β-hydroxybutyrate) in Bacillus





- Amorphous biopolymer accumulated as C and energy reserve material
- Presence of excess C in the environment while limiting nutrients (e.g. N)

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RATIONALE OF WORK

Why not use probiotic bacteria (ex: *Bacillus* spp.) containing amorphous PHB as biocontrol agents?

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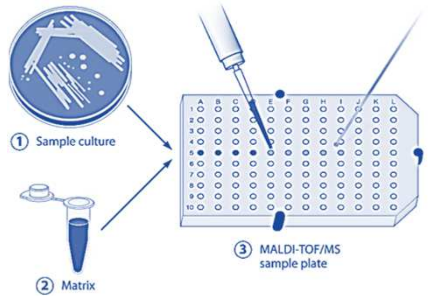
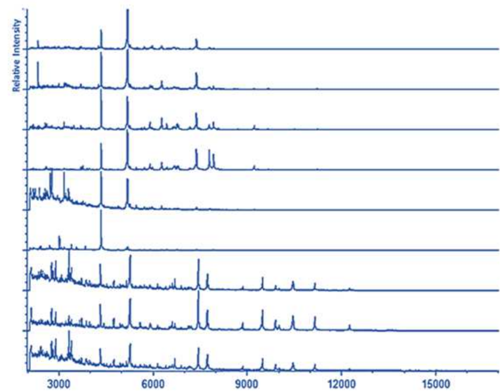
Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) for DEREPLICATION



50 isolates obtained



9 isolates selected from MALDI-TOF-based groupings

PHB quantification

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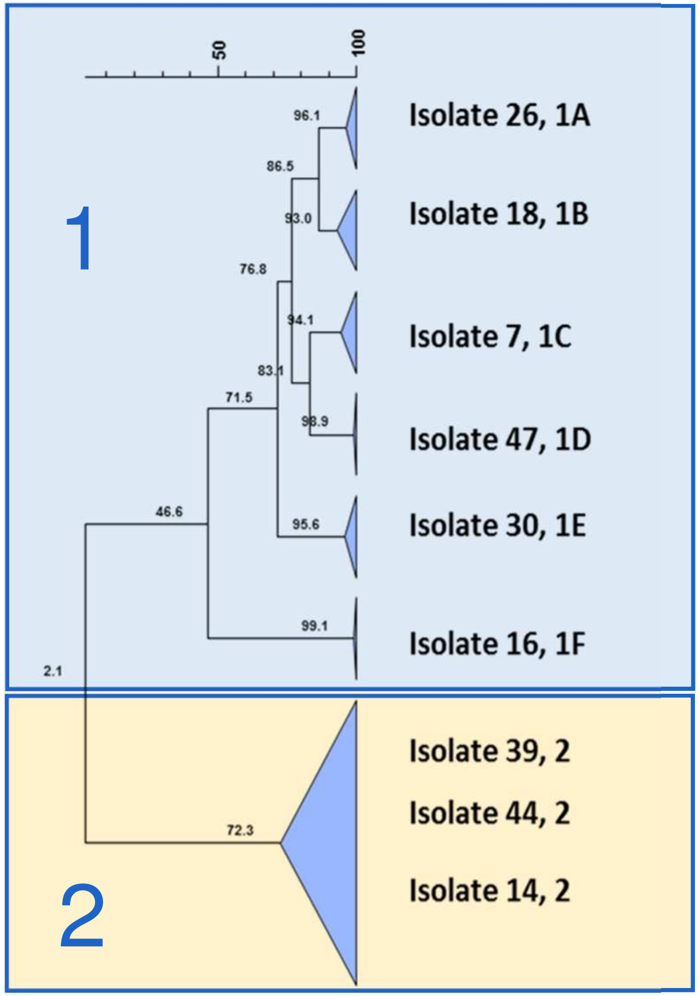
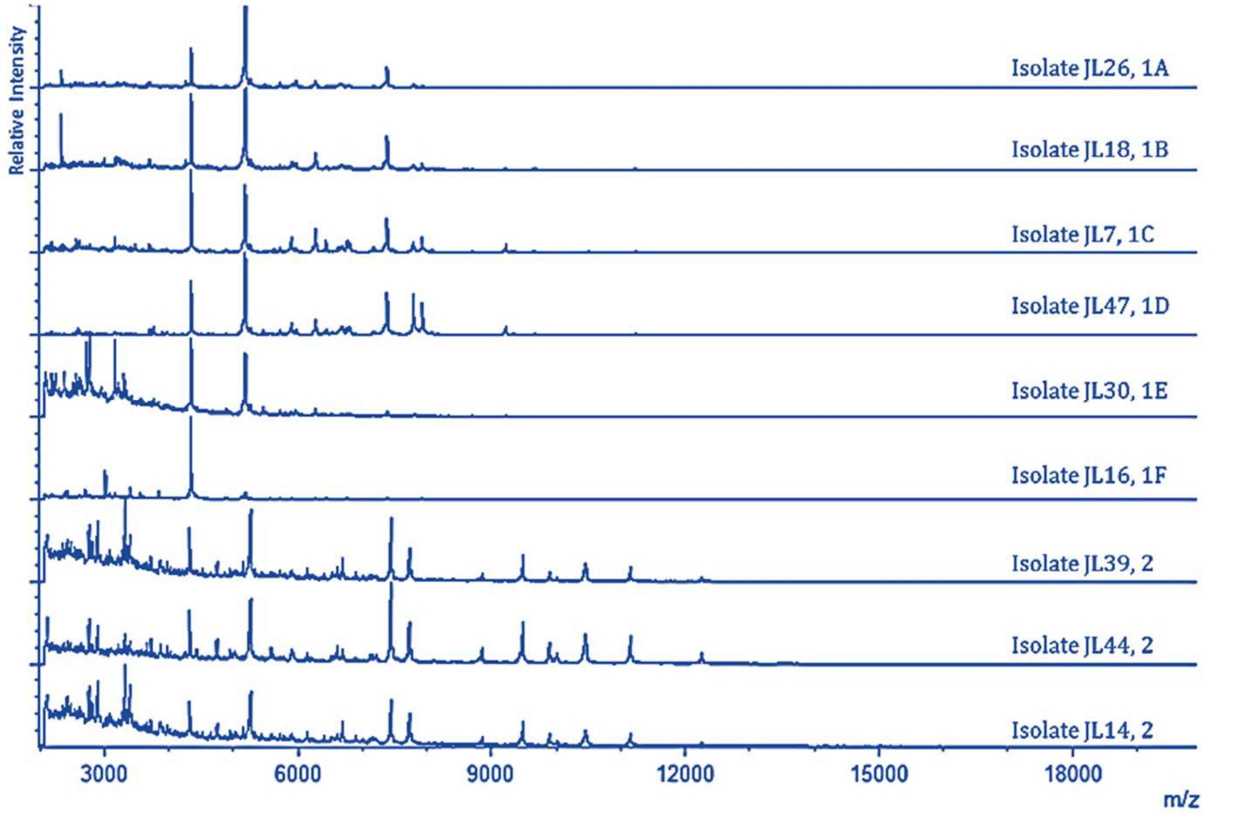
R1: RESULTS





