Effective use of probiotics in Aquaculture, lessons from South Asia

Immuno-Modulators in Aquaculture ‘IMAQulate’

Introduction

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University of Stirling, UK
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19 Nov 2018
Problem Background

• Growing international market for seafood driving small-holder intensification
• Diagnostics & health management a major challenge
• Restrictions on chemo-therapeutants driving demand for prophylaxis - linked to antimicrobial residue & AMR regulation
• Growing reliance on immuno-modulators including pre & probiotics of uncertain provenance & efficacy – especially shrimp (low cost vaccines for finfish in development)
• Poorly regulated market - burden of unjustified claims, poor quality assurance and advice on small-holders
Prophylactic Health Products (PHP) Scope

• **Probiotics:** live micro-organisms, administered in adequate amounts, conferring health benefit(s) on the host
  • **In-feed:** ‘gut micro-biome’
  • **Water & soil application:** ‘pond micro-biome’ – bioremediators

• **Pre-biotics:** substances promoting growth of beneficial microorganism in intestines (bacteria, fungi) , conferring host health benefits

• **Synbiotics:** nutritional supplements inc. combination pre & probiotics for synergistic effects (selective growth/ activation)

• **On-farm alternatives to commercial products:** ‘biofloc’, ‘fermentation’

1 Roberfroid 2007 2 WHO 2001
Research Foci

1. Inventory & pedigree analysis of commercial prophylactic health products (PHPs)
   - Primary focus on probiotics and prebiotics
   - Value chain surveys: chemical outlets, hatchery & farmer health management
   - Laboratory analysis: ingredient claims, AMR & anti-microbial contamination
   - Evolution of PHP supply chain, structure, regulation, market failures?

2. Cost-benefit assessment of selected ‘higher-pedigree’ PHPs in on-farm trials
   • PHP treatment during extended, bio-secure nursing
   • Post-treatment follow-up in grow-out ponds/cages

3. Development of novel prebiotics

4. Communication strategy: targeting small-scale intensive producers (primary beneficiary), PHP suppliers, regulators
<table>
<thead>
<tr>
<th>Country</th>
<th>Region</th>
<th>Shrimp</th>
<th>Finfish</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td>Andhra Pradesh</td>
<td><em>L. vannamei</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>West Bengal</td>
<td><em>P. monodon</em></td>
<td></td>
</tr>
<tr>
<td>Bangladesh</td>
<td>Khulna</td>
<td><em>P. monodon</em></td>
<td>Tilapia</td>
</tr>
<tr>
<td></td>
<td>Mymensingh</td>
<td></td>
<td>Pangasius</td>
</tr>
<tr>
<td>Kenya</td>
<td>Lake Victoria</td>
<td></td>
<td>Tilapia</td>
</tr>
</tbody>
</table>
India Shrimp Production Trends; Country & Region

Andhra Pradesh

L. vannamei

https://aquaaquaria.com/Aquaculture-Production
Industry Dynamics – Shrimp AP

Key Features of a Growth Industry
- Revenue grows faster than the economy
- Many new companies enter the market
- Rapid technology & process change
- Growing customer acceptance of product
- Rapid introduction of products & brands

Shrimp AP

Bangladesh Shrimp

Tilapia Kenya

Adapted: IBIS 2016
Growing Pains....

• Quantity Growth Phase:
  • 2009 experimental introduction *L. vannamei*
  • CAA reg. hatcheries/ farms, strict import SPF broodstocks authorized suppliers
  • +2014 expansion more unregulated.....
  • Land conversion from agriculture to shrimp ponds
  • Est. 5,000 hectares of illegal ponds in AP (MPEDA)
  • Farm level problems: seed quality, biosecurity, disease, antibiotic residues; esp. small-holders
  • EU reg. Oct 2016: Antibiotic tests on 50% Indian shrimp shipments (prev. 10%)

• Quality Growth Phase?: Improved regulation, statutory & market based
  • CAA aquaculture product registration
  • MPEDA farm registration
  • Inc. individual & group certification efforts
Shrimp Farms in AP: CAA Reg. 2017
Farming concentrations: Godaveri & Krishna Rivers
Year CAA Farm Registration by District & Size Class

- **10-163ha**
- **<10ha**
- **<5ha**
- **<2ha**
- **<1ha**
- **<0.5ha**

The graph shows the frequency of Year CAA farm registrations by district over various years, with specific regions highlighted.
CAA Registered Aquaculture Inputs 2017 Probiotics

Bangladesh
Supply chain Bangladesh

- 7 main importers
  - Diverse business interests
  - Train shops and farmers
- No regulation

![Diagram of supply chain with nodes and arrows indicating flow from manufacturer to importer, licensed distributor, dealer, retailer, intensive farmers, and traditional farmers. The diagram also indicates the flow outside BD and inside BD, with Foremost India highlighted.]

