Probiotics Cultures: A new alternative for the control of *Aedes aegypti*, *Anopheles albimanus* and *Culex quinquefasciatus* (Diptera: Culicidae)

Margarita Maria Correa, José David Mojica, Leonardo Rocha, Carolina Torres, e Ivan Dario Velez.
Control and Vectors

Insect of blood feeding

- Malaria, Dengue, Encephalitis, West Nile, Filariasis etc.

Prevention and control of mosquitoes population: urgent and immediate demand

Convencional control strategies: application of a wide spectrum of chemicals and pesticides that produce adverse effects

- High risk in humans and environment
- Appearance of a new generation of mosquito with resistant
- Increment in the price on new pesticides

Research on new Eco-friendly tools for the control of mosquito vectors

Biocontrol

Control by Biological Agents

- *Bacillus thuringenesis* var. *israelensis* (Bti) and *B. sphaericus* (Bs): high effectiveness with low concentrations and safe for non-breed organisms.
- Resistance has been registered with Bs in mosquito population (Nielsen-Leroux et al., 1995; Poopathi et al., 1999; Su and Mulla, 2004).

Mix of toxins act on different targets of the insect

- *Bacillus subtilis* / lipopeptides with huge biotechnology potential and biopharmaceutical application
- Several isoforms of surfactins, fengycin and iturin.

Microorganisms Consortia

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PROBIOTICS

Probiotics are live microorganisms which, when applied in adequate amounts deliver a health benefit to the host.

Worldwide Applications

USE

- Animal Health: Regulation flora intestinal
- Rehabilitation on waste areas: Solid Waste Management
- Odor control, composting, animal farming
- Agriculture, bioremediation, soil fertility, yield
- Disasters: Tsunamis and earthquakes
- Waste water treatments
- Pathogen control in water, soil, food.

Probiotic Species List

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Lactic Acid Bacteria</th>
<th>Purple non Sulfur Bacteria</th>
<th>Actinomycetes</th>
<th>Bacillus species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Lactococcus lactis</td>
<td>Rhodopseudomonas palustris</td>
<td>Lactobacillus plantarum</td>
<td>Bacillus subtilis var &quot;natto&quot;</td>
</tr>
<tr>
<td>Saccharomyces thermophilus</td>
<td>Lactobacillus acidophilus</td>
<td>Rhodopseudomonas spheroides</td>
<td>Lactobacillus delbrueckii</td>
<td>Bifidobacterium animalis</td>
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<tr>
<td></td>
<td>Lactobacillus bulgaricus</td>
<td></td>
<td>Lactobacillus casei</td>
<td>Bifidobacterium bifidum</td>
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<tr>
<td></td>
<td>Lactobacillus casei</td>
<td></td>
<td>Lactobacillus fermentum</td>
<td>Bifidobacterium longum</td>
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<tr>
<td></td>
<td>Lactobacillus delbrueckii</td>
<td></td>
<td>Lactobacillus plantarum</td>
<td>Lactobacillus acidophilus</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus fermentum</td>
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<td>Lactobacillus delbrueckii</td>
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<tr>
<td></td>
<td>Lactococcus lactis</td>
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<td>Lactobacillus casei</td>
<td>Bifidobacterium bifidum</td>
</tr>
<tr>
<td></td>
<td>Lactococcus lactis subsp. Diacetylactis</td>
<td></td>
<td>Lactobacillus fermentum</td>
<td>Bifidobacterium longum</td>
</tr>
<tr>
<td></td>
<td>Rhodopseudomonas palustris</td>
<td></td>
<td>Lactobacillus plantarum</td>
<td>Lactobacillus delbrueckii</td>
</tr>
</tbody>
</table>

Introduction

• Grown in multi-strain bacteria
• Very genetically diverse
• More resilient in the “real world”

Probiotics Consortia

• Laboratory grown as single-strains
• Limited genetic diversity
• More vulnerable in the “real world”

Pure Culture

Auroville, Dra. Margarita Correa
Compounds production path

- Organic Matter and Gases
  - Solar light, heat
  - Phototropic Bacteria
    - Sugars and other carbohydrates
    - Yeast
      - Growth promoter hormones, enzymes and antimicrobials
    - Lactic Acid Bacteria
      - Lactic acid
        - Sterilise compound, suppress harmful organic matter, accelerate organic decomposition
  - Aminoacids and nucleic acids
Initiative on the use of Probiotics