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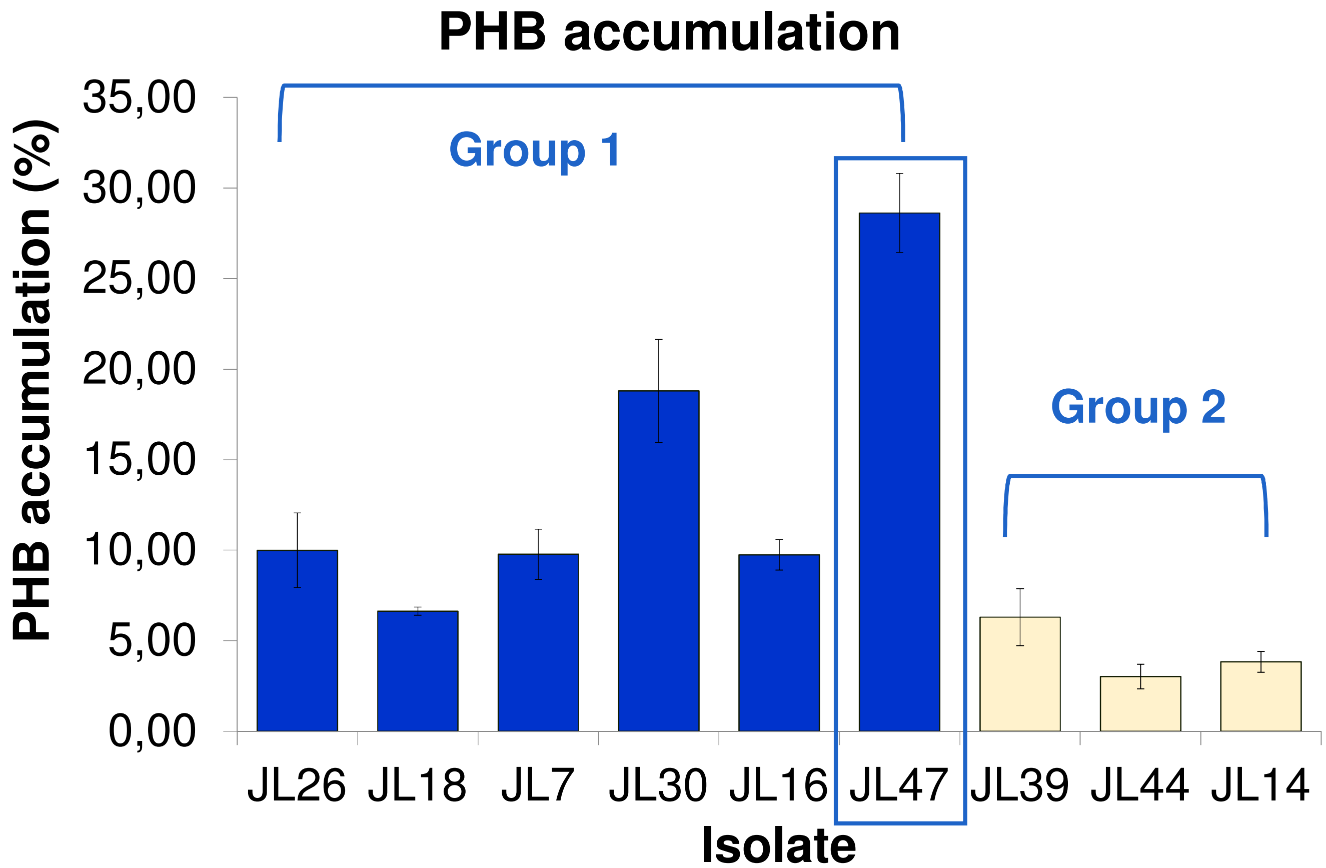
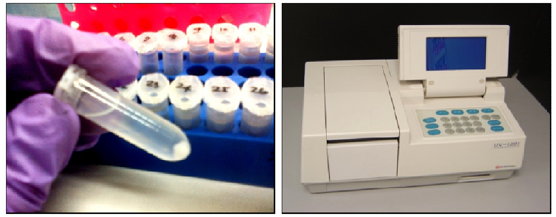




PHB assay

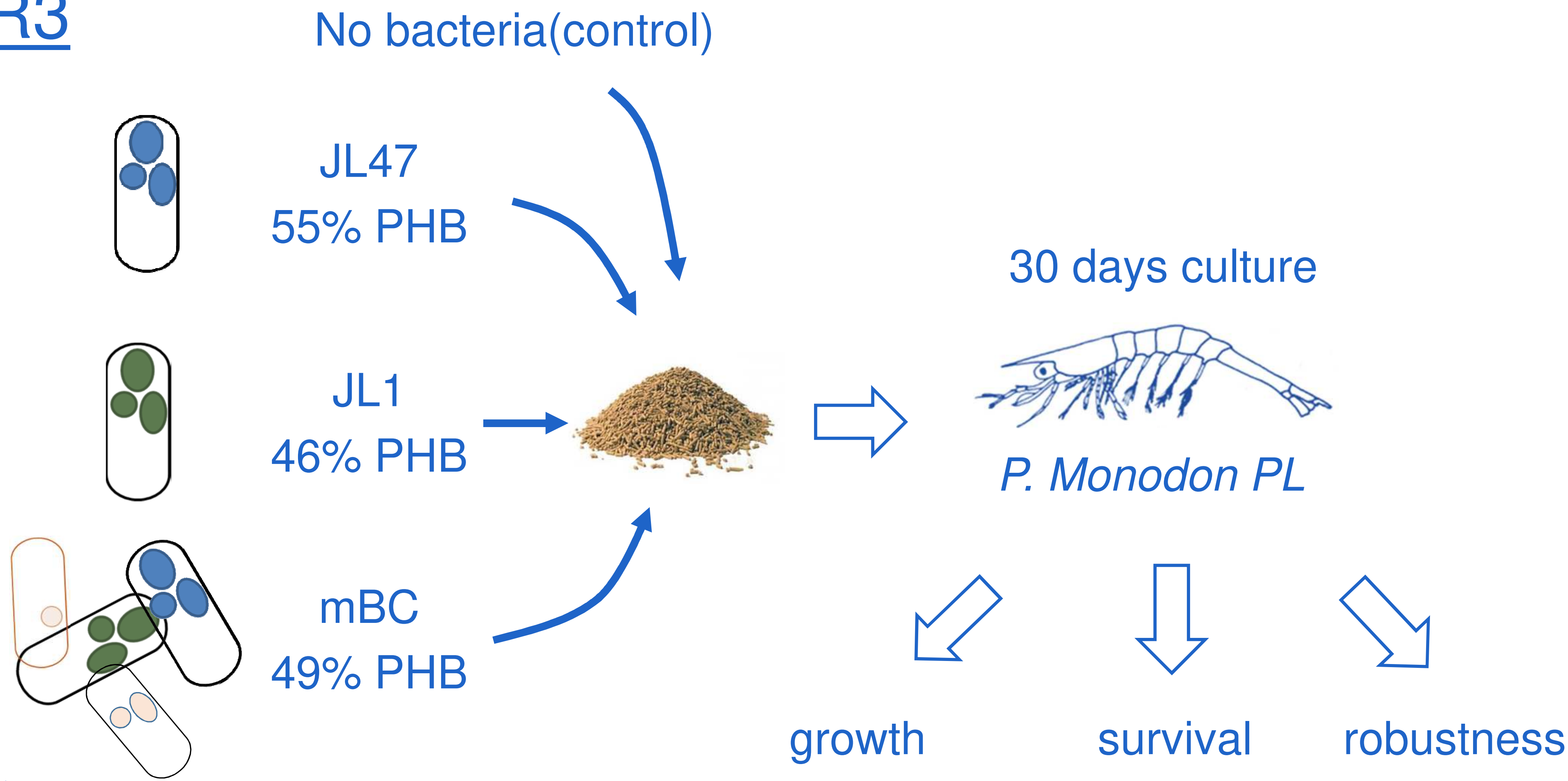
JL47 belongs to *Bacillus cereus* complex (16s rDNA identification)

24 h culture in LB +2% glucose

R3

DOES PHB-ACCUMULATING *BACILLUS* SP. JL47 ALSO WORK IN ACTUAL CULTURE SYSTEM?

