Aquafeed probiotics – Challenges and Opportunities (and Solutions)

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BIOMIN
Probiotics

...a tool to improve microbial balance

‘Live microorganism which when administered in adequate amounts confer a health benefit on the host’ – FAO/WHO 2002

Intestinal probiotics

- Immunity
- Nutrition
- Development of GIT

Pond probiotics

- Bioremediation (water)
- Bioaugmentation (soils)
- Biocontrol (pathogens)
Probiotics in aquafeeds

Google Scholar → ‘insert here’ probiotic aquafeed

Google Scholar

Articles

About 579 results (0.05 sec)

Bacterial probiotics

- Bacillus
- Lactobacillus
- Pediococcus
- Leuconostoc
- Lactococcus
- Enterococcus
- Carnobacterium

Bacillus spp.

- B. subtilis
- B. pumilus
- B. toyoi
- B. clausii
- B. cereus
- B. licheniformis
- B. amyoliquefaciens

Yeast

- Saccharomyces
- Debaromyces
- Rhodotorula
Challenges

1. Lack of reproducible results (efficacy)

2. Intestinal colonization of intestine

3. Application (Heat stability)

4. Registration
Enzyme production with *Bacillus*

Protease

Protease enzymes for protein digestion

High protein requirements of aquatic species

Protein is the most expensive part of the diet

Trends to use more plant proteins reduces digestibility
Enzyme production with *Bacillus*

Amylase

Amylase enzymes for carbohydrate digestion

Typically aquatic animals cannot utilize carbohydrates

If amylase producing *Bacillus* are ingested then they can contribute to carbohydrate digestion
Enzyme production with *Bacillus*

Cellulase

Cellulase enzymes for cellulose digestion

Cellulose is the most abundant organic polymer on Earth
- cell walls of plants
- cell walls of algae & phytoplankton
- oomycetes (water moulds)
Pathogen inhibition

Probiotics can produce substances that:
- Kill pathogens
- Inhibit pathogen growth

Probiotic

Bacteriocins/
acids

Pathogen

Inhibition zone

Pediocin

Reuterin

Enterin
V. parahaemolyticus inhibition

% viability of V. parahaemolyticus

V. parahaemolyticus
B. subtilis #3
B. subtilis # 4
B. subtilis # 5
B. subtilis # 2
B. subtilis # 6
B. subtilis # 1
B. licheniformis # 3
B. licheniformis # 2
B. licheniformis # 1
B. cereus # 1
B. cereus # 2
B. pumilus
Pediococcus acidilactici
Paracoccus pantotrophus
Enterococcus faecium
Lactobacillus reuteri
Ralstonia eutropha
Quorum sensing in aquaculture

Coordinate group activities:
Virulence
Survival
Bioluminescence
Swarming
Biofilm formation

Language = different chemical molecules
Bacterial languages

**Oligopeptide (gram +)**

*Staphylococcus, Clostridium, Listeria, Enterococcus*

H-Tyr-Ser-Thr-HN

group I *S. aureus* peptide thiolactone

**AI-2 (gram + & -)**

*Universal*

autoinducer 2 (AI-2)

**Acyl – Homoserine lactones AHLs (gram -)**

- N-acyl-L-HSL
- N-(3-oxoacyl)-L-HSL
- N-(3-hydroxyacyl)-L-HSL

**Diffusible signal factor (gram -)**

*cis-11*-methyl-2-dodecenoic acid diffusible signal factor (DSF)*

*Xanthomonas, Stenotrophomonas, Burkholderia, Xylella*

**Quinolone (gram -)**

2-heptyl-3-hydroxy-4-quinolone (PQS)

*Pseudomonas*

**Naturally ahead**
Natural quorum quenchers

Produced by:
- Algae
- Plants/phytogenics
- Bacteria

Enzymes

AHL lactonase - *Bacillus* spp.

AHL acylase (amidase)

Signal molecule

Lactonase

Acylase

Fatty acid + Homoserine lactone
Quorum quenching

Cut the ‘phone lines’

- Enzymes which degrade communication molecules

- No more ‘talking’ leads to…
  - Uncoordinated behaviour
  - Reduced virulence – disarms pathogens by interfering with gene expression
  - Easier to control/ kill
Quorum quenching - bioluminescence

Single cell - few molecules
**NO luminescence**

Many cells – threshold of molecules
**Luminescence!**

Destruction of signal molecules by other species

Many cells – too few molecules
**NO Luminescence**
**Bacillus sp. and quorum quenching**

*Bacillus* supernatant extract

**Quorum quenching**

*V. harveyi* BB120 $\rightarrow$ light production depends on QS
- More light = more bacterial ‘talk’
Probiotics and epithelial colonization

FACS flow cytometry

- Probiotics cultured in appropriate medium
- Bacterial cells harvested, washed and stained with CellTracker™ Green
- RTgill-W1 cells grown to subconfluence in wells
- Labelled bacteria added to epithelial cells at $10^7$ cells per well. Heat inactivated bacteria also added at same concentration
- Incubation
- Non-adhered cells were washed away and collected for FACS analysis
- Attached probiotics detached from epithelial cells with trypsin/EDTA
- Bacterial cells resuspended and fixed
- Analyzed with flow cytometer

P2 = Gill cells
P4 = Bacterial cells
P3 = Instrument ‘noise’
Probiotics and epithelial colonization

Lactic acid bacteria can adhere to epithelial cells…

- *Lactobacillus* best at attaching to epithelial cells, followed by *Pediococcus* and *Enterococcus*
- *Bacillus* cannot attach
- Whilst heat inactivated probiotics can attach, the efficacy is considerably lower than their ‘living’ counterparts
TLR recognizes intestinal pathogens via PAMPS.