[Logos of Novartis, Eon Group, Renata Limited, ACI, Fishtech, Square Pharmaceuticals Limited, and Eskayef Bangladesh Limited are displayed on the left side of the diagram.]
Probiotic ‘Pedigree’ Analysis

• Quality assurance indicators (9): ‘high – low’ risk assignment
  • Qualitative/ quantitative bacterial declarations (>1x10⁵)
  • Certification quality standards (ISO 9001, GMP, HACCP, ISO 22000)
  • Regulatory domain
  • CAA registration (India) = ‘antibiotic free’
  • AMR testing
  • Efficacy claims logic

• Laboratory analysis ‘high’ & ‘low’ risk products
  • Bacterial concentration (TSA culture) & composition (16s RNA)
  • Anti-microbial susceptibility (resistance) & contamination testing
    
    Amoxicillin, Oxytetracycline, Erythromycin, Vancomycin, Doxycycline, Chloramphenicol, Furazolidones, Oxalic Acid, Florfenicol (n=15)
## Analytical Results (n = 68)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>‘Low’ Risk</th>
<th>‘High’ risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declared probiotic species</td>
<td>Bacillus spp., Lactobacillus species</td>
<td>Bacillus spp., Lactobacillus spp., Nitrobacter spp.</td>
</tr>
<tr>
<td>Concentration (CFU/g)</td>
<td>1.25x$10^4$ to 4.25x$10^9$</td>
<td>0 to 2.4x$10^{10}$</td>
</tr>
<tr>
<td>Other spp.</td>
<td>NA</td>
<td><em>Escherichia vulneris</em> (human pathogen), <em>Propionibacterium acne</em> (causes acne), Unidentified - gram –ve &amp; +ve spp.</td>
</tr>
<tr>
<td>Antibiotic resistance</td>
<td>0%</td>
<td>40% (<em>Amoxicillin, Erythromycin, Vancomycin</em>)</td>
</tr>
<tr>
<td>Antibiotic contamination</td>
<td>NA</td>
<td>3 products – 1 CAA registered!</td>
</tr>
</tbody>
</table>

Bryant Basuki, Toms Joseph
Farmer Health Survey Research Hypotheses

1. Shrimp health management perceptions, capacities & practices are vary between enterprises operating at different production scales

2. Probiotic efficacy is correlated with salinity gradients operating from coastal (full oceanic salinity) to inland sites adjacent to tidal rivers (brackish conditions)
### Farm Scale

<table>
<thead>
<tr>
<th>Farm Scale</th>
<th>n</th>
<th>Business Ownership</th>
<th>Farm Management</th>
<th>Salaried labour (FTE)</th>
<th>Third-party Certification</th>
<th>Government (CAA &amp; or MPEDA) registration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>137</td>
<td>Farmer</td>
<td>Owner</td>
<td>≤ 3</td>
<td>No</td>
<td>Yes/ No</td>
</tr>
<tr>
<td>Medium</td>
<td>55</td>
<td>Farmer</td>
<td></td>
<td>4 to 10</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Large (not certified)</td>
<td>23</td>
<td>Joint-venture/Corporate</td>
<td>External salaried</td>
<td>&gt;10</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>3rd-party Certified</td>
<td>38</td>
<td>Corporate/ JV/contracted</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

### Hatchery Scale

<table>
<thead>
<tr>
<th>Hatchery Scale</th>
<th>n</th>
<th>Vertical &amp;/or horizontal integration</th>
<th>Broodstock maturation tanks</th>
<th>Third-party Certification</th>
<th>Government (CAA &amp; or MPEDA) registration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small NoReg</td>
<td>4</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Small</td>
<td>7</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>26</td>
<td>Yes/ No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Large</td>
<td>10</td>
<td>Yes</td>
<td>Yes</td>
<td>No/ Yes</td>
<td></td>
</tr>
</tbody>
</table>
Prophylactic v Therapeutic Use of Probiotics by Hatchery Scale – Central AP 2018

- 2017 probiotic-use: L = 100%, M = 88%, S = 36%

- Small greater propensity for therapeutic use & lower recurrent historic use rate

- All probiotics applied to water (no in-feed app.)