R3



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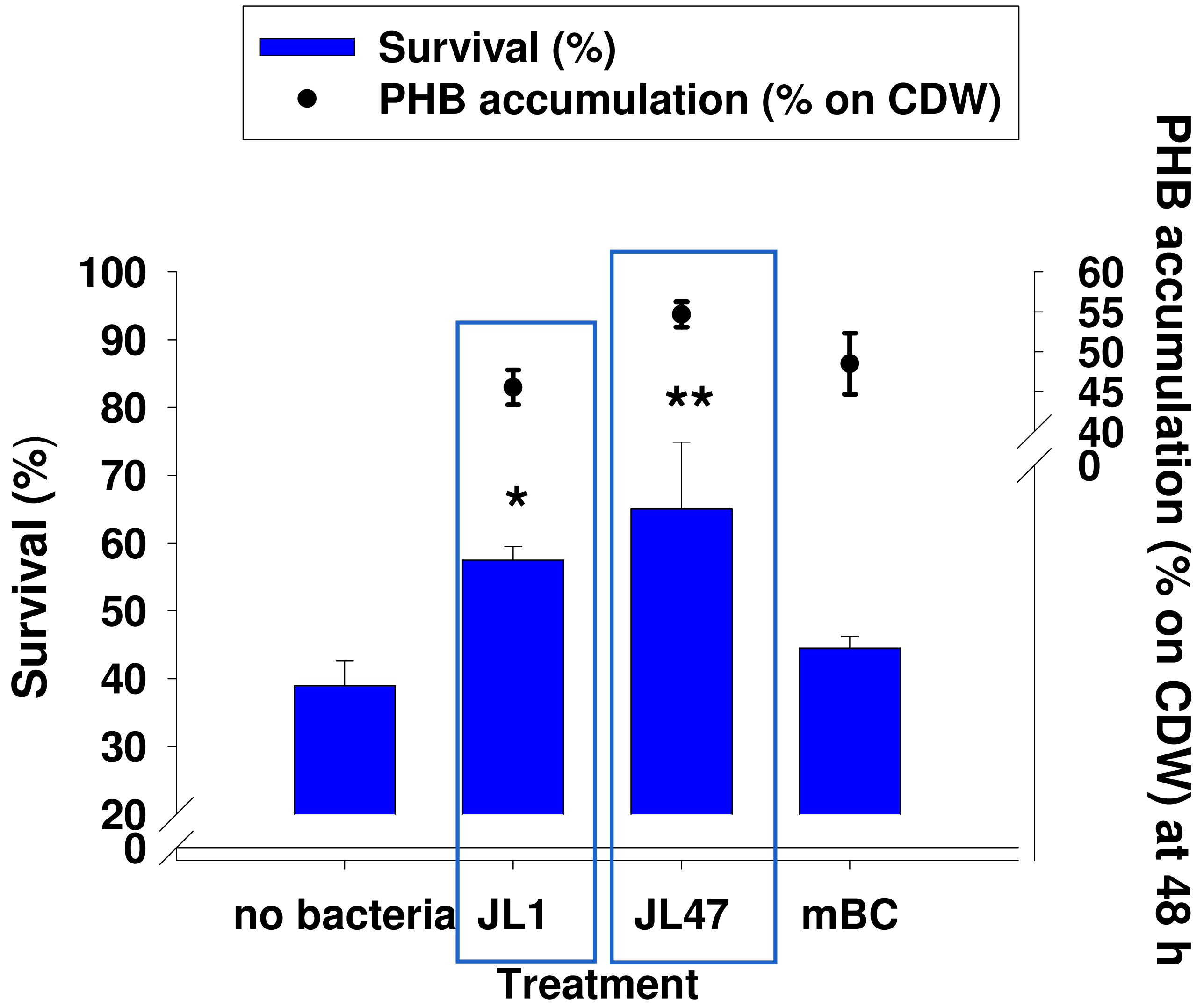
R3: RESULTS

SURVIVAL



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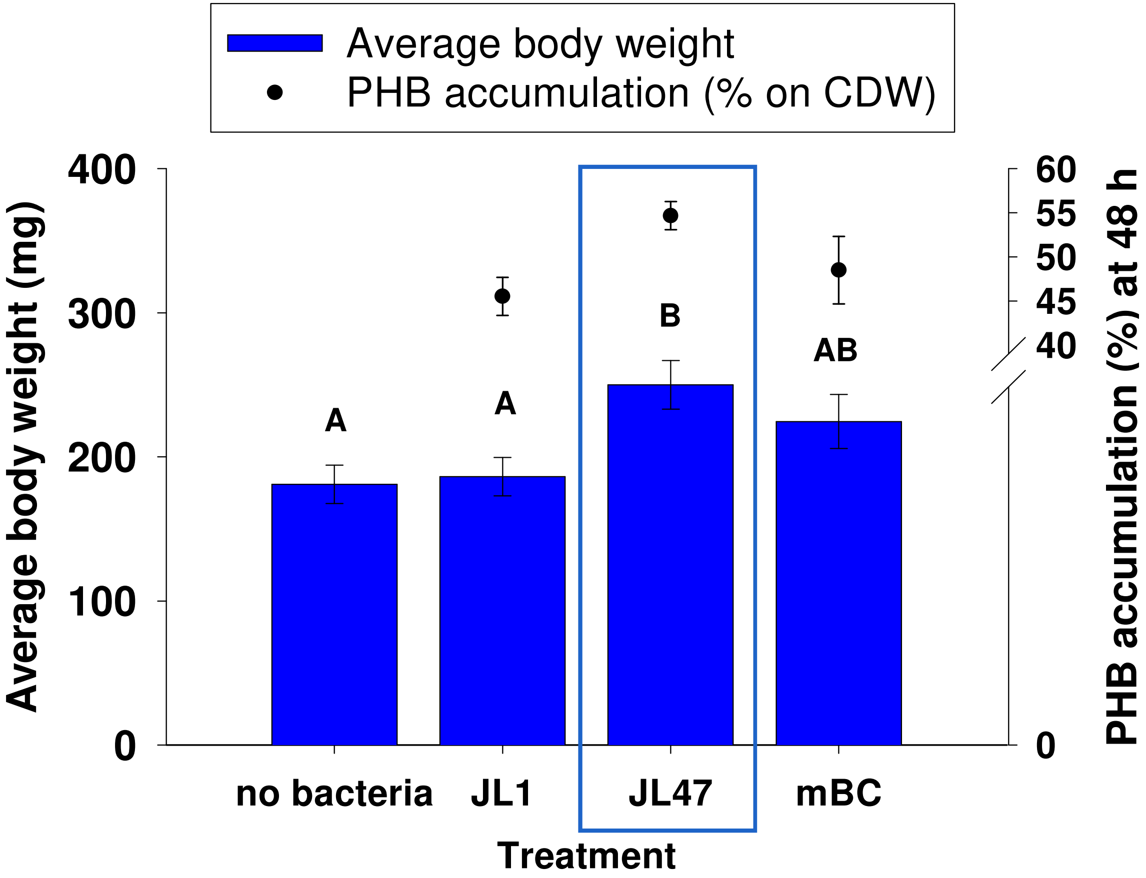
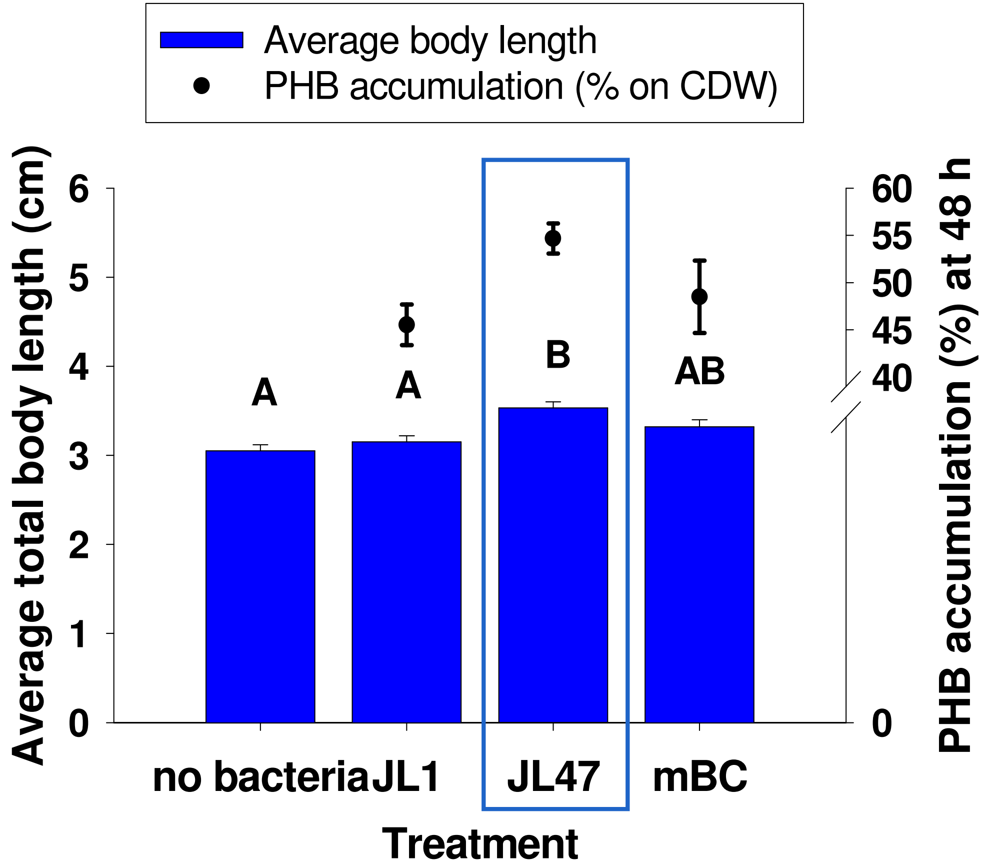
R3: RESULTS

