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UGent Aquaculture R&D Consortium

MICROBIAL MANAGEMENT IN FISH AND SHELLFISH LARVICULTURE: FROM GNOTOBIOTIC EXPERIMENTS TO APPLICATIONS

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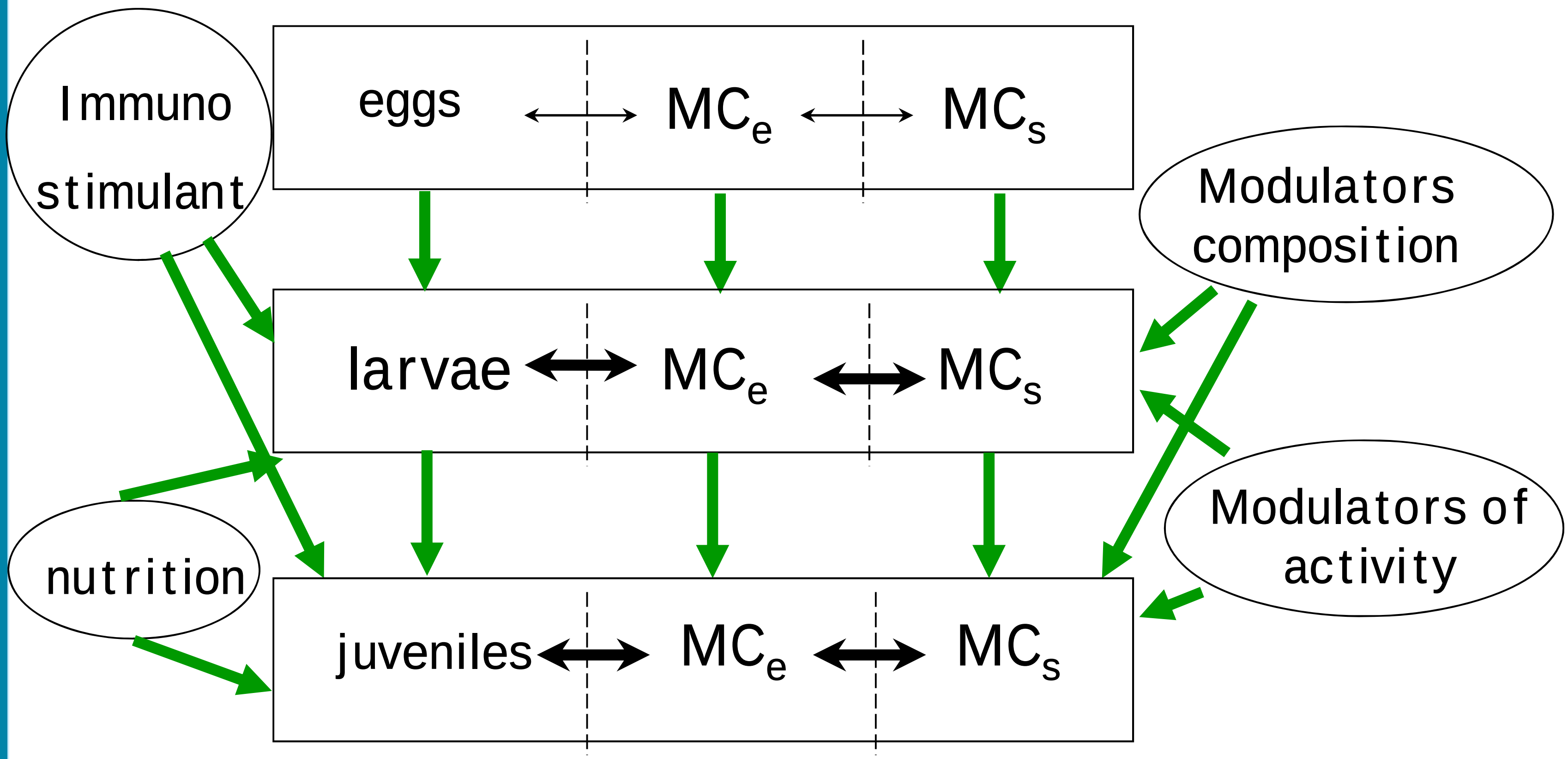
Starting point

- Aquaculture target organisms live in an environment conducive to the proliferation of micro-organisms because
  - Feed
  - Excretion products
- Reproducibility?
- Reliability?

Conceptual framework

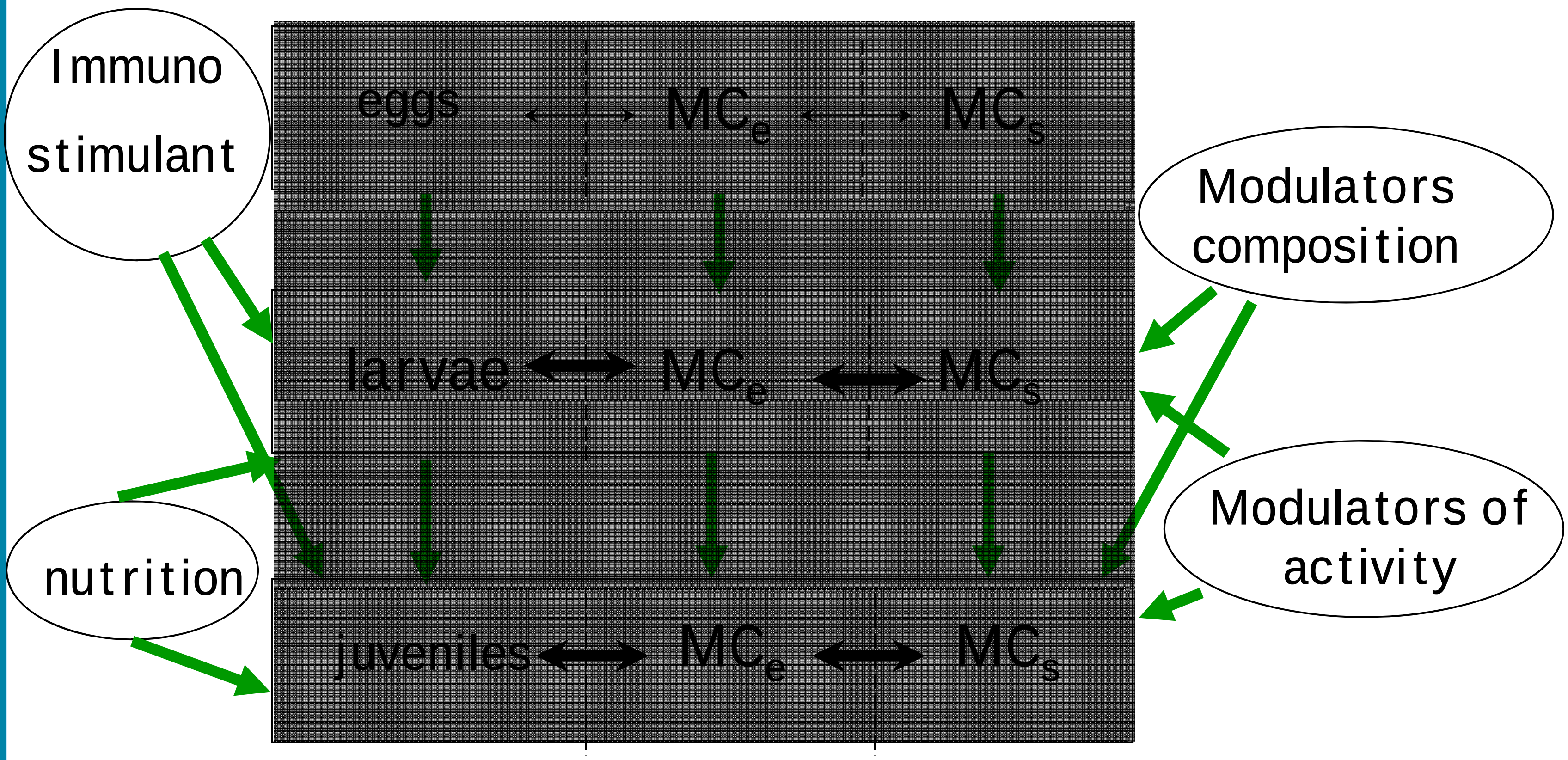


Microbial community composition

- Culture dependent techniques
  - Culturable phenotype
  - Unculturable phenotypes

- Culture independent techniques

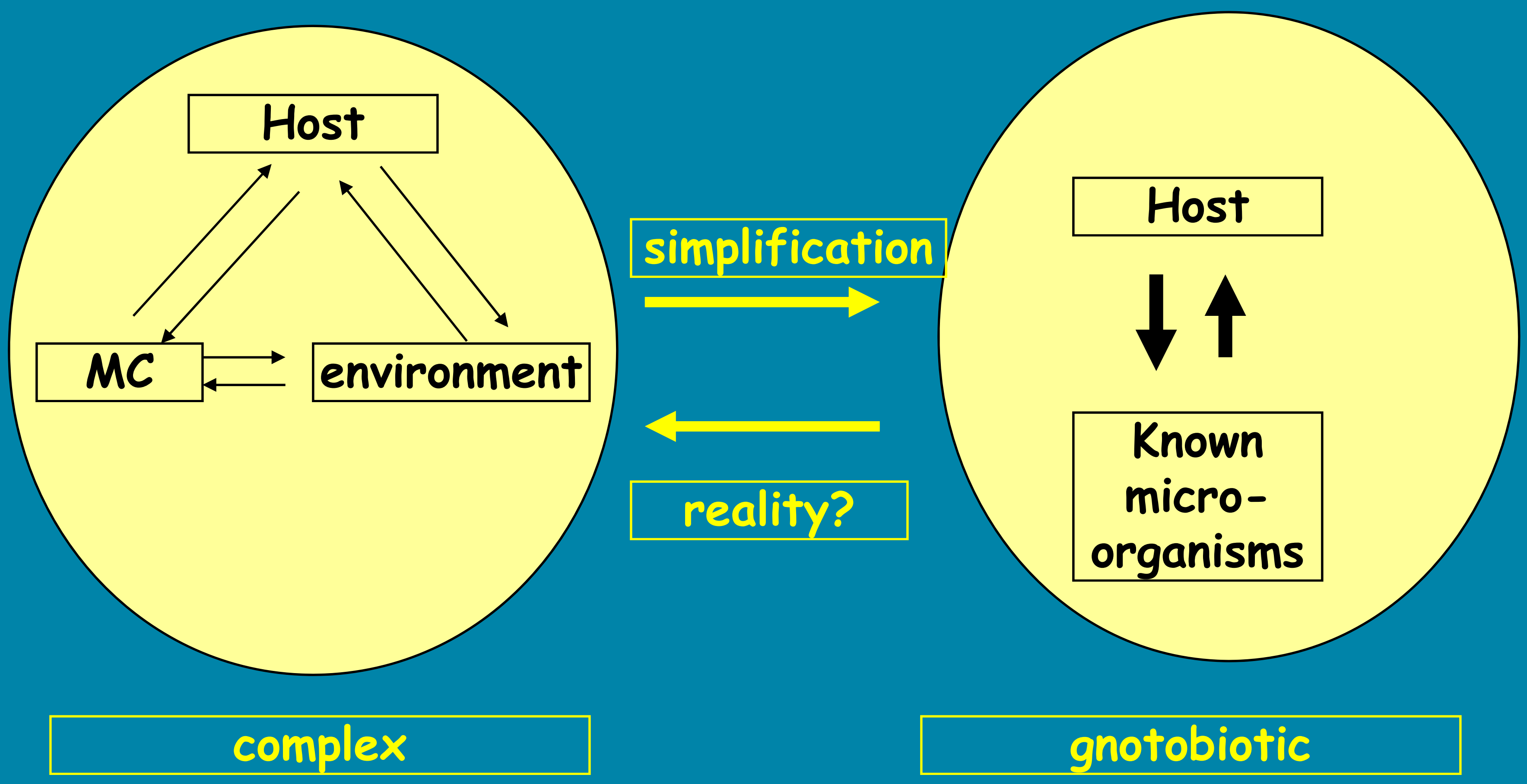
Conceptual framework



Experimental approach:

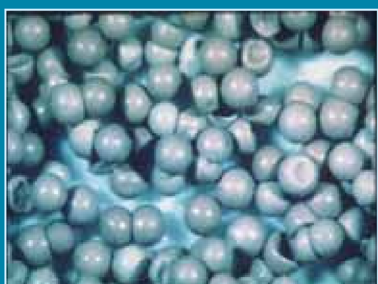
- Gnotobiotic systems
  - Artemia
  - Brachionus
  - Seabass
- Non-gnotobiotic verification

How to study host-microbial interactions?