<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Cost/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inve (Thailand) Ltd - Pro-W</td>
<td>$15.11/kg</td>
</tr>
<tr>
<td>Inve (Thailand) Ltd - Sanolife Mic</td>
<td>$51.11/kg</td>
</tr>
<tr>
<td>Avivet Nutritional Services Pvt Ltd - Bactocell 100</td>
<td>$14.47/kg</td>
</tr>
<tr>
<td>Aristogene Biosciences Pvt Ltd - Vibrioshield</td>
<td>$17.10/kg</td>
</tr>
<tr>
<td>Nurture Aqua Technology - Epicin Hatchery</td>
<td>$14.47/kg</td>
</tr>
<tr>
<td>Nurture Aqua Technology - Epicin-D</td>
<td>$17.10/kg</td>
</tr>
</tbody>
</table>
No Survey Farms Reporting Probiotic Use by Type & Manufacturer – Central AP Summer Crop 2017

Srinivasa Cystine & CP dominance
Dry Season Mean Pond Salinity (ppt) by Total Farm Culture Area - farms with multiple water sources

Ground water (borehole) – 88% farms (n = 230)
Surface water (canal, river, tidal creek, sea) – 66% farms (n = 172)
Ground & surface water – 58% farms (n = 151)

On-farm trials – 8ppt
Extended PL nursing final mean weights 33 DoC

Nursery Trial Final Average Weights (g) & SE Bars

- **Biofloc**: 0.38
- **WaterProbiotic**: 0.34
- **FeedProbiotic**: 0.30
- **FeedSynbiotic**: 0.29
- **FeedPrebiotic**: 0.28
- **FeedWaterSynt**: 0.27
- **Control**: 0.25
Conclusions & Recommendations

• Greater propensity for therapeutic v prophylactic use by small-holders
• Correlated with diagnostic-capacity limitations
• System-specific intensification thresholds for logical application
• Pedigree analysis:
  • Regulators: risk-based sampling approach for anti-microbial contaminants/ AMR
  • Farmers: inferences on product efficacy
• ASC standards revision/ group certification
• Biofloc biosecurity & production performance benefits (inc. AHPND)
  • Incorporation in extended Biosecure nursing
Thankyou
Contact: fjm3@stir.ac.uk
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IV Summary & Recommendations

Ananda Hotel Inn, Bhimavaram

19 Nov 2018
Probiotic analysis – concern areas

• Unsubstantiated (or fraudulent) efficacy claims
  • EU only 1 probiotic EFSA endorsed for aquaculture (salmon) in Europe!

• Genotype x environment - interaction problems

• Environmental and occupation health risks
  • Anti-microbial resistance genes
  • Contamination - human pathogens, allergens
  • Contamination with antimicrobials?
Most common microorganism declarations

**Bioremediation (water &/ or soil)**
- *Bacillus* spp. – anti-microbial & nitrate assimilation properties
- *Thiobacillus* spp. – nitrate dependent sulphur oxidization (*d. nitrificans*)
- Nitrifying bacteria – NH$_4$ and NO$_2$ oxidation (gram –ve)
- *Aspargillus* spp. – extracellular enzymes, citric acid

**In-feed/ gut probiotics**
- *Lactobacillus* spp – lactic acid, bacteriocins
- *Pediococcus* spp. – lactic acid bacteriocins
  - both homofermentative (carbohydrates to lactic acid/ no alchohol) – silage inoculants
- *Saccharomyces* (yeast) spp. - Immunomodulators
Risk indicators (Pedigree Analysis)

- Mis-spelling of active ingredient/species names
- Over-extensive inclusion of multiple microbial species
- Quantitative bacterial declarations ($<1 \times 10^6$)
- Lack of certified quality standards (ISO 9001, GMP, HACCP, ISO 22000)
- Regulatory domain?
- CAA registration (India) = ‘antibiotic free’
- AMR testing
- Efficacy claims logic
Indeterminate indicators

- Inconsistency in qualitative declarations (genus/ species/ strain) across products designated ‘high’ or ‘low’ risk on other indicators
- Similar inconsistency in quantitative (concentration) declarations
  - Relatively few products with species-wise declarations
  - Many give total concentrations (CFU/mg)
  - ‘High’ risk products most likely to provide no concentration data
Farmers should be wary of...