GROWTH





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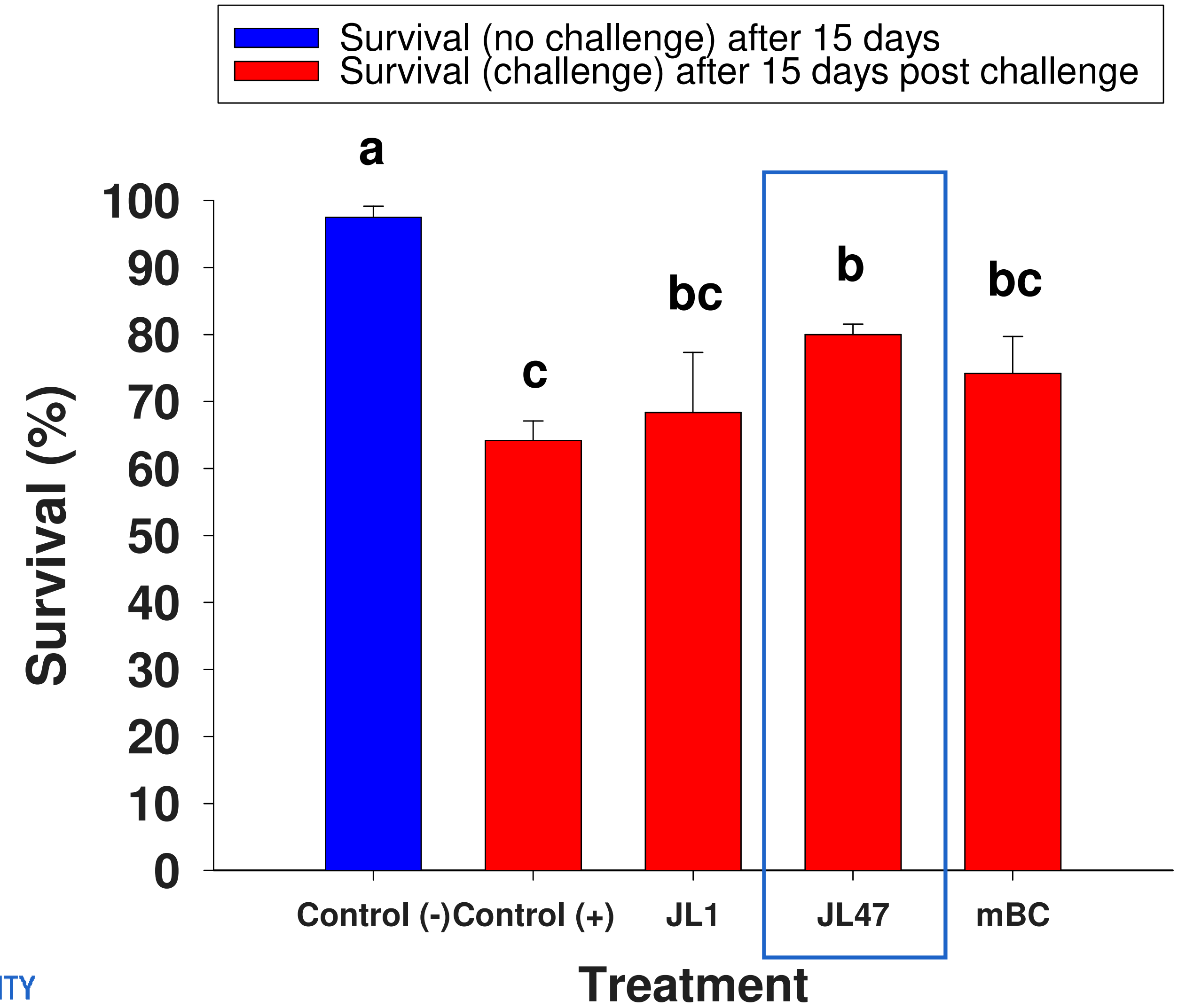
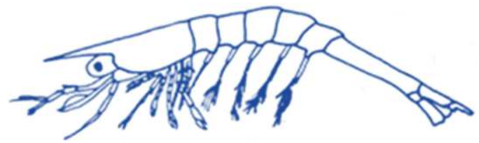
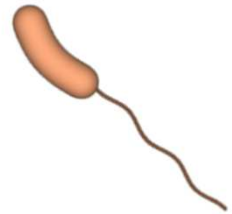
R3: RESULTS



ROBUSTNESS



Stressor
(NH_4Cl)



Vibrio campbellii 21363
(10^6 cells mL^{-1})

R3: SUMMARY OF RESULTS

Treatment	PHB accumulation (%)	Growth	Survival	Robustness
JL47	55	**Yes**	**Yes**	**Yes**
JL1	46	**No**	**Yes**	**No**
Mixed bacterial culture (mBC)	49	**No**	**No**	**No**
No bacteria	0	**No**	**No**	**No**

R3: CONCLUSIONS

- Only the PHB-accumulating *Bacillus* sp. JL47 improves the **survival**, **growth** & **robustness** of *P. monodon* PL in actual culture conditions in nursery tanks
- The result is in parallel to the previous *in vivo* result in gnotobiotic *Artemia*

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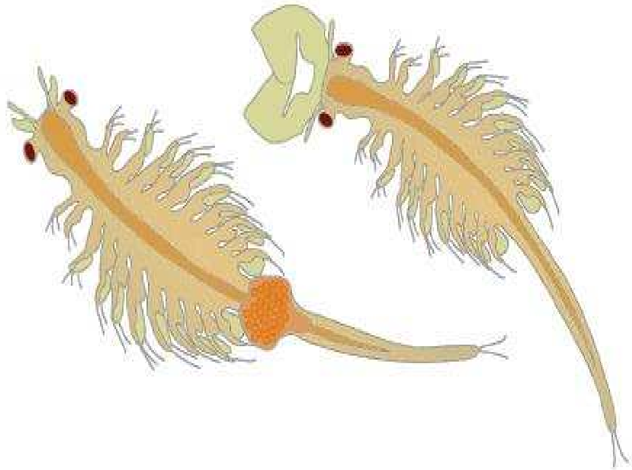
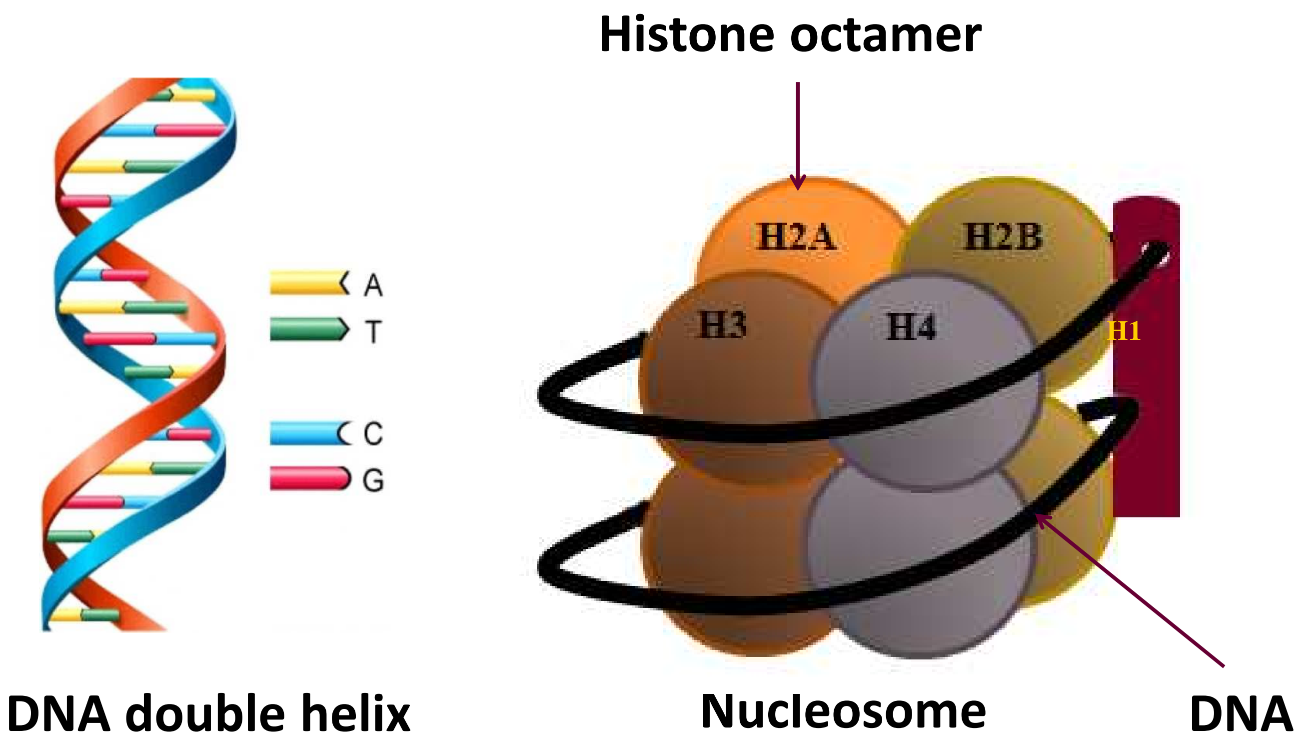
Artemia as model organism for epigenetic studies