Gnotobiotic Artemia: GART

Decapsulation



Hydrated cysts

Non-axenic conditions

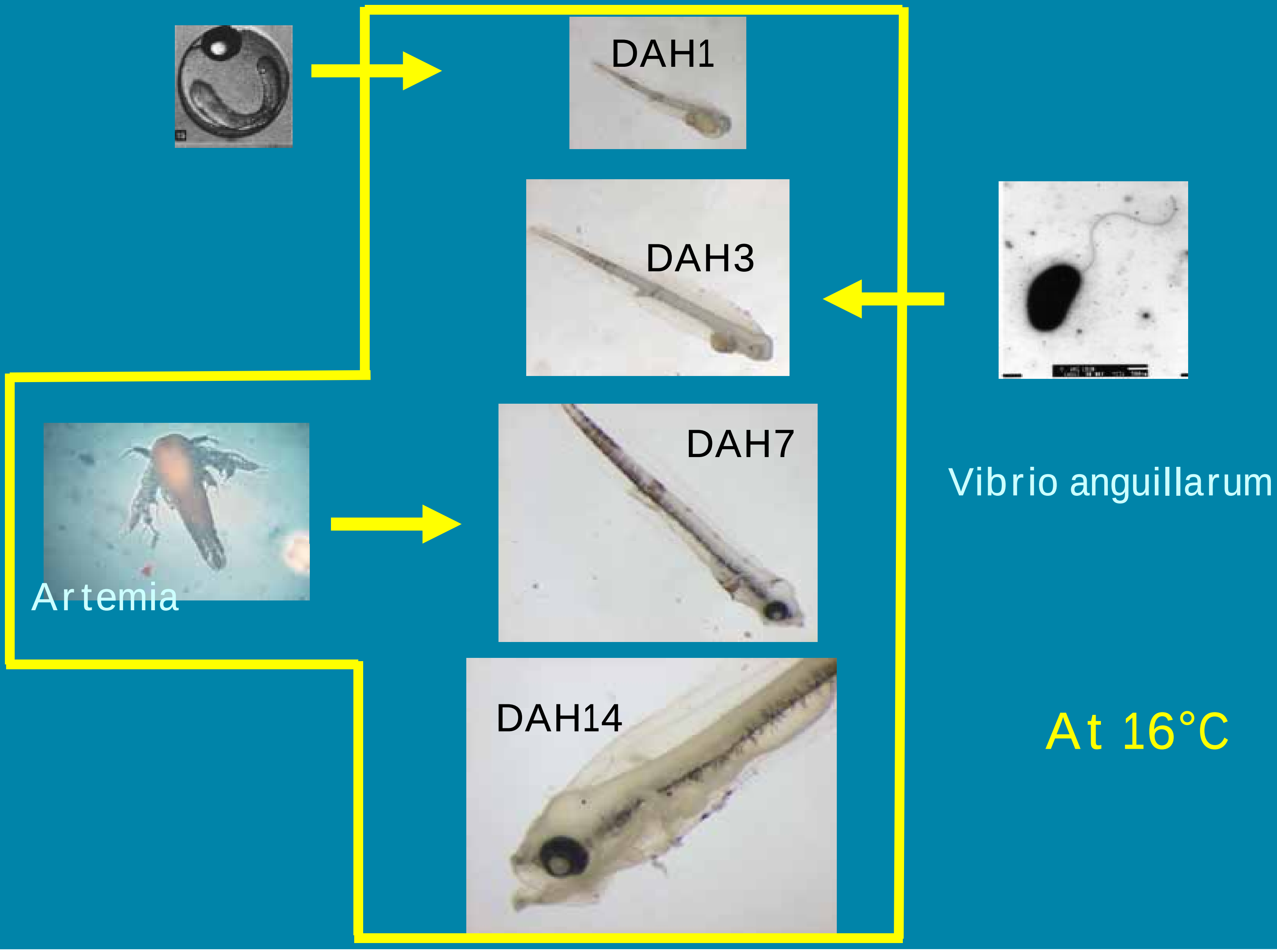
Decapsulated cysts

20-50 nauplii counted for the Experiments

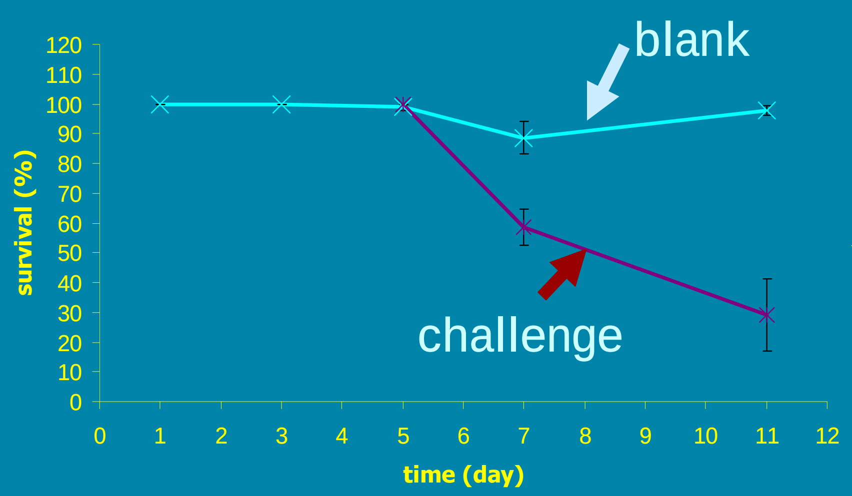
Axenic conditions

Gnotobiotic challenge: add Vibrio

Gnotobiotic Artemia –seabass food chain



Gnotobiotic Artemia –seabass food chain



Virulent

Vibrio anguillarum

Strain HI610

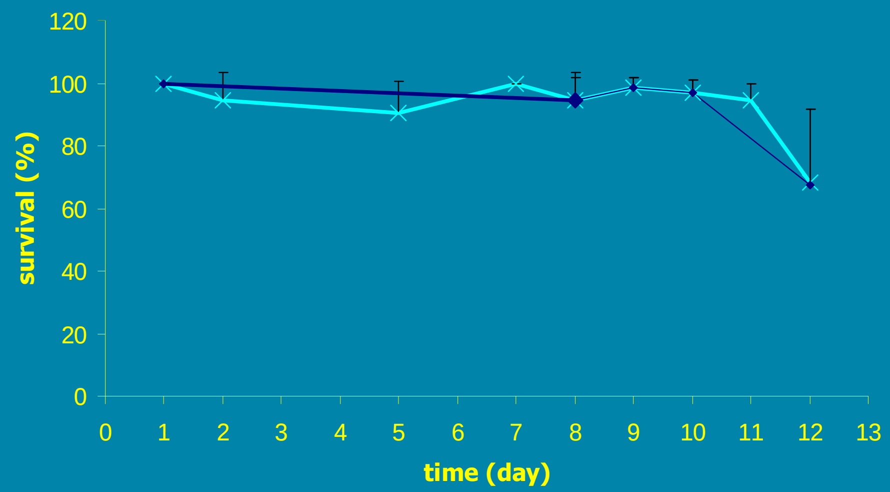
serovar O2a

Avirulent

Vibrio anguillarum

Strain 43

serovar 01



Steering host-microbial interactions

- *Stimulating the host's immune response*
  - yeast cell wall-bound glucan
  - heat shock proteins
- *Influencing microbial numbers or activity*
  - polyhydroxybutyric acid
  - quorum sensing

Yeast cell wall-bound glucan as immunostimulant?

Isogenic yeast strains with altered cell wall composition

Strains	Effect on the cell wall composition	
mnn9	mannoproteins ↓	β-1,6 glucans ↑, chitin ↑
mnn6	phosphomannose ↓	
fks1	β-1,3 glucans ↓	chitin ↑, mannoproteins ↑
Knr4		
kre6	β-1,6 glucans ↓	
chs3	Chitin ↓	glucan ↑, mannoprotein ↑
gas1	Reduced a cell wall protein (GPI), β-1,3 glucans ↓	defective architecture, chitin ↑
WT	No mutation – control yeast	

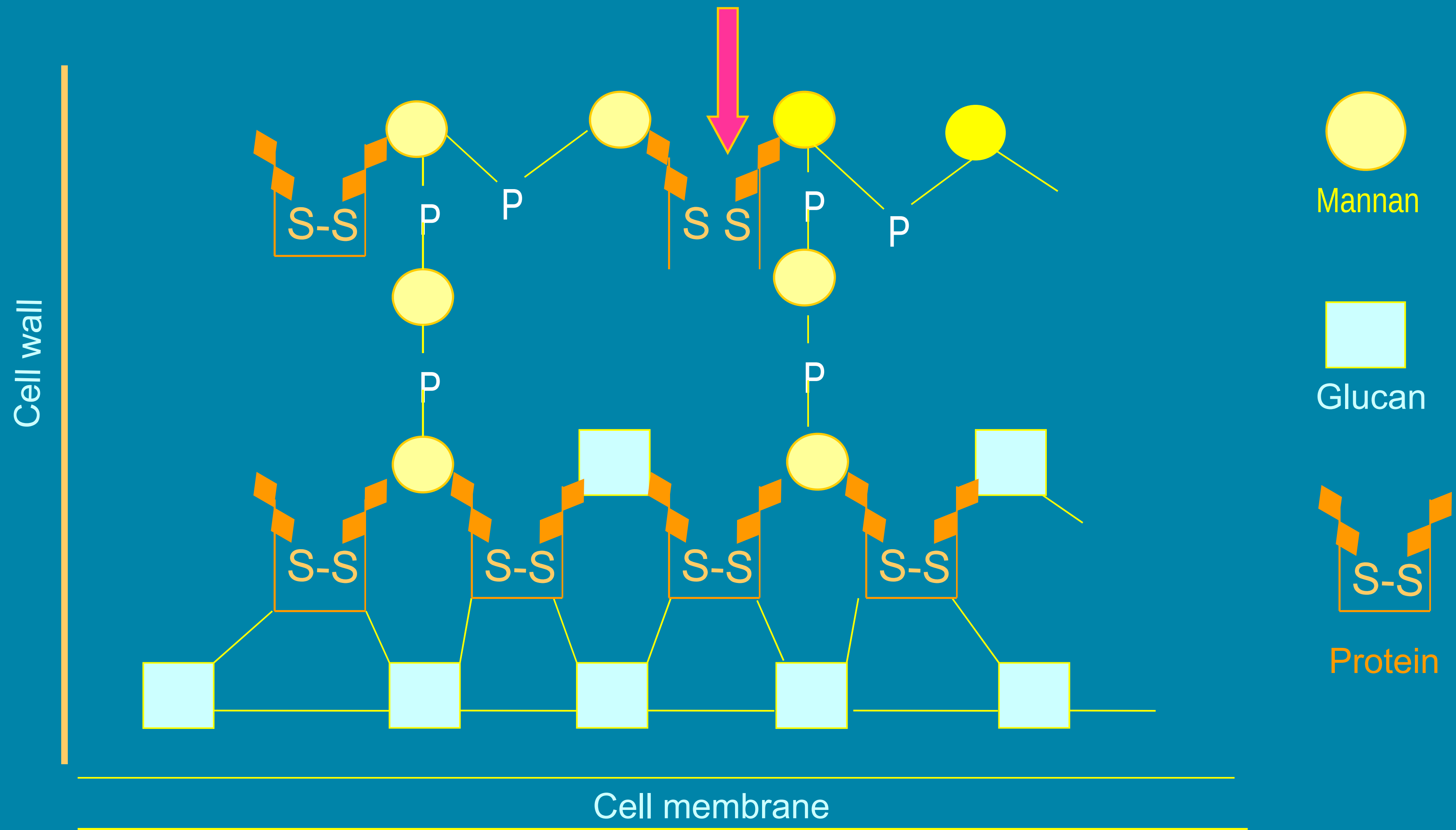
Altered cell wall composition of yeast

	WT-exp YNB	mnn9 -exp YNB
Mannoproteins	43%	16%
β (1,6) glucans β (1,3) glucans	55%	75%
Chitin	2%	9%