• Products declaring > 4-5 microbial spp.
• Functionally incompatible active ingredients combinations e.g.
  • aerobic & anaerobic spp.
  • gut probiotic spp and bioremediation spp.
• Low declared bacterial concentrations <1x10^6 CFU/ml
  • Noting many analysed product concentrations lower than declarations
Chose robust species with known modes of action e.g. *Bacillus subtilis*

- Produces:
  - Extracellular bacteriocins
  - Enzymes for nitrate assimilation & respiration
- Spores highly durable v heat, toxic agents, lytic enzymes, UV radiation
- DNA repair e.g. v UV (but mutation & ARG risk)
- Rapid germination and growth cycles (lag & log phase)
- Optimal temp 25-35 °C (mesophilic)
- Optimal salinity 0-10ppt (↑ ppt ↓ germination & metabolic rates)
  - Compare with low salinity tolerance of lactobacillus spp.
- Aerobic i.e. most suited to well-mixed aerated ponds
Nitrifying bacteria?

• Both studies: no detection of declared nitrifying bacteria spp.
• Very slow log & exponential phase times & limited efficacy in water conditions with high suspended organic loadings or low DO levels
• No inclusion in any sample products determined as lower risk
• Gram –ve spp. - greater facility for ARG transfer than Gram +ve spp.
• Farmers should be wary of claims associated with such products
Occupational health risks for ‘high risk’ products

Opportunistic pathogens
• *Brevundimonas diminuta*: pleuritis
• *Escherichia vulneris*: diarrhoea, sepsis, immune suppression
• *Escherichia coli*: faecal indicator organism
• *Propionibacterium acne*: acne
• *Alcaligenes faecalis*: skin soft tissue infection

Allergens/ toxicogenic spp.
• *Aspergillus niger*: mycotoxins, aspergillosis, otomycosis

Potentially pathogenic?
• *Streptococcus* spp., *Clostridium* spp. *Vibrio* spp.

PPE use in manufacture, handling farm standard operating protocols
Anti-microbial resistance in *bacillus* spp. & other isolates

- Culture isolates from 25 probiotics evaluated: 15 CIFT, 10 UoS

  - **CIFT** 150 bacillus spp. isolates:
    - Only 4% entirely resistant to panel of 15 antibiotics
    - 96% with penicillin resistance (ARG implications)
    - 11% with high resistance to chloramphenicol (beta-lactamase: acquired resistance)

  - **UoS** 3 resistant isolates from 2 of 5 ‘high risk’ products sampled (all undeclared Gram –ve spp.)
    - Two unidentified spp. resistant to vancomycin
    - *E. Vulneris* resistant to ampicillin, vancomycin, erythromycin
    - Gram –ve spp: high propensity to exchange DNA between same & different species
      i.e. greater ARG transmission risk & human health concern
ARG & Human Health Risk

• The World Health Organisation (WHO) categorises antimicrobials used in human health as:
  1. ‘critically important’ – includes vancomycin
  2. ‘highly important’ – includes chloramphenical
  3. ‘important’ to human health – e.g. pencillin

• Further analysis required to differentiate between
  • innate/ intrinsic vs acquired/ adaptive resistance
  • Transfer risks through association with mobile genetic elements (plasmids, transposons)
Antimicrobial contamination

• **UoS** – 1 of 5 ‘high’ risk products – exhibiting antimicrobial activity despite lack of any culture isolates

• **CIFT** – 3 of 58 products
  - Inc. 1 CAA registered product!

• Further analysis required to determine antibiotic types

• CAA ‘antibiotic free’ product sampling currently voluntary basis

• Oversight costs: proliferation of products & batch variability

• Risk based sampling/ unannounced audits as part of regulatory process?
Other Regulatory recommendations

ARG risk mitigation
• Founder stocks subject to ARG screening
• Deposit in secure culture collection with accession numbers
• Manufacture quality assurance to prevent contamination
  • e.g. GMP certification – with 3rd party auditing and accreditation

Product labelling (to permit more informed consumer choice)
• Further declaration/ response curves to environmental stressors
  • Salinity, temperature, pH
• Qualitative: microbial composition to at least species level
  Quantitative: [CFU] by species
Next steps

• Exchange of samples between labs to assess replicability of findings
• Further sampling of ‘high risk’ products to assess batch variability
e.g. contamination with human pathogens linked to poor manufacturing quality assurance?
• Evaluation of other PHPs: prebiotics, phytobiotics, immunodulators
  • e.g. risk of use of antibiotics as growth promoters?
• Biofloc – Extension micro-tank systems to small-scale farmers for bio-secure extended nursing (to 20 DoC w/o water exchange)?