"Micro organisms for macro problems»

Introduction

Was decided to extend the research to Arthropods of medical importance

<table>
<thead>
<tr>
<th>Year</th>
<th>Sample People</th>
<th>% Incidence</th>
<th>Total Cost Medicine</th>
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<tr>
<td>2003</td>
<td>2,609</td>
<td>12.23</td>
<td>US $5,500</td>
</tr>
<tr>
<td>2004 start</td>
<td>657</td>
<td>2.40</td>
<td>US $1,200</td>
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<tr>
<td>Probiotics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>391</td>
<td>2.26</td>
<td>US $841</td>
</tr>
<tr>
<td>2006</td>
<td>640</td>
<td>0.68</td>
<td>US $617</td>
</tr>
<tr>
<td>2007</td>
<td>280</td>
<td>0.95</td>
<td>US $237</td>
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</tbody>
</table>
General Objective

To evaluate the efficacy of Probiotic cultures over immatures phases of mosquito *Aedes aegypti, Anopheles albimanus* and *Culex quinquefasciatus* in laboratory conditions.
Specific Objectives

• To establish the susceptibility in immature phases of *A. albimanus, Ae. aegypti* and *Cx. quinquefasciatus* in different probiotic concentrations

• To determine the Lethal Concentration fifty (LC$_{50}$) and ninety (LC$_{90}$) of Probiotic cultures for larvae of *A. albimanus, A. Aegypti* and *Cx. Quinquefasciatus*

• To demonstrate the reproducibility of a product with low cost and eco friendly as biological control alternative
Methodology

1. Breed immature forms
   - Larvae separation and account
   - Probiotic dilutions preparation

2. Probiotic applications
   - Measurement (1) of physicochemical factors at the beginning of the experiments

3. Follow up and registration of mortality (24, 48, 72 and 120 hours)
   - Measurement (2) of physicochemical factors at the end of the experiments

4. Experiment conditions: average temperature: 28 ºC ± 2 and Relative humidity 70 ± 5 %
   - Experiement repetitions / specie: 10 (4000 larvae / test)
Mortality (%) vs Concentration

Results

- Susceptibility $A.\ aegypti < C.\ quinquefasciatus < A.\ albimanus$ after 120 hours of application.
Lethality ($LC_{50}$) after 120 hours

- *A. aegypti* more susceptible with less probiotic concentration
- Geetha et al., 2007: Bti *C. quinquefasciatus* more susceptible (4 ng/ml) and *Anopheles stephensi* less susceptible (18 ng/ml)
## Physicochemical factors *Aedes aegypti*

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Measurement</th>
<th>pH</th>
<th>O₂ (%)</th>
<th>Conductivity (uS/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 %</td>
<td>1</td>
<td>3,22 a</td>
<td>68,06 c</td>
<td>578,68 e</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3,22 a</td>
<td>39,06 d</td>
<td>623,68 e</td>
</tr>
<tr>
<td></td>
<td>C -</td>
<td>7,03 b</td>
<td>66,87 c</td>
<td>66,93 f</td>
</tr>
<tr>
<td>12 %</td>
<td>1</td>
<td>3,21 a</td>
<td>64,62 c</td>
<td>660,06 e</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3,18 a</td>
<td>28,87 d</td>
<td>641,43 e</td>
</tr>
<tr>
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<td>C -</td>
<td>7,13 b</td>
<td>70,12 c</td>
<td>66,66 f</td>
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<tr>
<td>14 %</td>
<td>1</td>
<td>3,20 a</td>
<td>65,50 c</td>
<td>718,18 e</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3,18 a</td>
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<td>C -</td>
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<td>69,50 c</td>
<td>65,73 f</td>
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<tr>
<td>16 %</td>
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<td>3,20 a</td>
<td>68,93 c</td>
<td>785,18 e</td>
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<tr>
<td></td>
<td>2</td>
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<td>751,75 e</td>
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<td>C -</td>
<td>7,22 b</td>
<td>69,12 c</td>
<td>65,91 f</td>
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</table>