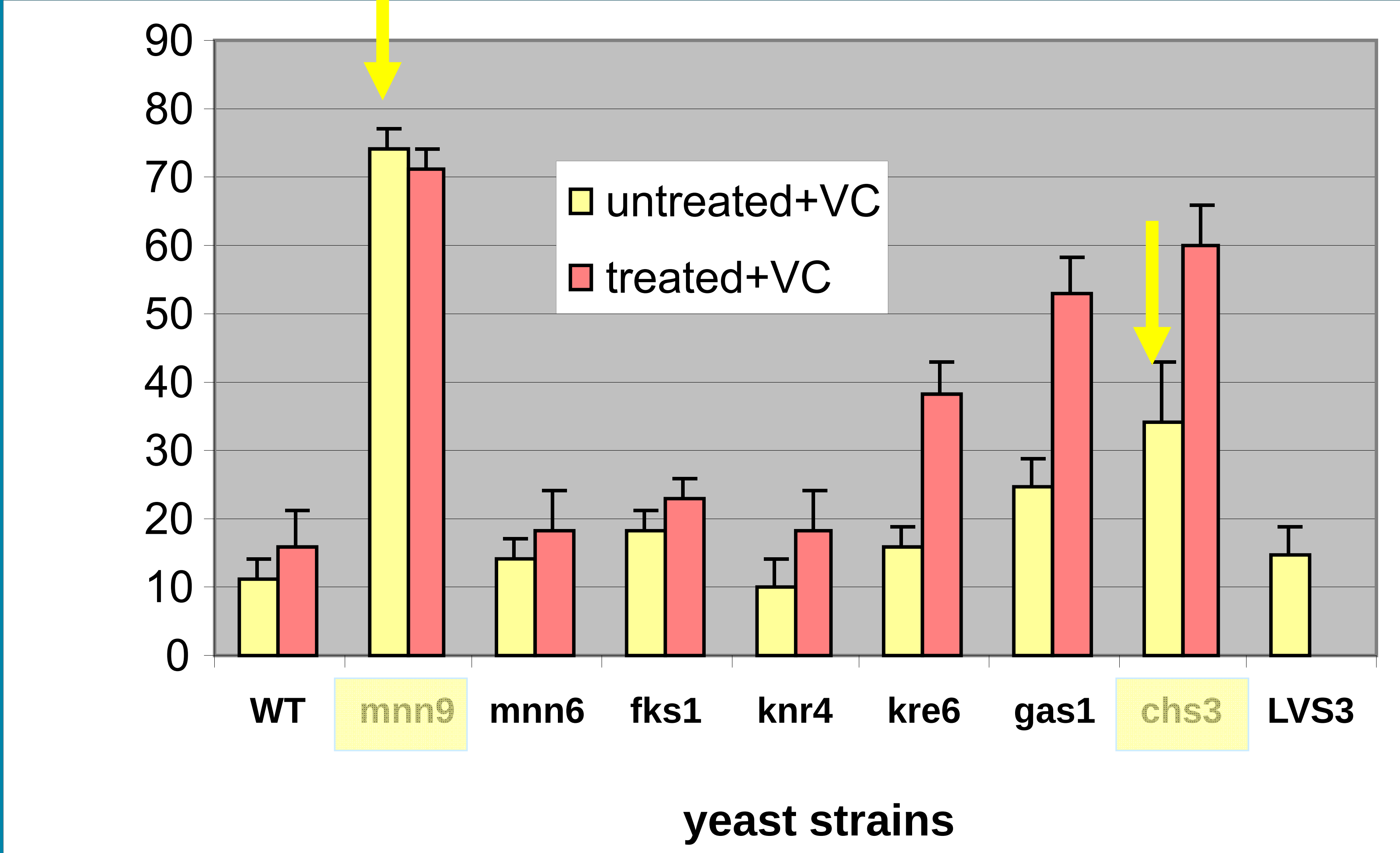
(adapted from EUROCELLWALL project from EU)

- ✓ Since β-glucans are well-known immunostimulants, possibly the mnn9 yeast acts as immunostimulant, allowing *Artemia* to be protected against pathogens