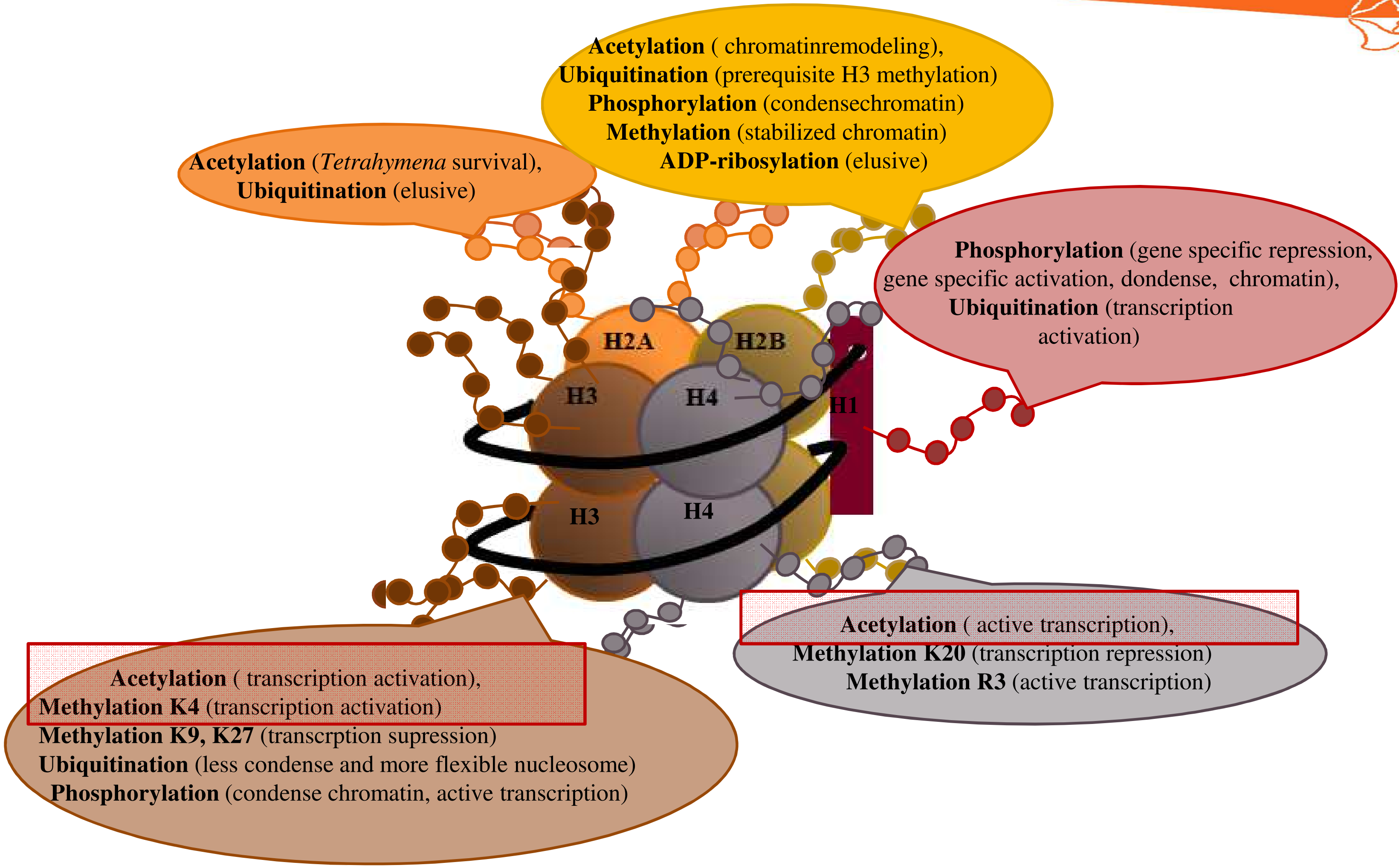
- Short generation time – 3 generations in about 6 months
- Genome is sequenced
- Easy and cheep to maintain and manipulate
- Produce large numbers of offspring
- Several parthenogenetic strains



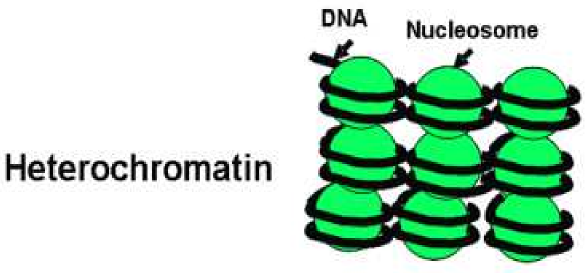
Life code

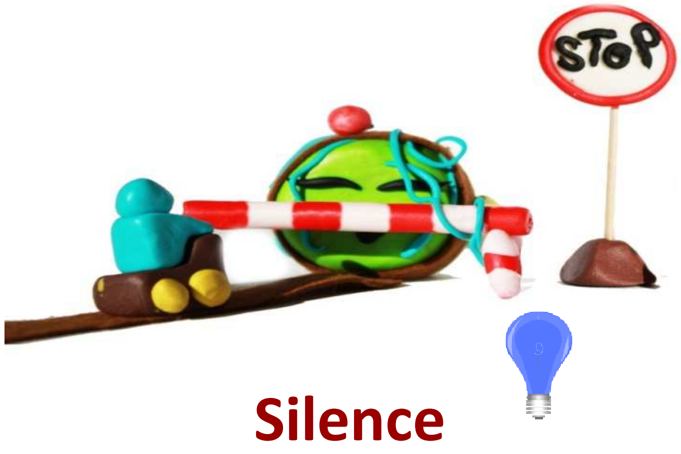

Histone octamer
A
T
C
G
H2A
H2B
H3
H4
H1
DNA double helix
Nucleosome
DNA

Histone tail modification



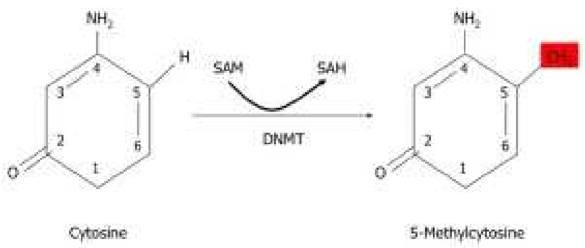
Histone tail modification





DNA methylation

DNA methylation



- Addition of methyl group to 5´ cytosine on CpG sites
- Heterochromatin formation
- Reduced chance of transcription