Different letters indicate significative statistic differences p< 0,0001. Test of Tukey. 1: Measurement of the factor at the beginning, 2: Measurement of the factor at the end of the experiment, C -: Control without application of probiotics.
## Physicochemical factors *Anopheles albimanus*

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Measurement</th>
<th>pH</th>
<th>O₂ (%)</th>
<th>Conductivity (uS/m)</th>
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</thead>
<tbody>
<tr>
<td>10 %</td>
<td>1</td>
<td>3,23</td>
<td>a</td>
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<td></td>
<td>2</td>
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<td></td>
<td>C -</td>
<td>6,60</td>
<td>c</td>
<td>63,25 f</td>
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<td>12 %</td>
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<td>3,26</td>
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<td></td>
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<td>c</td>
<td>66,00 f</td>
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<tr>
<td>14 %</td>
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<td>3,26</td>
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<td></td>
<td>C -</td>
<td>6,71</td>
<td>c</td>
<td>64,87 f</td>
</tr>
</tbody>
</table>

Different letters indicate significative statistic differences $p< 0.0001$. Test of Tukey. 1: Measurement of the factor at the beginning, 2: Measurement of the factor at the end of the experiment, C -: Control without application of probiotics.
Discussion

• Susceptibility varies according to the species. A. aegypti more susceptible than A. albimanus.

• LC$_{50}$ of the probiotic is high. In *B. subtilis* (5-25 ul/ml) (Geetha et al., 2007)

• Cultivation of probiotic: synergistic effect and offers less option to resistance of mosquito populations to the biolarvicide, genetically more diverse

• Physico-chemical factors (pH, temperature, solar exposure and age of the larvae) influence the effectiveness of the formulations with bacteria or toxins against mosquito larvae (Mulla, 1985; Becker et al., 1992; Mittal et al., 1995; Nayar et al., 1999).

• Lipopeptids of *B. subtilis*: insensitive to sunlight and equally effective to kill larvae. Advantage over the conventional biolarvicides *Bti*, and *Bs*.

• Ecofriendly alternative and without lethal effects compared with pesticides.
• The effectiveness *Bs.* and *Bti.* against larvae of anopheline mosquitoes is reduced about 10 times in laboratory tests to 21°C compared with tests, carried out at 31°C (Mittal, 2003).

• Studies with raw lipopeptids indicated a reduction in the larvicidal power around 4% by 23°C, on its original activity that occurs at 35°C (K. Das & A. K. Mukherjee, 2006).

• Strains of *B. subtilis* high thermostable power between two strains evaluated after periods of warming 100°C by 60 minutes (K. Das & A. K. Mukherjee, 2006).

• Toxins of proteins of *Bti* and *Bs* are highly sensitive to sunlight (UV radiation). Exposure to sunlight 6 hours reduces the strength larvicide near the 50 and 75% respectively (Mittal, 2003).

• The probiotic effect up to a month and shelf life about a year
Explanation for the Mechanism of Action

- Microorganisms
  - Production Alcohols, Esters and other metabolites
    - Esterilization effect (OH+)
    - Disminution of the pH
      - Digestive system of the larvae is disturbed
        - Death
  - Other effects NO known

Cycle Lipopeptides: B. subtilis
Conclusions

• Susceptibility presented variations according to the species (\textit{A. aegypti} < \textit{C. quinquefasciatus} < \textit{A. albimanus}) after 120 hours of application.

• \textit{Aedes aegypti} more susceptible to the probiotic, mortality > 90 \%; \textit{A. albimanus} mortality \sim 80\% and less susceptible. Only \textit{C. quinquefasciatus} effect depending on dose.

• \text{LC}_{50} for \textit{Aedes aegypti} was the lowest (6.26 \%) and statistically different to that obtained for the other species.

• \textit{Aedes aegypti}, presented less variation in physico-chemical factors indicating greater effectiveness to the larvicide.
Perspectives

• Studies in no target organisms: fishes and insects

• Studies on the mechanism of action and the synergist effect

• Ecofriendly alternative

• More field studies that will bring more information in the behaviour of the natural probiotics and larvae/pupa control on wild mosquito.
Acknowledgement

• Medical and Molecular Enthomology Unit (UEMM)

• Dra. Margarita Correa (Auroville)

• To PECET for allow the research and development of the different graduate theses