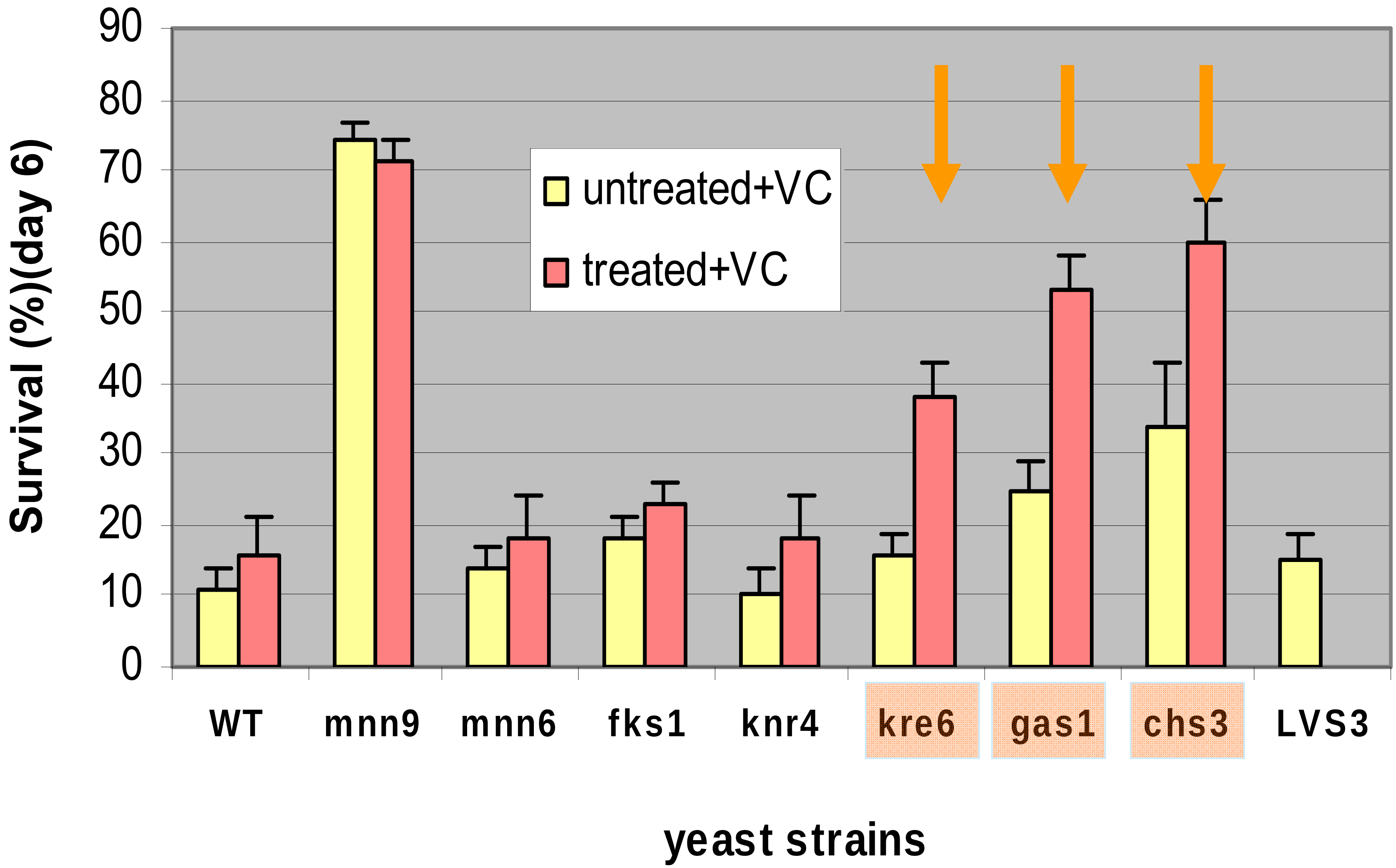
Yeast treatment with 2-mercapto-ethanol



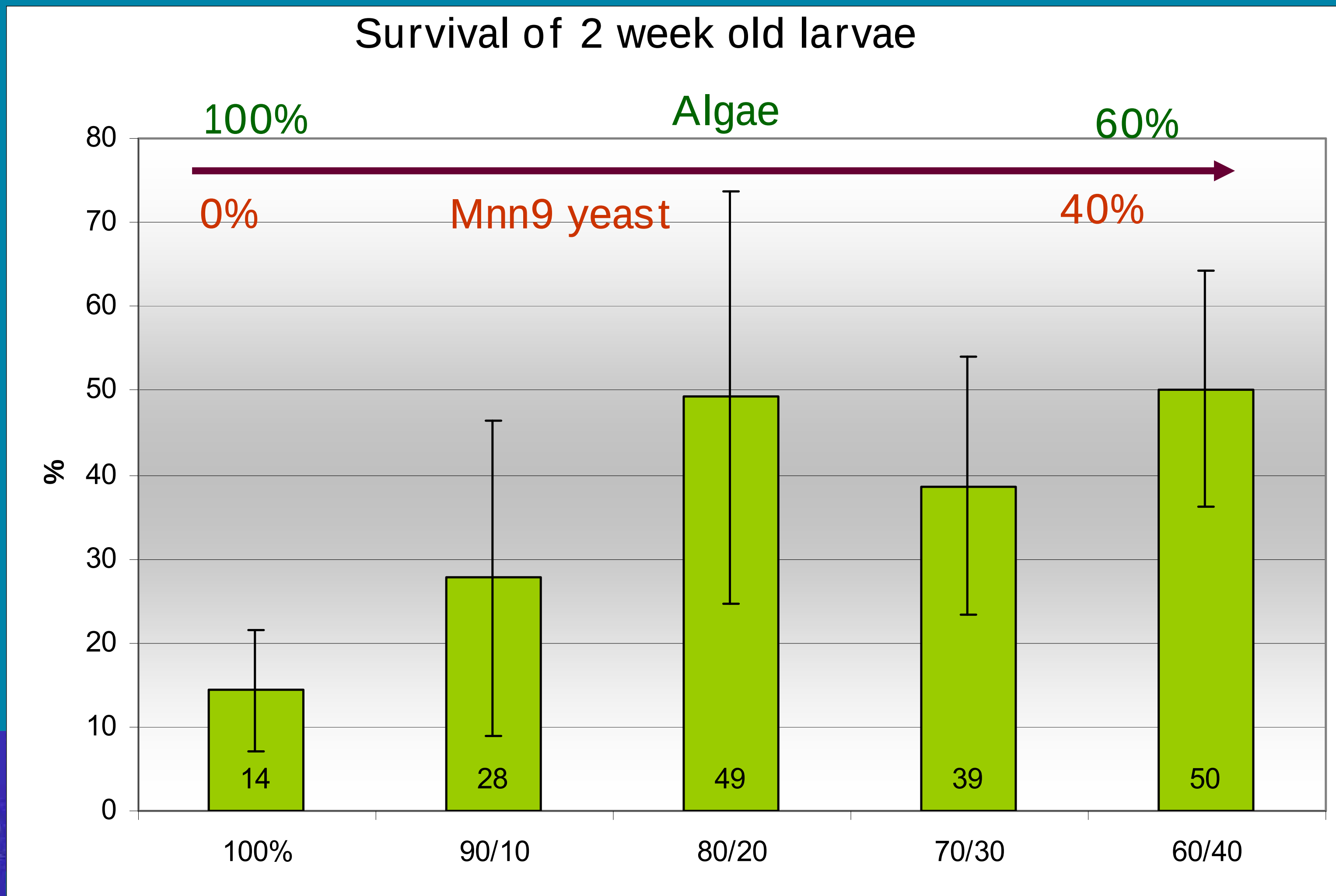
Artemia survival in GART



Artemia survival in GART



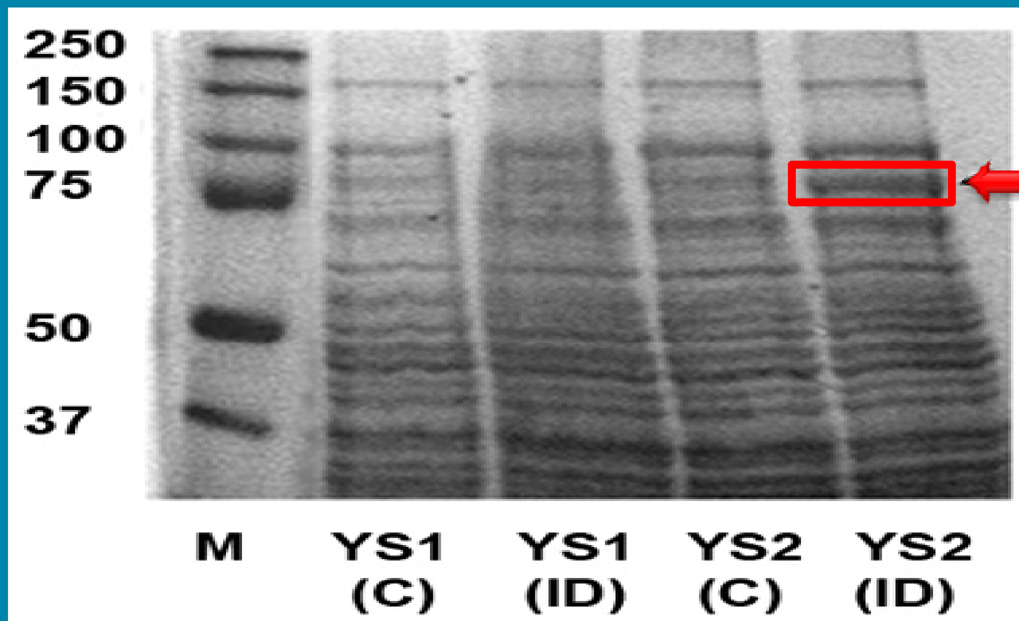
Non-gnotobiotic: mussel larvae



Heat shock proteins as immunostimulants?

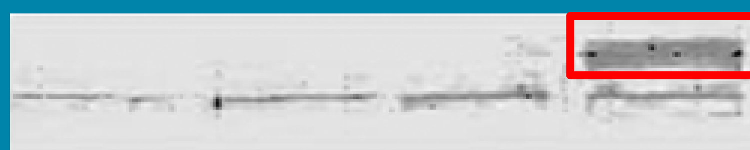
DnaK (HSP70) overexpression:

E. coli strain YS2



DnaK

WB →

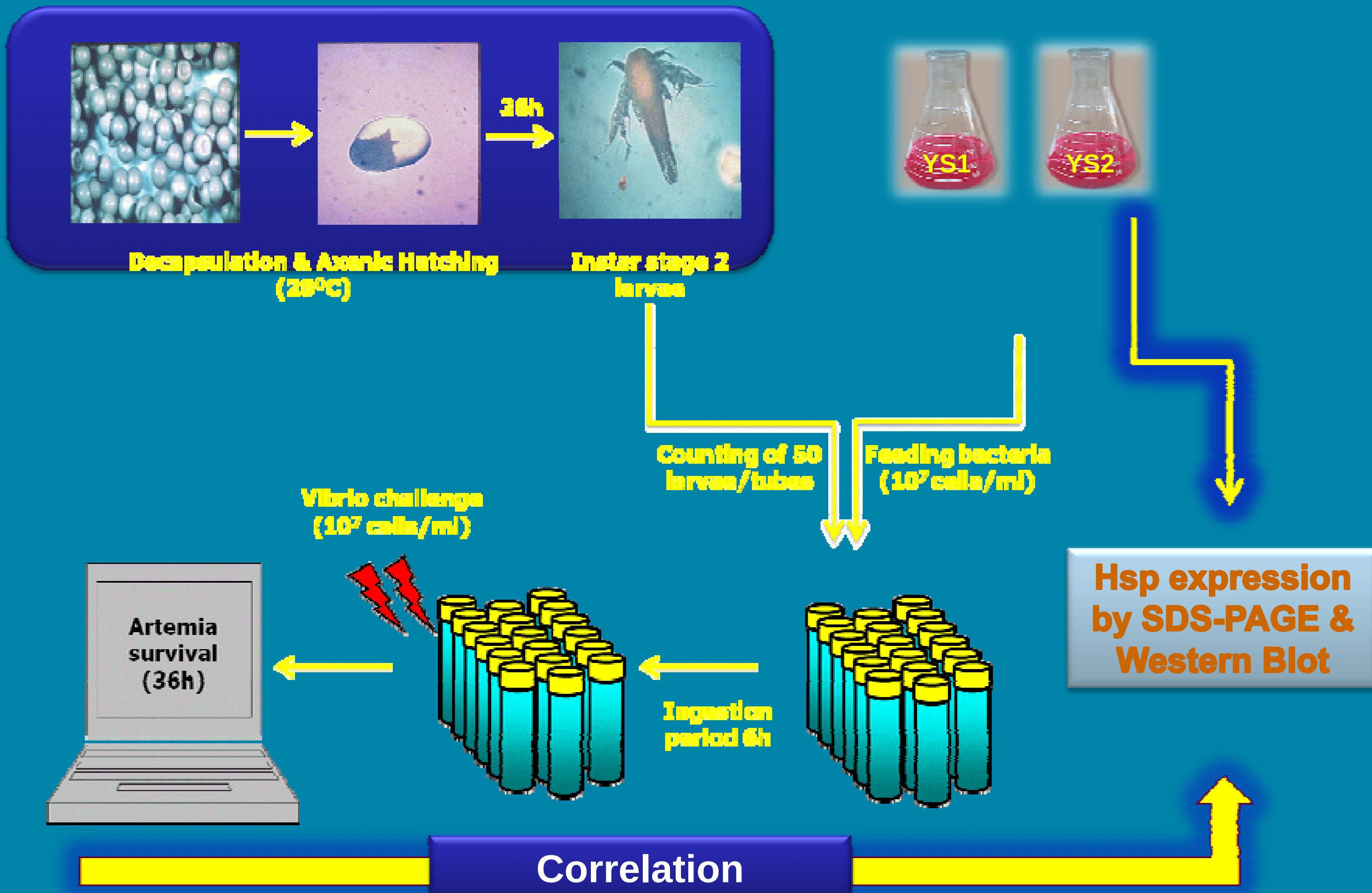


DnaK

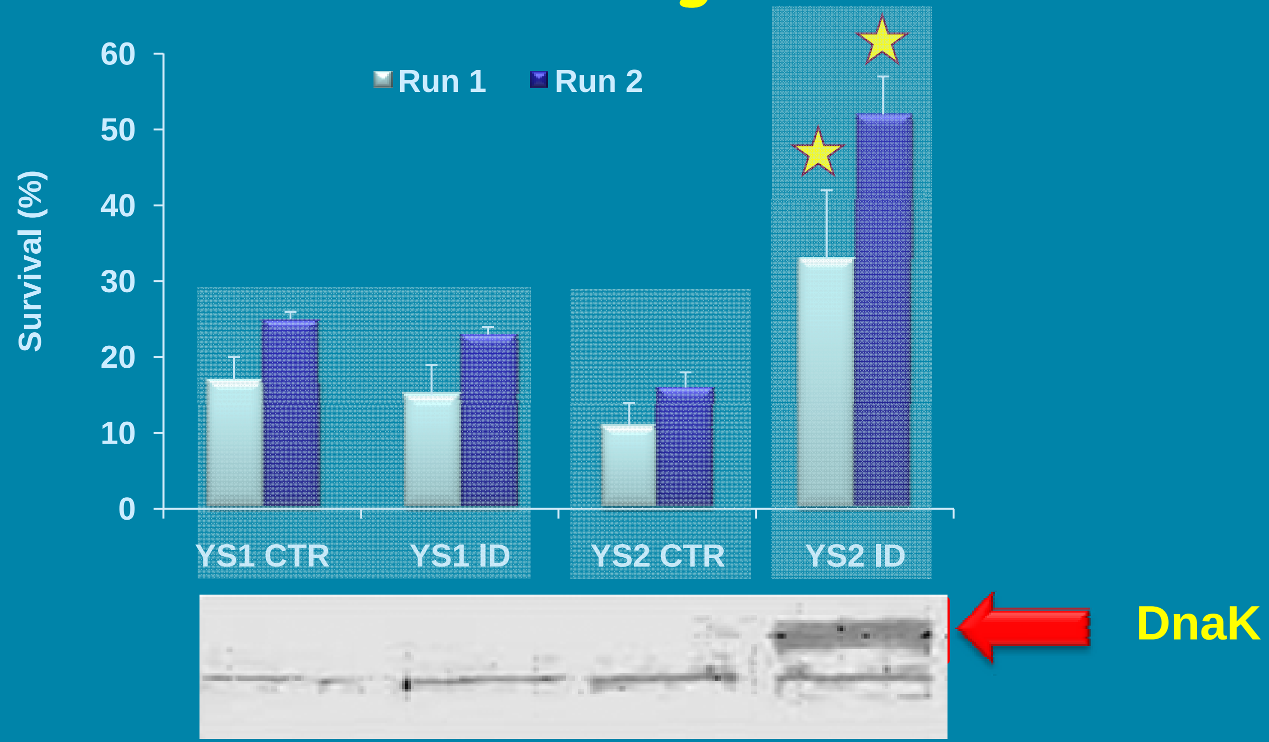
YS1: control strain, no DnaK overproduction