Silenced gene

Artemia as model organism for epigenetic studies

Bisexual

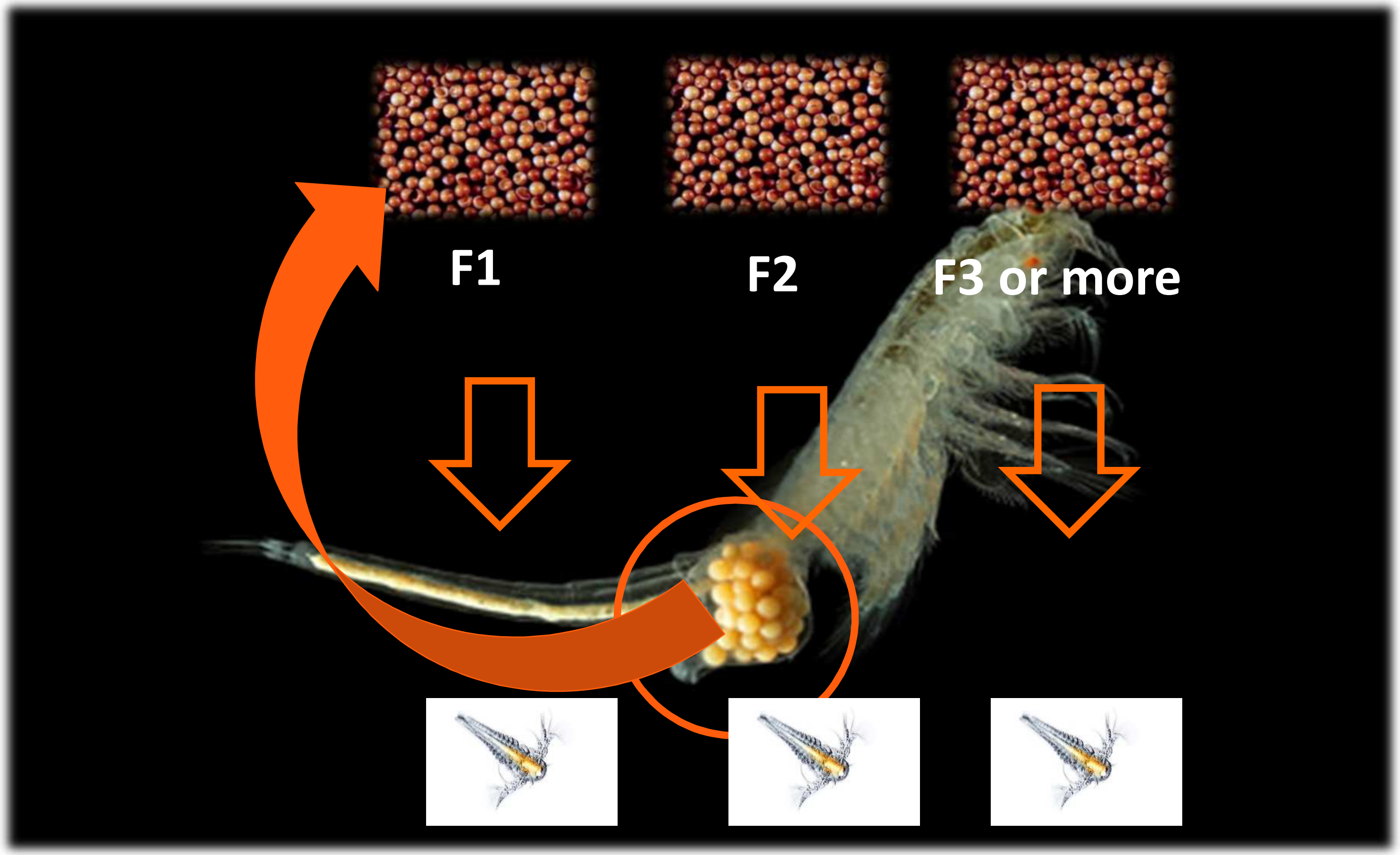
Wild population maternal/paternal heredity

Parthenogenesis

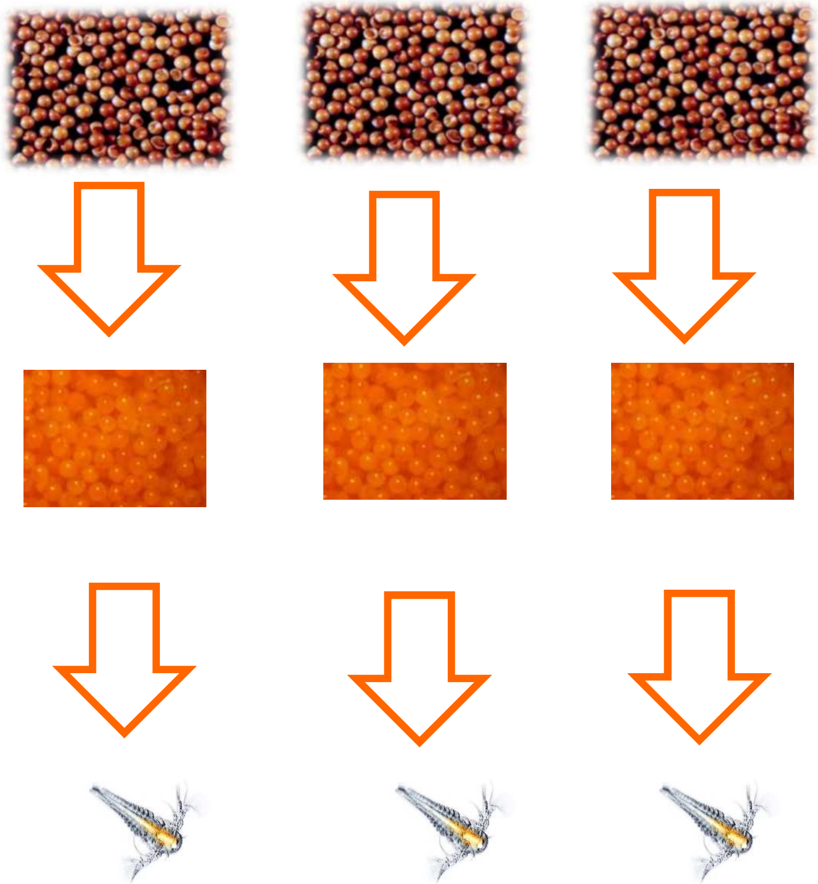
NO MALE

Clonal population started from a single female

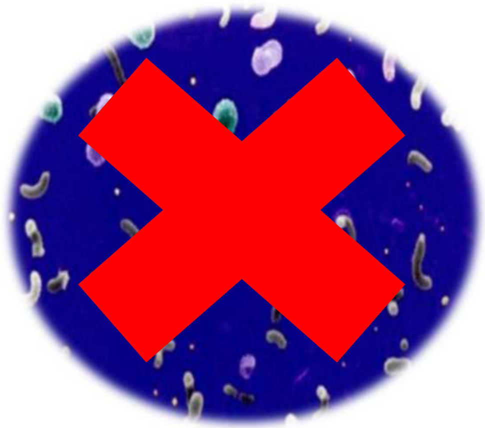
Cyst (dormant egg) production – common garden experiments