When TLR’s are activated, adaptor proteins such as MYD88 are recruited.

MYD88 recruitment initiates IκB activation and it is converted to the transcription factor NFκB.

NFκB enters the nucleus where it switches on genes resulting in the transcription of pro-inflammatory cytokines.
Probiotics in fish – *in vivo* trial

Improved growth performance...

**Treatments:**
1. Basal diet
2. Basal diet + *AquaStar® Growout* (3g kg\(^{-1}\))

**Duration:** 6 weeks

**Species**
*Nile tilapia (Oreochromis niloticus)*

**Initial weight:** 29g

**Replication:** \(n = 3\)

- Faster growth – quicker time to market
- Bigger fish
- Better feed efficiency
  - ↓ feed costs
  - ↓ waste = ↑ water quality
- Higher profitability!

**Location:** Plymouth University
Probiotics in fish – *in vivo* trial

…stronger intestinal barrier…

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Aquastar®</th>
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<tbody>
<tr>
<td>Goblet cells (per 100 µm)</td>
<td>4,77 ± 1,46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7,55 ± 2,49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IEL’s (per 100 µm)</td>
<td>29,50 ± 4,59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40,95 ± 7,04&lt;sup&gt;b&lt;/sup&gt;</td>
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IELs (Intra epithelial leucocytes) – white blood cells which will attack pathogens if they breach the epithelia

Goblet cells produce intestinal mucus – this provides a physical and chemical barrier

↑ Goblet cells + ↑IELs = ↑ Intestinal barrier = ↑ Protection = ↓ Disease
Probiotics in fish – *in vivo* trial

...and improved immunity

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Intestine</th>
<th>Head kidney</th>
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<tbody>
<tr>
<td>TLR2</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>TNFα</td>
<td>↑</td>
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<tr>
<td>IL-1β</td>
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<td>IL-10</td>
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Immunity gene involved in the recognition of pathogens

Pro-inflammatory genes

Anti-inflammatory genes
Probiotics in fish – *in vivo* trial

...interpreting the data

- Up-regulation of TLR2 may result in **improved recognition of pathogens**, particularly Gram-positive (*Streptococcus*).

- Up-regulation of pro-inflammatory genes is indicative of a fish with **superior immune readiness**.

- Up-regulation of anti-inflammatory genes prevents an **excessive inflammatory response** and is also linked with **mucosal tolerance**.
Probiotics in shrimp – *in vivo* trial

- **Objective:** Evaluate the effect of commercial probiotic in Pacific white shrimp through different feeding regimes
- **Treatments ($n = 7$):** Defined below
- **Duration:** 12 weeks
- **Feeding:** 6 times per day up to satiety
- **Initial weight:** $1.13 \pm 0.01$g
- **Challenge:** *V. parahaemolyticus* (IM injection $4.8 \times 10^4$ CFU)
- **Location:** ACAN, Vietnam

<table>
<thead>
<tr>
<th>Group</th>
<th>Group code</th>
<th>AquaStar® Growout</th>
<th>Feeding regime</th>
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<tr>
<td>Control</td>
<td>1</td>
<td>-</td>
<td>Control feed - continuously fed in a separate RAS</td>
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<tr>
<td>Continuous</td>
<td>2</td>
<td>3g/kg Feed</td>
<td>Supplemented feed - continuously fed</td>
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<td>Pulse 1</td>
<td>3</td>
<td>3g/kg Feed</td>
<td>Pulsed: 1 week fed with supplemented feed - 1 week fed with control feed</td>
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<tr>
<td>Pulse 2</td>
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<td>3g/kg Feed</td>
<td>2 week fed with supplemented feed - 2 week fed with control feed</td>
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<tr>
<td>Pulse 3</td>
<td>5</td>
<td>3g/kg Feed</td>
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Probiotics in shrimp – *in vivo* trial

Improved growth performance...

![Graphs showing final weight, weight gain, SGR, and FCR with different treatments labeled as a, b, or ab.](image-url)
Probiotics in shrimp – *in vivo* trial

...and better disease resistance

**BIOMIN recommendation:** continuous probiotic supplementation significantly improves growth AND maximizes disease resistance
Heat stability - solutions

Overcoming heat stability…

• *Bacillus* probiotics have built in ‘protection’ via spores
  …but benefits may be limited to growth
  …surviving feed manufacture does not guarantee germination in intestine

• Dead or inactivated probiotics
  …but benefits are limited to immunity (and not always!!)

• Encapsulation
  …but applications in extrusion are limited

• **Post pellet application**
  …but adds extra step in feed manufacture
Improving the environment to benefit the animal
Summary

- Probiotic species generally dictates the benefits that one can expect

- *Bacillus* spp. can produce enzymes, but probiotic capacity is strain specific

- LAB can control pathogens and colonize epithelia where they modulate immunity and improve disease resistance

- Multi–species approach maximizes host benefits
  - Nutrition
  - Intestinal barrier function
  - Immunity
  - (Environment)

- Heat stability problem can be overcome – PPLA offers users greatest flexibility