YS2: positive strain, DnaK overproduction by arabinose induction

Experimental design using GART

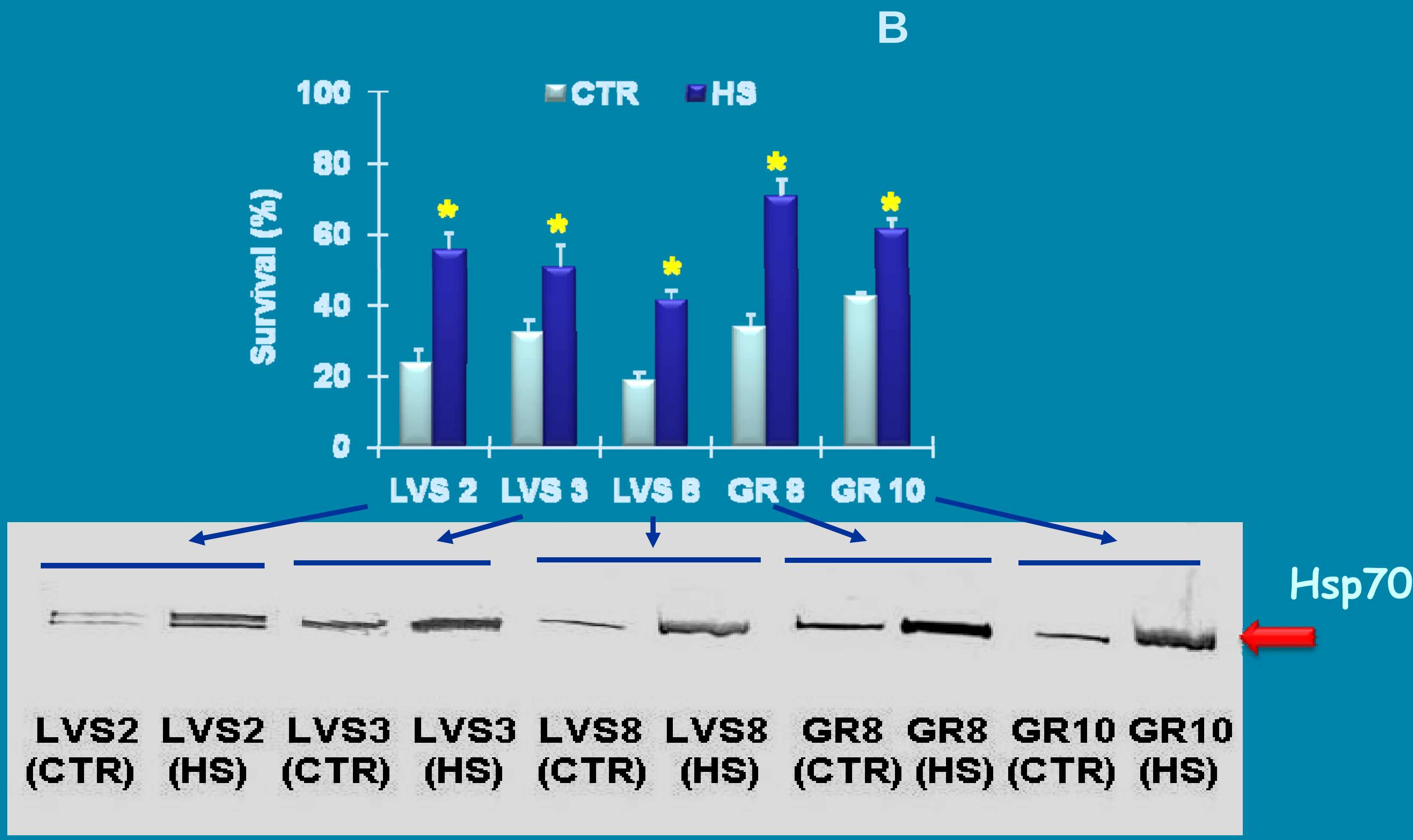


Enhanced resistance by DnaK feeding in a Vibrio challenge

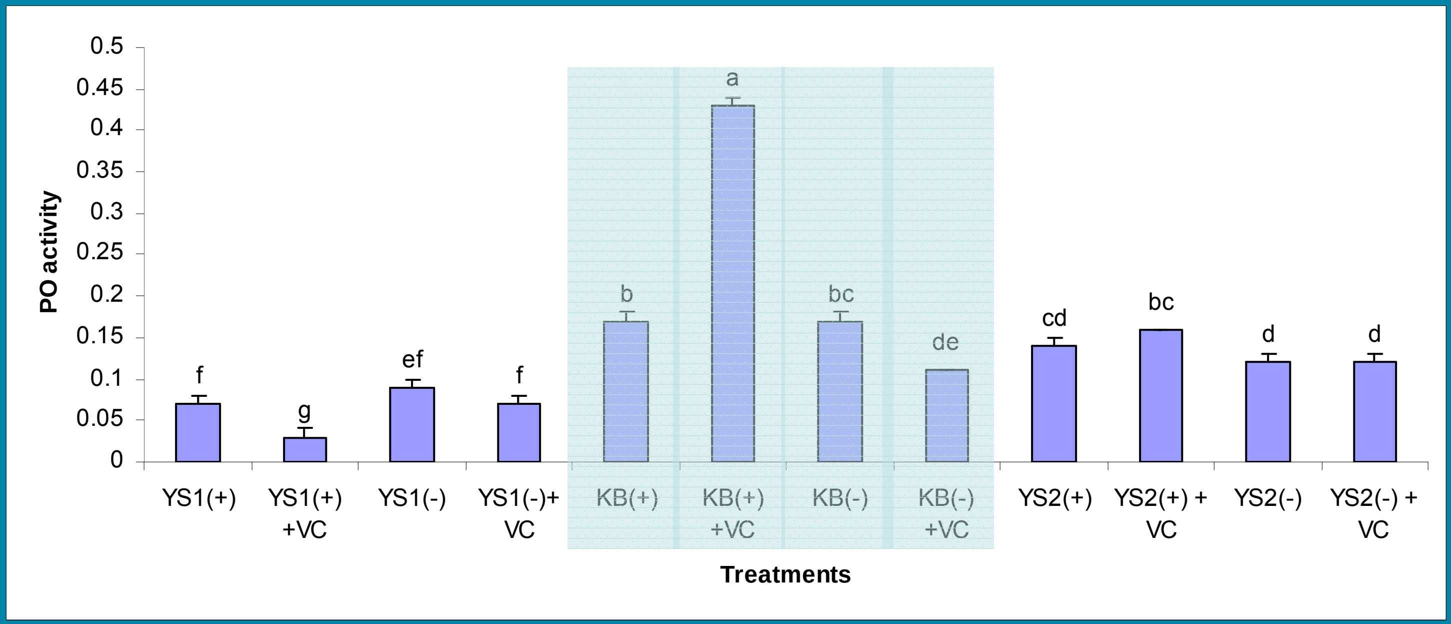


- Survival of *Artemia* larvae fed either induced or non-induced negative control strain YS1 was low.
- Survival of non-induced YS2 strains as in negative control
- A significant increase in survival in larvae fed with arabinose-induced DnaK overproducing YS2 were exposed to *V. campbellii*

Enhanced resistance by DnaK-homolog feeding in a Vibrio challenge



Phenol oxidase activity: 12 hours after challenge



- Effect of feeding HSP70
- Effect of challenge with *Vibrio campbellii*
- Effect of feeding HSP70 and challenge with *Vibrio campbellii*

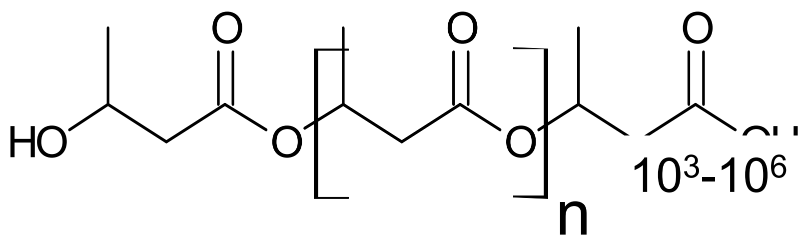
CONCLUSIONS HSPs

- **Exogenous HSPs feeding possibly triggers the *Artemia* innate immune response, producing anti-inflammatory activity (PO) which suppresses infection**
- **Sofar, no confirmation under non-gnotobiotic conditions**

POLY-β-HYDROXYBUTYRATE (PHB)

POLY-β-HYDROXYBUTYRATE (PHB)

- Linear polymer of β-hydroxybutyric acid



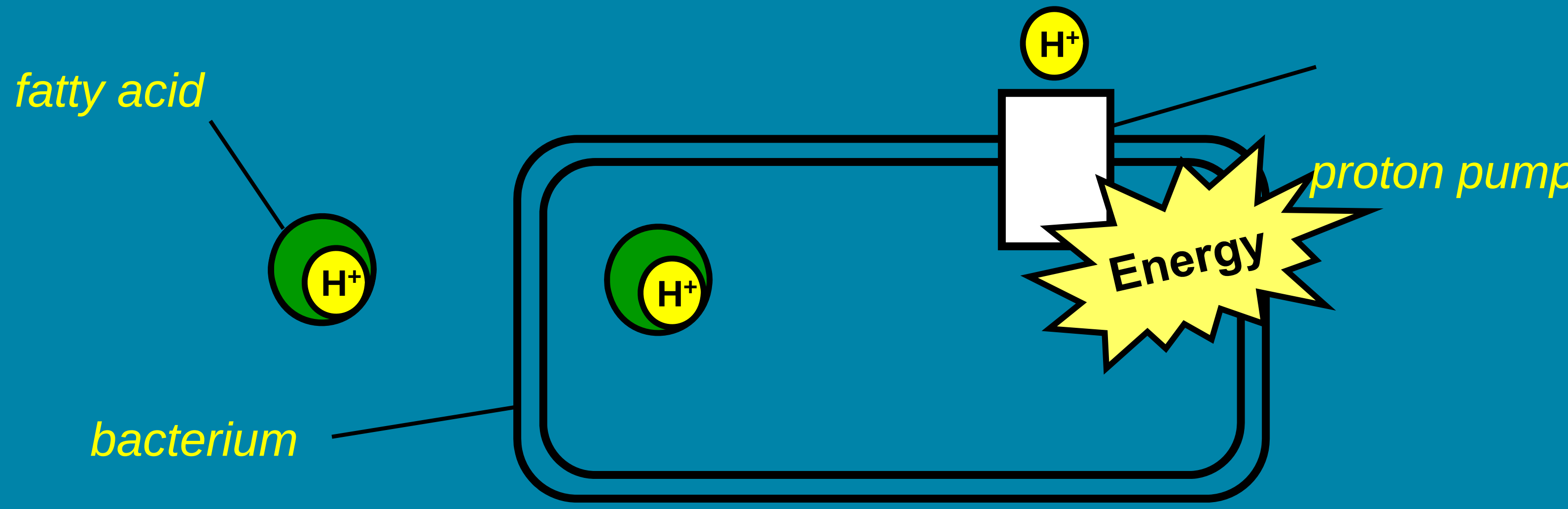
Could PHB also be used to protect *Artemia* from luminescent vibriosis?

POLY-β-HYDROXYBUTYRATE (PHB)

- Carbon and energy storage compound in bacteria
- Produced under nutrient limitation and excess carbon source
- Used as biodegradable plastic
  - Inert material (very slow desintegration)
  - Less stable in presence of digestive enzymes
  - Degraded by extracellular PHB depolymerases produced by some bacteria (e.g. Comamonas) and fungi