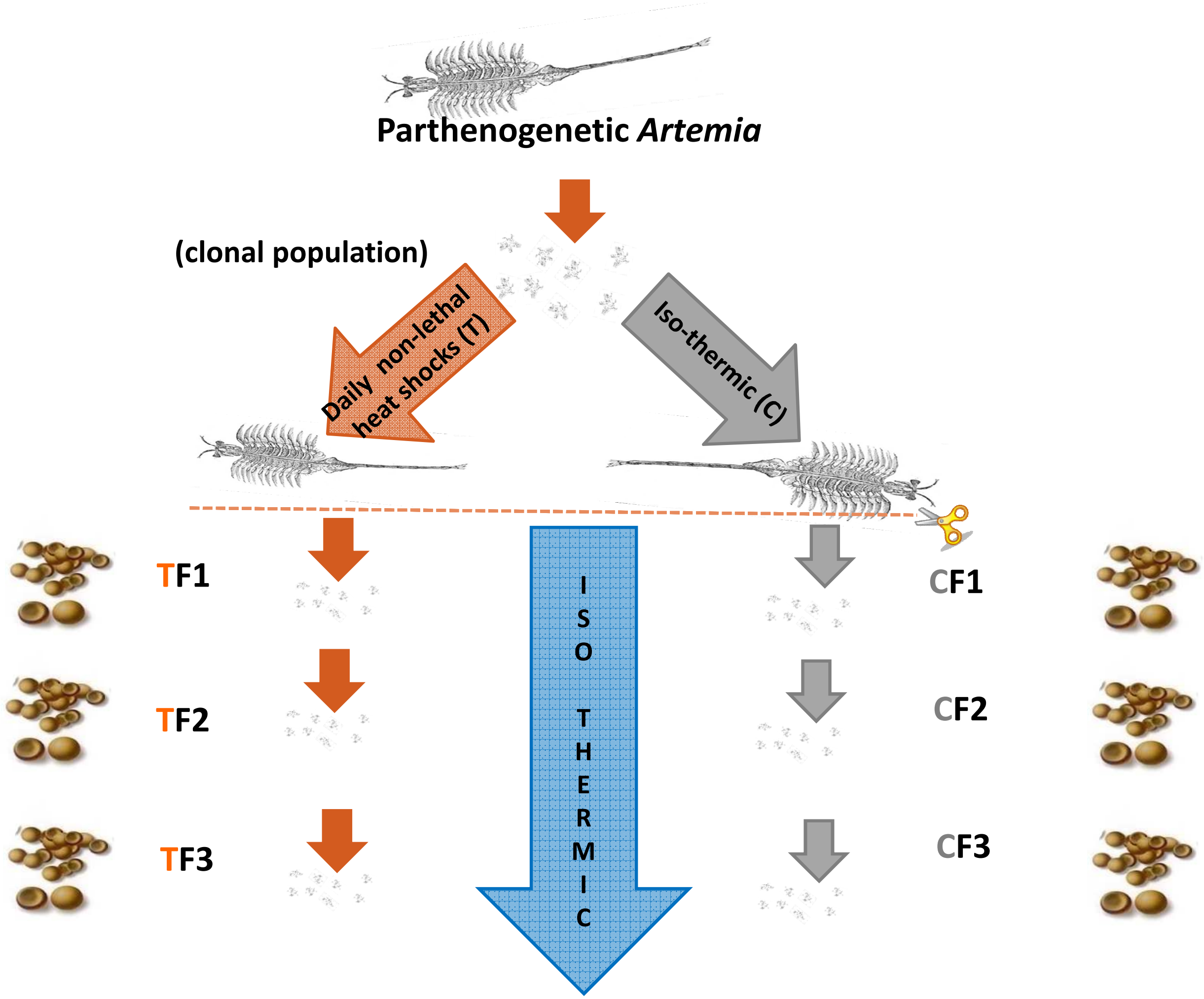
Common garden experiments – axenic /gnotobiotic



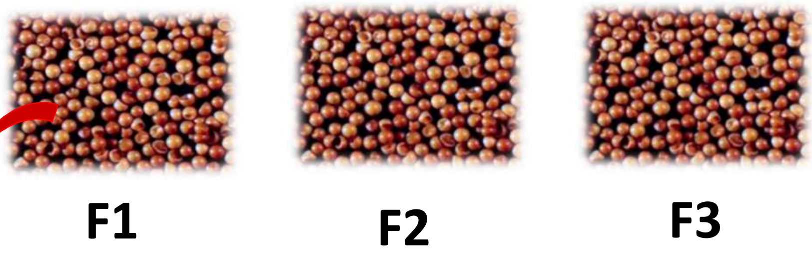
Chemical removal of the embryo outer shells
Sodium hydroxide
hypochlorite solution

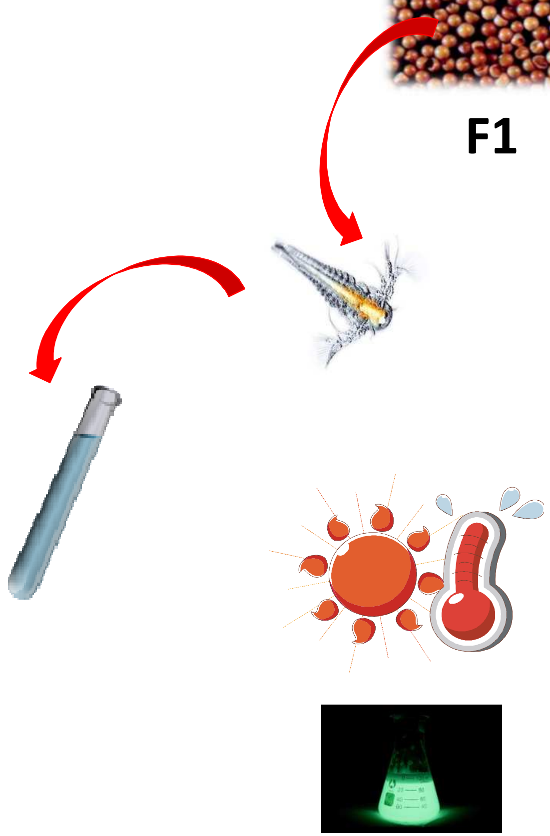


Experimental set up



Common garden test - Verifying stress-resistant phenotypes





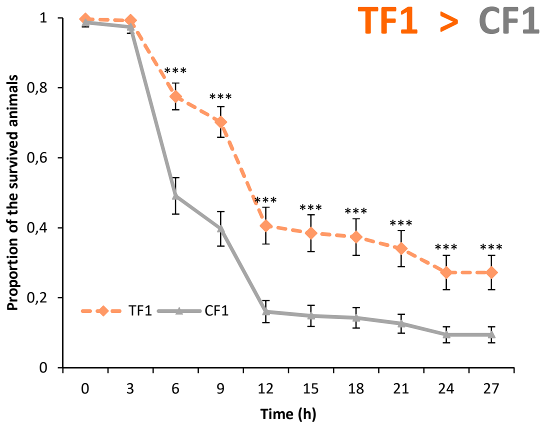
20 instar II nauplii in 6 replicates

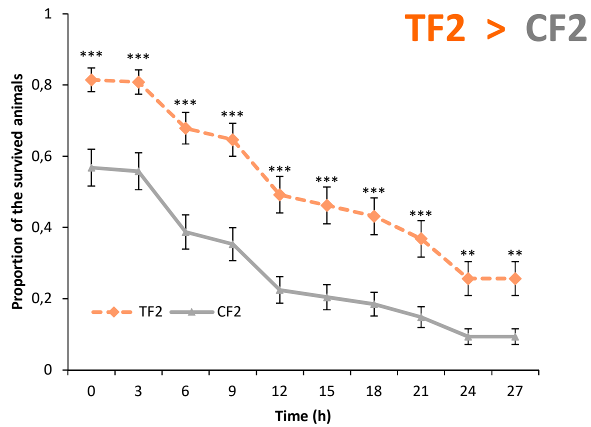
42°C for 15 min

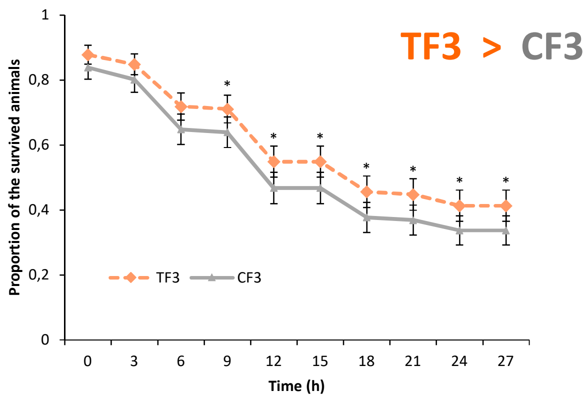
Or

Vibrio campbellii **10^7 cells/ml**

Thermo-tolerance test







gnoto

V. campbellii resistance

Conclusion:

- phenotypic association between thermotolerance and pathogen resistance

- Transgenerational inheritance of the phenotype

gnoto

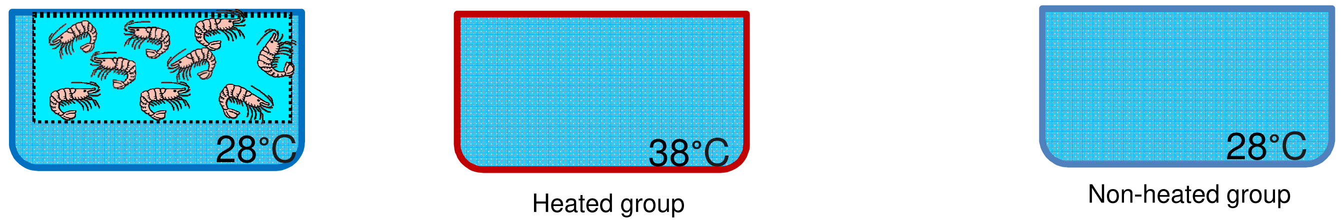
The FASEB Journal • Research Communication

Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model

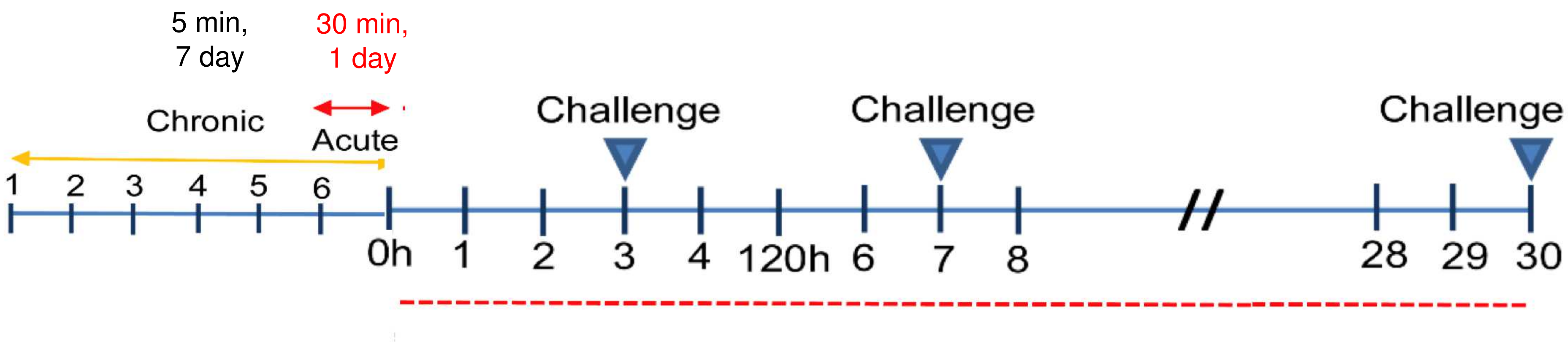
Parisa Norouzitallab,*[,†] Kartik Baruah,*[,†] Michiel Vandegehuchte,[‡] Gilbert Van Stappen,*[,†] Francesco Catania,[§] Julie Vanden Bussche,[‖] Lynn Vanhaecke,[‖] Patrick Sorgeloos,*[,†] and Peter Bossier*[,†,1]

*Laboratory of Aquaculture, [†]*Artemia* Reference Center, and [‡]Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Ghent, Belgium; [§]Institute for Evolution and Biodiversity, University of Münster, Münster, Germany; and [‖]Laboratory of Chemical Analysis, Ghent University, Merelbeke, Belgium

Application in the field: VANNAMEI



Schematic of heat treatment and challenge experiments.

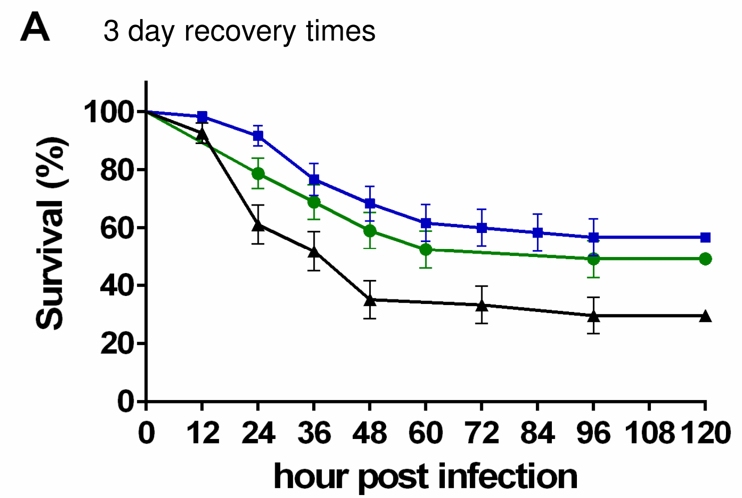


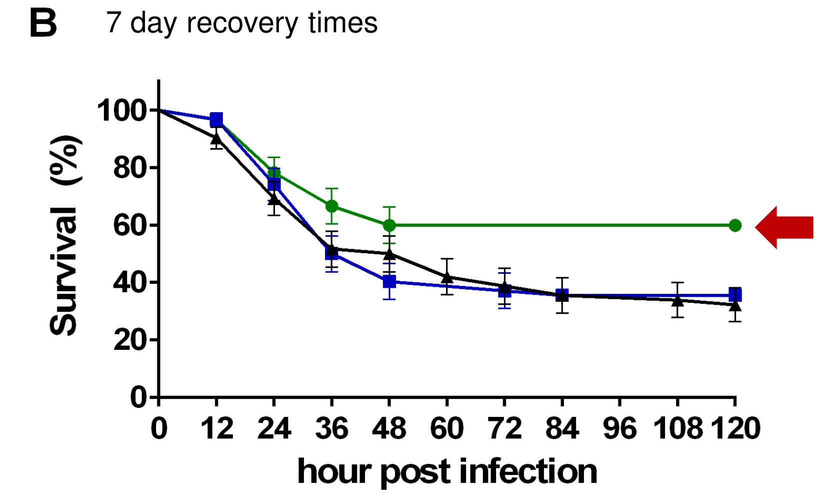
Junprung et al., 2017

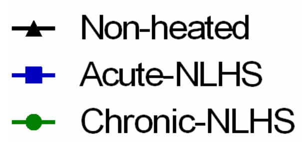
Wisarut Junprung, Premruethai Supungul, Anchalee Tassanakajon, HSP70 and HSP90 are involved in shrimp tolerance to AHPND-causing strain of after non-lethal heat shock, Fish & Shellfish Immunology, Volume 60, 2017, Pages 237-246

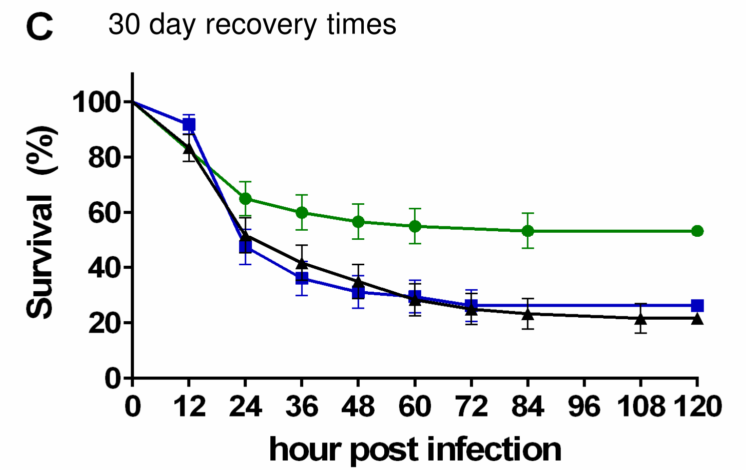
NLHS shows high survival rate upon VPAHPND infection in *P. vannamei* .

Junprung et al., 2017

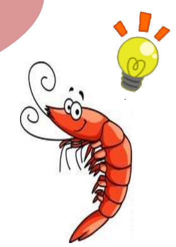








NLHS induces *P. vanamei* resistant to VPAHPND infection.



Wisarut Junprung, Premruethai Supungul, Anchalee Tassanakajon, HSP70 and HSP90 are involved in shrimp tolerance to AHPND-causing strain of after non-lethal heat shock, Fish & Shellfish Immunology, Volume 60, 2017, Pages 237-246

Non lethal heat shock (NLHS)

- A NLHS protects crustacean against against pathogens
- In Artemia this works transgenerational

MCM strategies

- Quorum sensing interference
  - Bacillus
  - Compounds
- Probiotics
  - Bacillus
  - PHB containing Bacillus
- Shrimp heat shock conditioning
- COMBINATIONS?

Thank you for your attention

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