Impact of live versus dead bacteria on survival, growth and gene expression in Atlantic cod *(Gadus morhua)* larvae.

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Introduction

The use of probiotics is being investigated as a strategy to increase microbial control in cod farming and to reduce problems concerning the production of high quality larvae. The present work intends to explore the effects of live versus dead probiotic candidates on the survival, growth and gene expression in gnotobiotic Atlantic cod larvae.

Materials and methods

The cod eggs were disinfected once before arrival, and upon arrival washed in filtered (0.2 µm Micropore filter) autoclaved seawater (FASW). The eggs were then disinfected once more,for 10 minutes in FASW with glutaraldehyde (final concentration 400 mg l-1) The eggs were washed in FASW, and transferred to large petri dishes with FASW added 10 ppm ampicillin and rifampicin. The larva were reared in 2 litre bottles (30 larvae l-1) with aeration, and fed bacteria free rotifers, *Brachionus* "Nevada", from day 3 to day 17. Bacteria free *Isochrysis* sp. algae was added to achieve green-water, and also served as feed for the rotifers.

Three treatments were tested: bacteria free larvae (Control), live *Microbacterium* sp. ND2-7 (two replicates abbreviated LM1 and LM2) and dead *Microbacterium* sp. ND2-7 (abbreviated DM). The bacteria were added to a total concentration of 10^6 CFU ml-1.
On day 17 post hatching, larvae were sacrificed using metomidate (0.5g l-1, lethal dose). Length of individual larvae was determined by measurements from phtotes taken. RNA was isolated and the gene expression of 8 different genes was quantified by means of real-time PCR. The results were normalized to the expression of the housekeeping gene *β-actin*.

We also conducted a short term experiment with 3 different probiotic candidates, testing various mixtures of these, one known pathogen served as negative control. The larvae in this experiment were reared in 50ml bottles (30 larvae 50ml-1), without aeration and feeding. The experiment was terminated after 5 days. The expression of two genes, *C3* and *Cyp1a1*, was quantified by real-time PCR.

Selected genes for qPCR

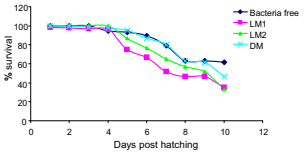
Immune response
C3
Serum lectin (CLECT)
Metabolism
Tryptophanyl t-RNA synthetase (Wars)
Angiopoietin (Fiaf)
Farnesyl diphosphate synthetase (Fdps)
Stress response
Gluthatione peroxidase (Gpx)
Cell adhesion
Integrin (Itgb1bp3)
Xenobiotic metabolism
Cyp1A1

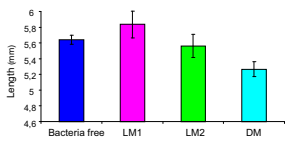
Rearing bottles, first feeding experiment

Results

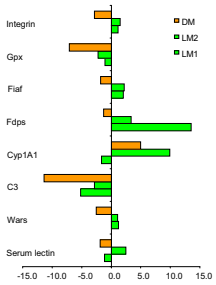
First feeding experiment

On day 17 post hatching the survival was higher in the control treatment (57 %), than in the treatments with live *Microbacterium* sp. (35 and 33 %). There were no significant difference between the control and the treatment with dead *Microbacterium* sp. (47 %). At day 17 the larvae from the first feeding experiment given live *Microbacterium* sp. (LM1) were significantly longer than the larvae from the treatment with dead *Microbacterium* sp. There were no differences in larval length between the other treatments.





qPCR-results, first feeding experiment

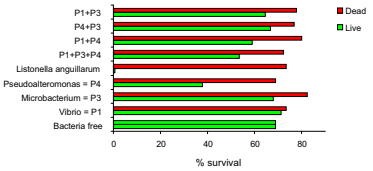


Compared with the bacteria free control treatment, the expression of most of the selected genes were up regulated with addition of live bacteria, and down regulated with addition of dead bacteria.

For *Cyp1a1* and *Serum lectin* the fold changes varies also between the replicates with live bacteria.

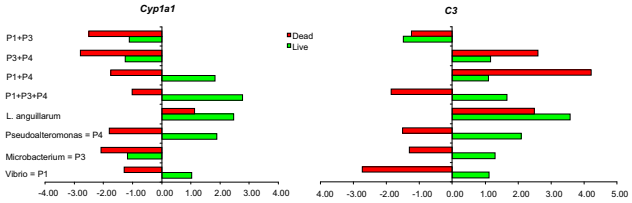
Short term experiment

The survival was higher for the treatments with dead probiotic candidates than for those with live probiotic candidates.



qPCR-results, short term experiment

Fold change in gene expression relative to the bacteria free control treatment.



Cyp1a1 seems to be down regulated by the addition of dead bacteria, with the exception of dead *L. anguillarum*.

C3 seems to be up regulated by the addition of live bacteria, with the exception of live P1+P3.

Conclusions

For both experiments, the survival was slightly reduced by the presence of live bacteria. In addition the expression of the selected genes seems to be up regulated by live bacteria and down regulated by dead bacteria.

There is some variation in gene expression between the replicates used in the first feeding experiment, this indicates that although the microbial conditions were the same, other environmental factors were probably different enough to influence gene expression.

In the short term experiment the treatment with live *Pseudoalteromonas* sp. gave lower survival compared to the other treatments, indicating that this might not be a suitable probiotic for cod larvae. Increasing the number of strains added did not improve survival compared to the addition of one strain. However as this system is very simplified compared to the "real world", the impact on survival of these probiotic candidates needs further investigations.

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