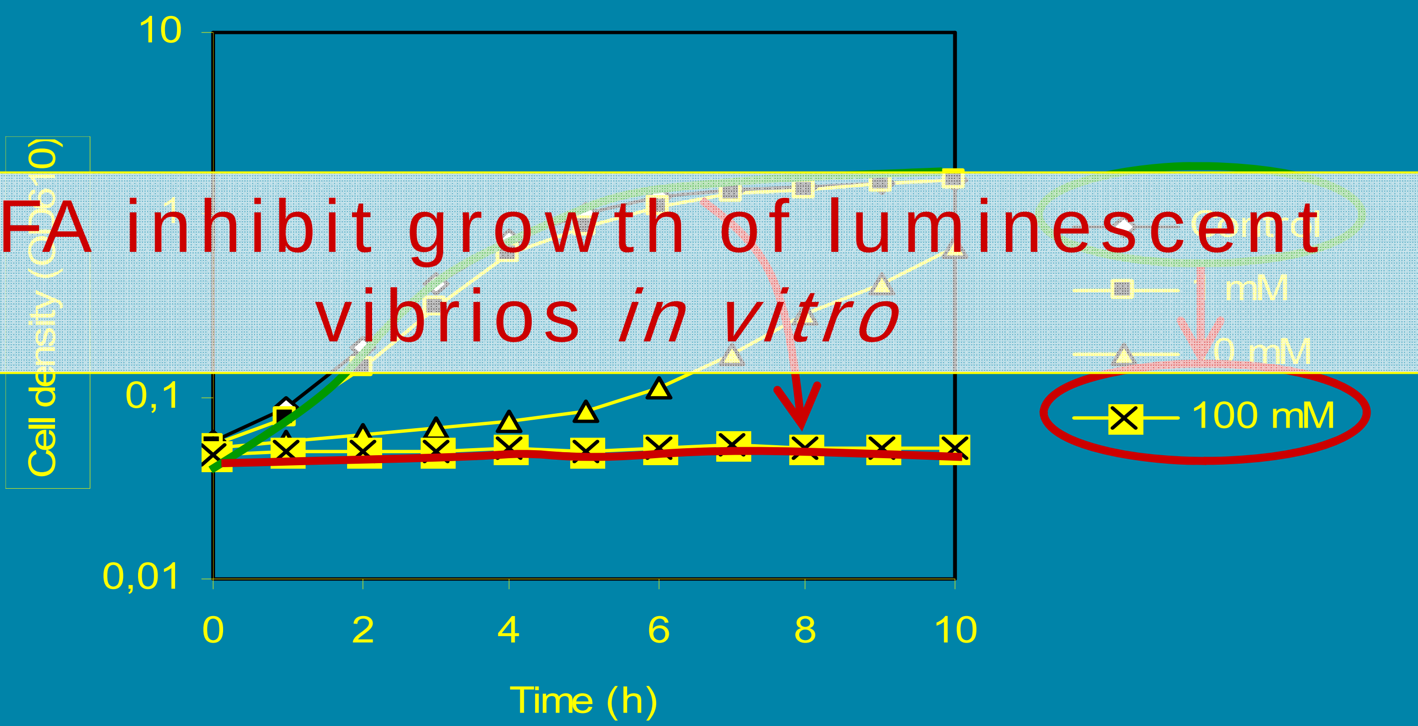
SHORT-CHAIN FATTY ACIDS

- Short-chain fatty acids (SCFA): formic, acetic, propionic, butyric and valeric acid
- Known to inhibit growth of enteric bacteria (*Salmonella, Klebsiella, Escherichia coli*)
  - Acidification of cytoplasm
  - Energy needed to keep internal pH optimal
  - Effect is pH-dependent (lower pH → higher effect)



SCFA in vitro: Vibrio inhibition

- Formic, acetic, propionic, butyric and valeric acid: all similar effect; effect is pH-dependent
- Example: valeric acid (pH 6)



SCFA inhibit growth of luminescent vibrios *in vitro*

SHORT-CHAIN FATTY ACIDS

Short-chain fatty acids (SCFA): formic, acetic, propionic, butyric and valeric acid

- Energy needed to keep internal pH optimal

Hypothesis: SCFA could protect shrimp from luminescent vibriosis.

- Effect is pH-dependent (lower pH → higher effect)

Source of energy for intestinal mucosa

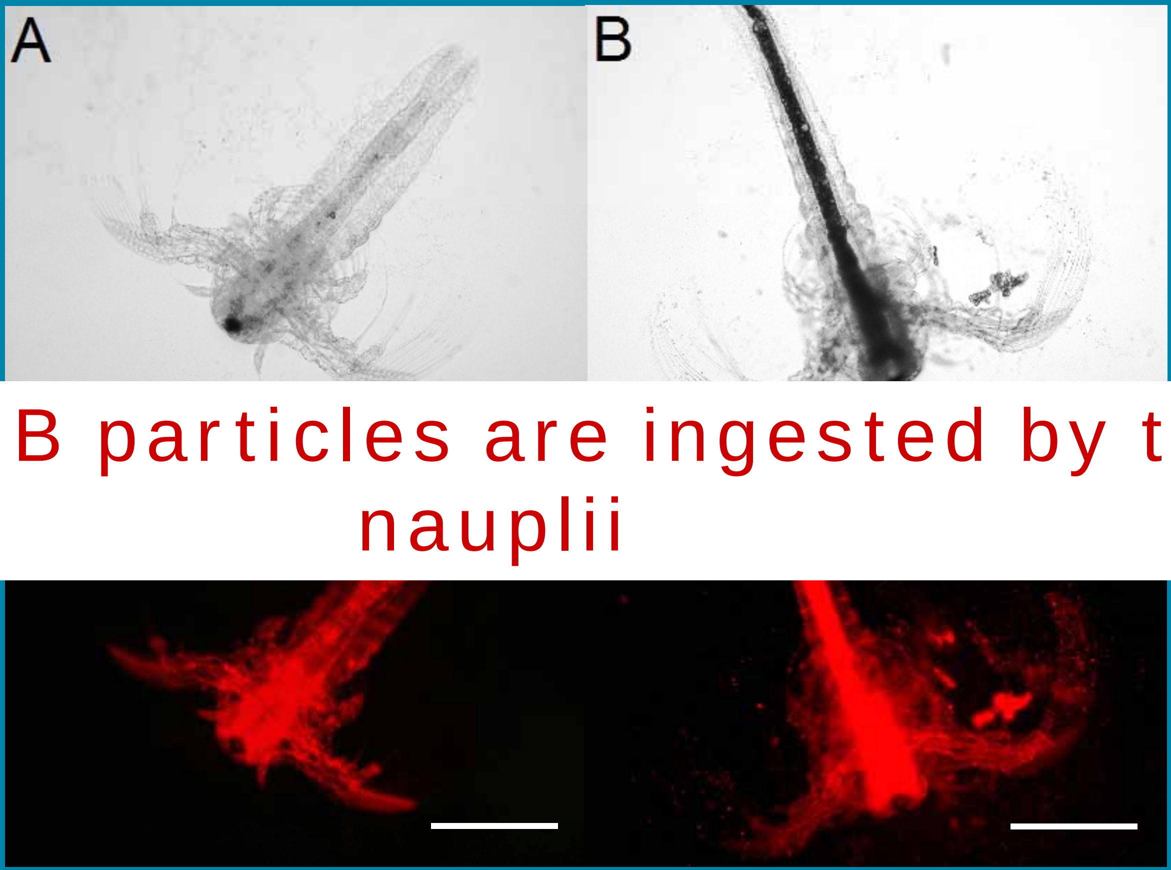
- Mucosa functions better
- More resistant to infection

PHB UPTAKE BY *ARTEMIA*

- Starved nauplii without feed or with PHB particles

Light microscopy

Fluorescence microscopy (Nile Blue)

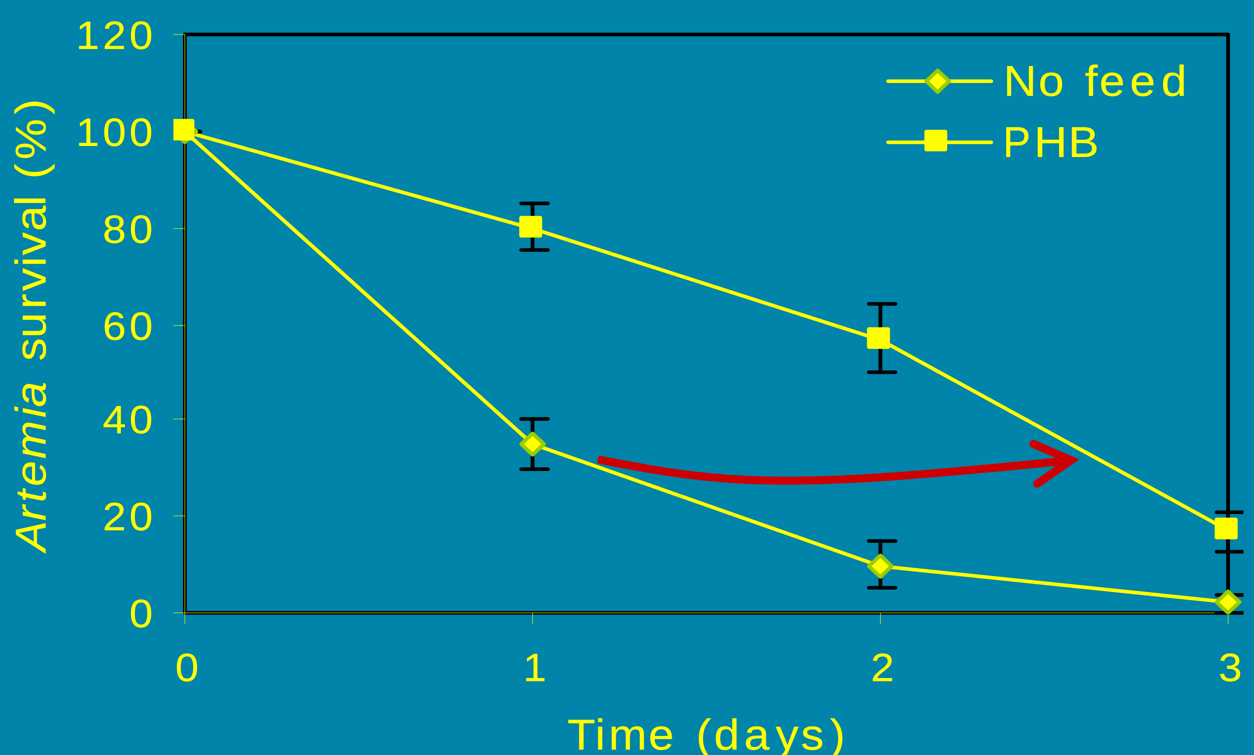


The PHB particles are ingested by the nauplii

(bar = 250 µm)

EFFECT ON STARVED ARTEMIA

- Sterile *Artemia* nauplii: no feed added or only PHB particles (1 g/l) a



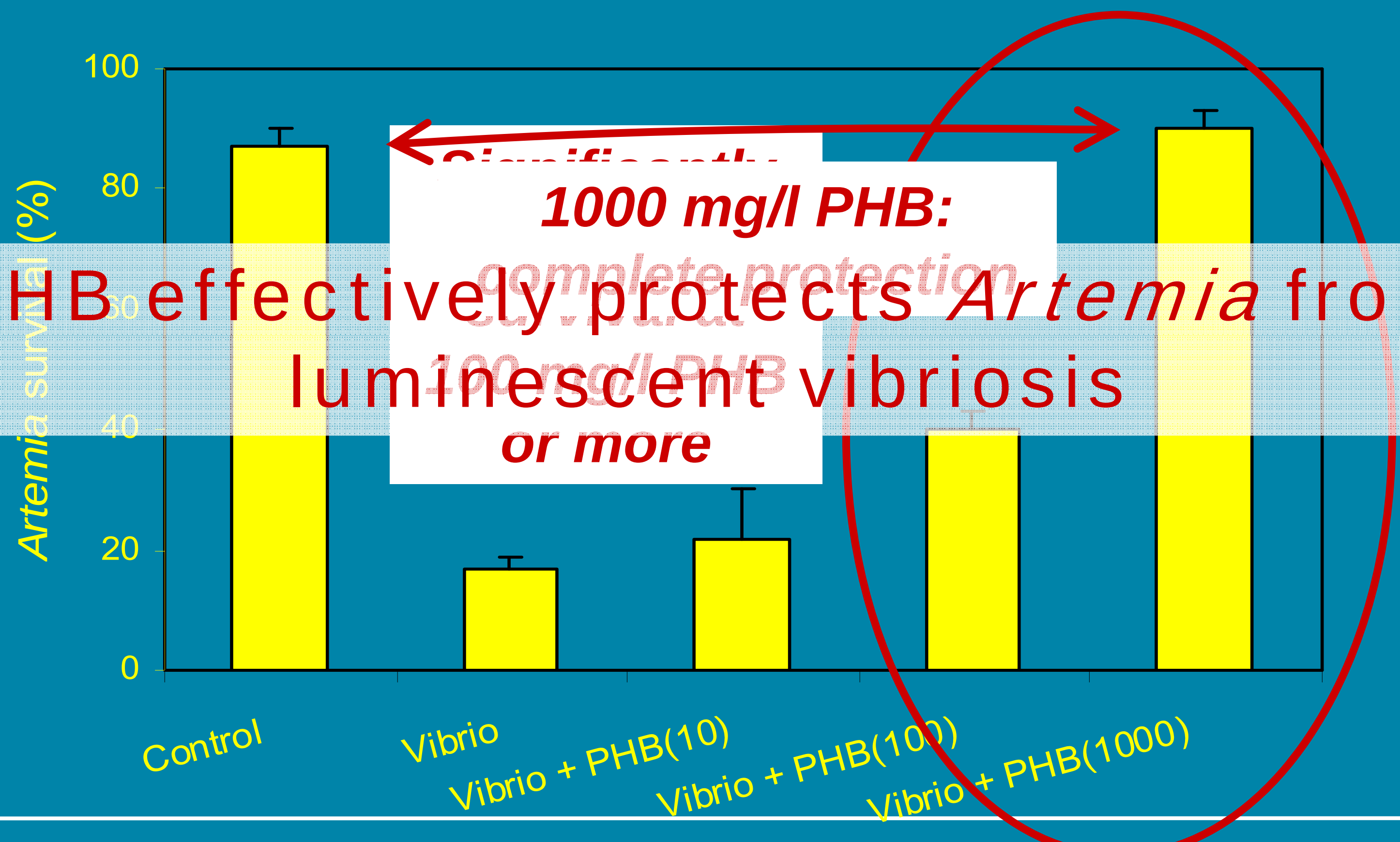
Longer survival with PHB particles

The nauplii can obtain energy from the particles

The particles must be (partially) degraded in the gut

EFFECT in GART: *Artemia* survival

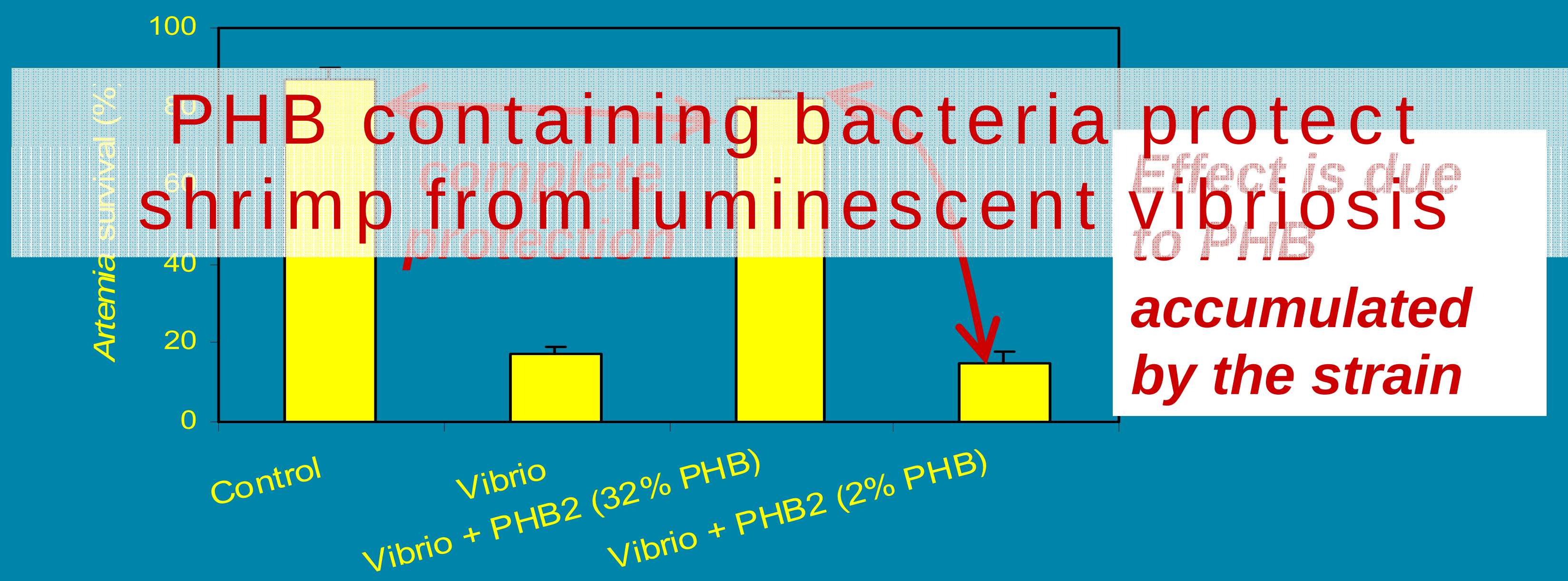
- *Artemia* nauplii challenged with *Vibrio campbellii*
- PHB particles added to culture water at start of test



PHB effectively protects *Artemia* from luminescent vibriosis

PHB-ACCUMULATING BACTERIA

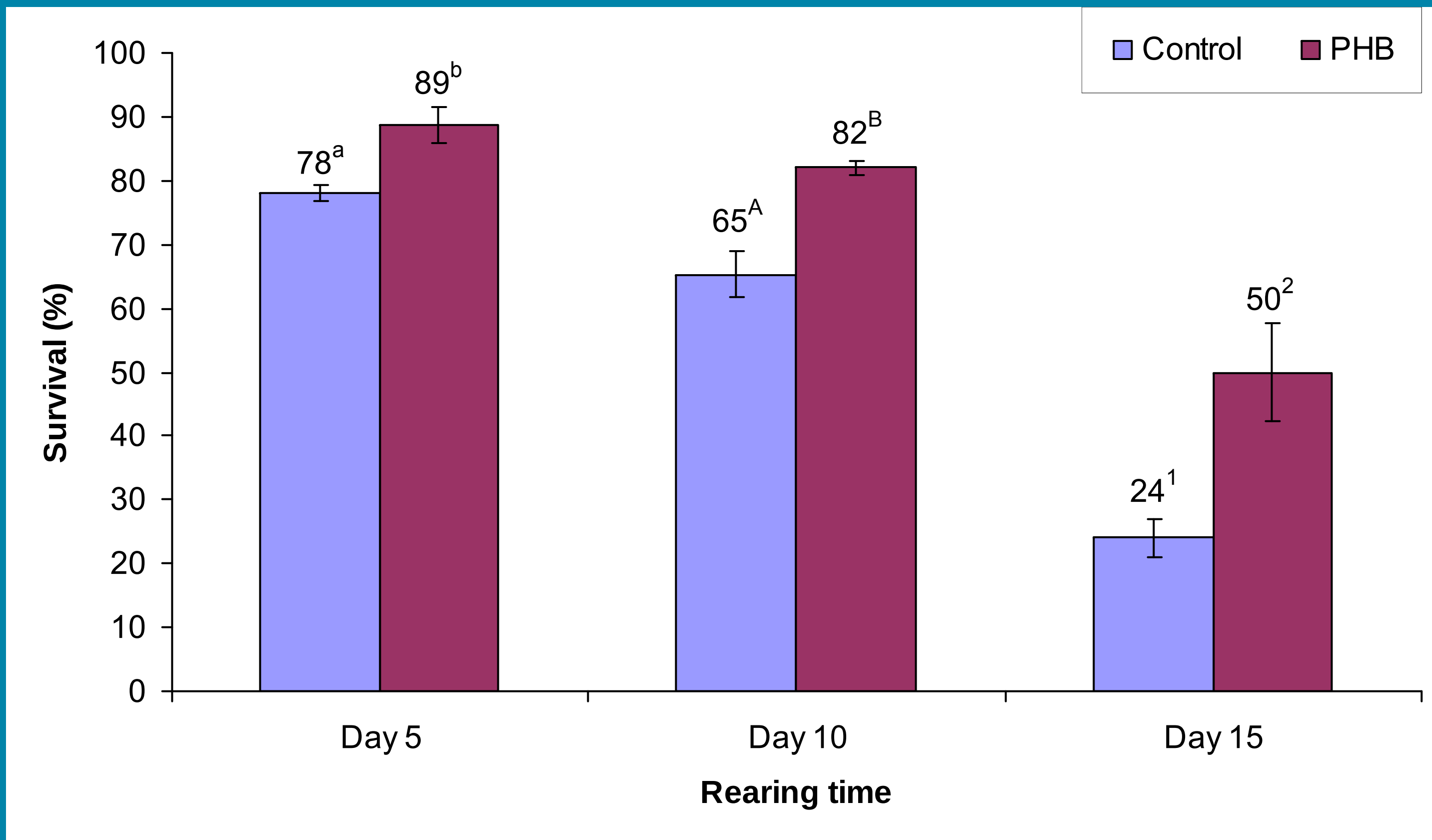
- *Brachymonas* strain PHB2 isolated from PHB-accumulating enrichment culture
- Fed-batch enrichment with PHB → 32% on VSS
- Strain PHB2 added at 10^7 cells/ml (~ 10 mg/l PHB)



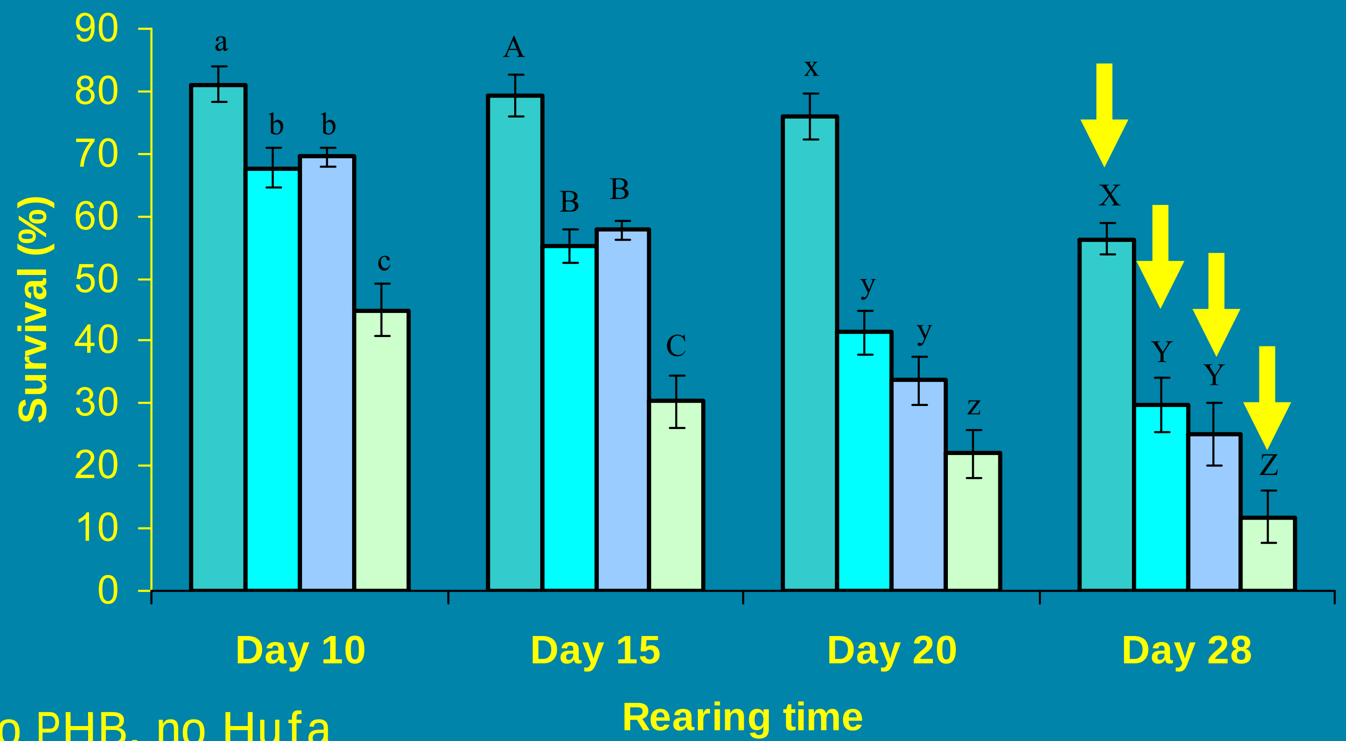
PHB: non-gnotobiotic

An *Artemia – Macrobrachium* food chain example

Macrobrachium larval survival feeding on PHB enriched *Artemia* nauplii



PHB combined with a classical HUFA enrichment



- No PHB, no Hufa
- No PHB, Hufa
- PHB, no Hufa
- PHB, Hufa

CONCLUSIONS PHB

- PHB particles protect *Artemia* from luminescent vibriosis
- In Artemia, PHB offered complete protection

  at 1 g/l
- In Artemia, PHB-containing bacteria also completely protected at dosage ~ 10 mg PHB/l
- Positive effects in the aquaculture food chain: but further verification is needed.

Quorum sensing

What is Quorum Sensing (QS)?

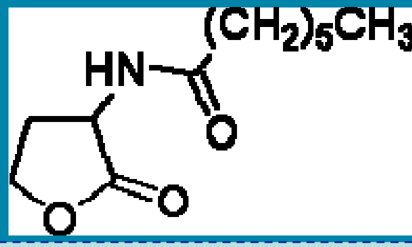
- QS: a mechanism by which bacteria regulate gene expression in response to their population density by producing, releasing and detecting small signal molecules (quorum sensing molecules) (Fuqua *et al.*, 1997).

- QS: process of bacterial cell-to-cell communication/conversation with signal molecules

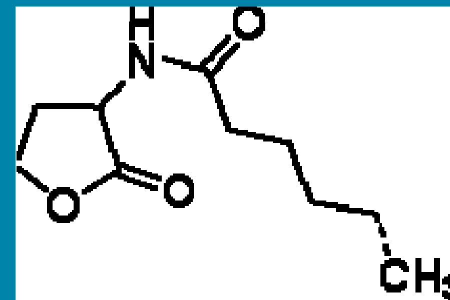
What type of processes are under the control of QS?

- Many bacterial behaviors are regulated by quorum sensing
  - luminescence
  - Symbiosis
  - Virulence
  - Antibiotic production
  - Biofilm formation

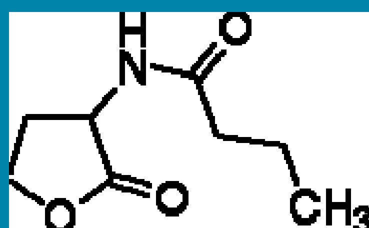
QS molecules: acyl homoserine lactones (AHL)



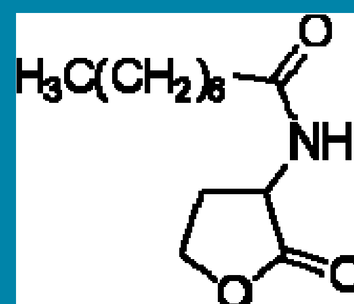
N-Heptanoyl-DL-homoserine lactone (C7-HSL)



N-Hexanol-DL-homoserine lactone (C6-HSL)



N-Butyryl-DL-homoserine lactone (C4-HSL)



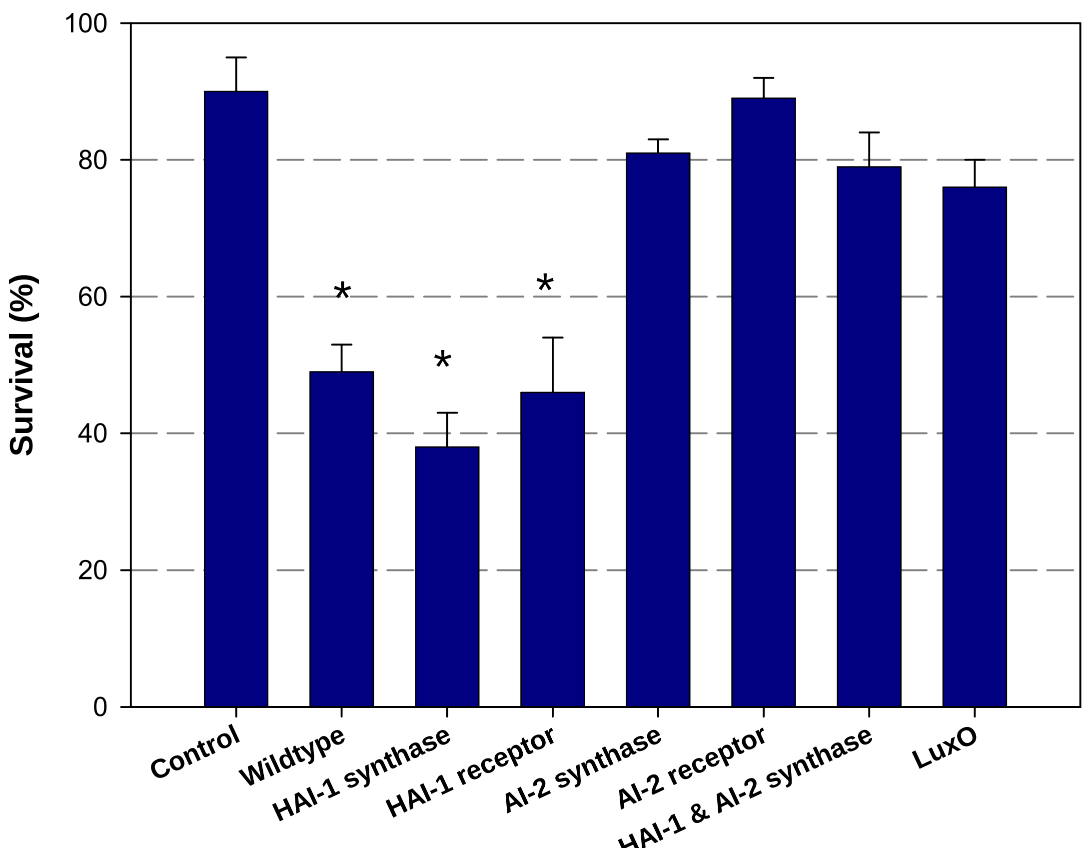
N-Octanoyl-DL-homoserine lactone (C8-HSL)

What is Quorum sensing (QS)?

Can we demonstrate that quorum sensing is important in microbial interference with (larval) stage of aquatic animals?

Artemia survival after challenges with mutants

of the wild type Vibrio harveyi strain BB120



*: significantly different from control (P< 0,01)

Conclusions

- QS regulates (AI-2-mediated) the virulence of the *V. harveyi* strain towards *Artemia*
- In this case, gnotobiotic conditions facilitate in the proof of principle

Quorum sensing: non-gnotobiotic

An *Artemia – Macrobrachium* food chain example

Effect of AHL mixture

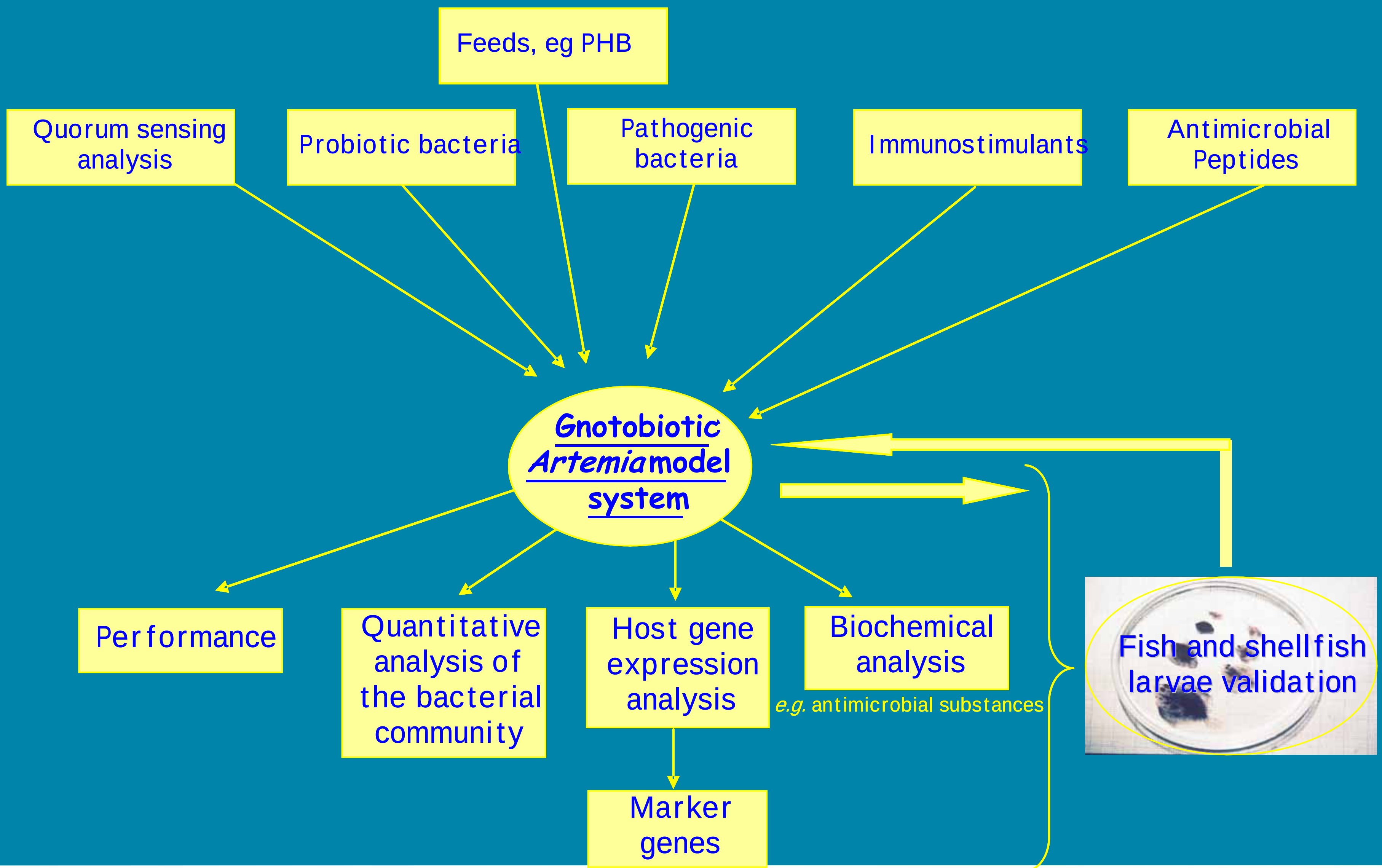
Treatments	Survival	LSI
Control	70.0± 4.2[b]	5.3 ± 0.4[b]
AHL_{mix1}	49.2 ± 2.6[a]	4.8 ± 0.3[a]

- Survival of *Macrobrachium* larvae on day 7 post-hatch, (mean ± SD, n = 6).
- daily AHL addition of ***1 mg/l***

Quorum sensing: conclusions

- QS is important in host-microbial interactions in the aquatic environment
- Data on <u>in vivo QS molecule concentration</u> are mostly lacking, necessary to further substantiate QS importance for an aquaculture setting

General conclusions



Acknowledgements

- BOF Ugent
- IWT
- VLIR
- EU
- BTC
- Staff of Ugent Aquaculture consortium

THANK YOU

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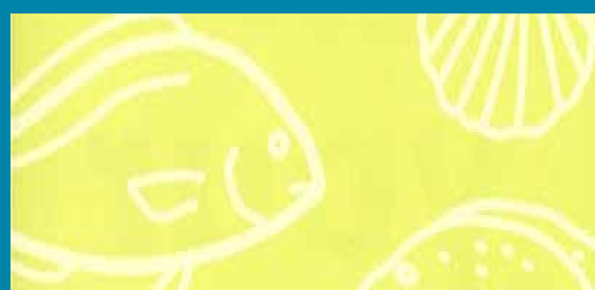

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