Use of Symbiotic Bacteria to Reduce Mass-Rearing Costs and Increase Mating Success in Selected Fruit Pests in Support of SIT Application

Report of the First Research Coordination Meeting of an FAO/IAEA Coordinated Research Project, held in Vienna, Austria, from 21 to 25 May 2012

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BACKGROUND

Flies and moth pests cause significant and widespread damage to fruit and vegetable production. Insecticide application remains the predominant method of controlling these pests. Indeed, these high value crops are the main targets of agrochemicals, receiving currently 32% of all applications in agriculture. Despite this growing dependence on agrochemicals, suppression of the pest populations is frequently inadequate. In addition, due to regulation, pest resistance, environmental and human health concerns, there is an increasing demand for the replacement of the intensive use of these chemicals by environmentally friendly, effective and sustainable methods, within integrated management approaches. Chief among these is the sterile insect technique (SIT) and related biological control applications.

The efficacy of the SIT is determined by the quality of insects mass-reared in production facilities, irradiated and released in the field. Constraints on the quality of these insects are manifest at every stage of production and major efforts have been made to assure quality within reasonable economic limits. Indeed, the increased use of the SIT is frequently limited by cost-benefit considerations, as the mass-rearing of target insects, their delivery and release in prime condition may be in some situations prohibitively expensive. Accordingly, efforts to streamline the SIT process, combining improved quality of sterile insects with reduced production costs should enable the increased application of this approach.

In the past, efforts to improve sterile insect quality for fruit fly pests have focused on colonization, mass-rearing, quality control and pre-release handling. In the current proposal, we seek to extend these approaches to manipulating the diverse microorganisms associated with the fruit pests targeted in SIT operations to protect fruit and vegetable crops.

Insects are indisputably the dominant multicellular organisms in terrestrial habitats. As such they maintain intricate and complex interactions with other organisms in their habitat. Some of these interactions, such as those between insects and plants, or insects and vertebrates, have been extensively studied. On the other hand, the associations between insects and microorganisms, while pervasive and of paramount ecological and evolutionary importance, are only gradually being understood. Insects depend on symbiotic associations with a variety of microorganisms, which affect many aspects of host biology and physiology including nutrition, immunity, mating behaviour and reproduction.

The insect symbiotic associations are currently artificially divided into three categories: The first category includes symbionts that provide nutrients such as amino acids and vitamins to their hosts through mutualistic associations. The second category includes symbionts that provide their hosts with the ability to survive heat stress, to develop resistance to parasitic wasps and/or microbial pathogens, and to exhibit altered host plant preference. The third category includes symbionts that manipulate the reproductive properties of their hosts, inducing phenomena such as parthenogenesis, feminization, male-killing and cytoplasmic incompatibility (CI), which is a kind of male sterility.
**Symbiotic microorganisms and the SIT**

Symbiotic organisms can be important at all stages of the SIT. We seek to resolve four key questions related to them:

1. Can symbionts help reduce the cost of production and increase mass-reared sterile insect quality?
2. How are symbiotic associations affected by radiation and can they be ameliorated?
3. Can they be used as probiotics during the pre-release period to improve sterile insect quality?
4. Can they be used to develop novel pest control tools, complementary to the SIT?

These questions are expanded in the following paragraphs.

1. **Costs of production and increased quality**

   Under natural conditions, fruits used by larvae have extremely low amino acid contents. Ovipositing females inject their eggs into fruit along with bacteria that fix atmospheric nitrogen and others that break down the fruit to produce nutrients essential for larval growth. The mass-rearing process frequently disassociates the reared insects from their native microflora, allowing the proliferation in larval media of opportunistic microorganisms that may not be beneficial. Yeasts to provide nourishment, and chemicals to suppress opportunistic microorganisms, represent by far the largest cost of larval diets. Adding endogenous symbiotic bacteria to the artificial larval and adult diet may significantly:
   - reduce mass-rearing costs by eliminating the need for yeasts and chemicals
   - prevent the growth of deleterious microorganisms
   - improve mass-rearing efficiency and quality of the insects produced

2. **Effects of radiation**

   Evidence suggests that radiation of mass-reared flies can disrupt symbiotic associations by favouring some bacterial species and suppressing others. Understanding the effects of radiation may enable us to design responses that address them in a manner that optimizes the SIT efficiency.

3. **Probiotics**

   In nature, symbiotic bacteria become established in the gut of adult flies. These appear to play an important role in the reproductive success of males. The complement of bacteria present in released males following mass-rearing and irradiation may differ from their wild counterparts enough to impede their performance. There is preliminary evidence that restoring the symbiotic bacteria, prior to release, can significantly improve their sexual performance. Enriching the sterile insect diet of the Mediterranean fruit fly with the naturally occurring bacterium, *Klebsiella oxytoca*, significantly improved sterile male mating competitiveness in the laboratory and in field cages. In addition, bacterially enriched sterile males inhibited female receptivity to re-mating more efficiently than sugar fed males and survived longer periods of starvation. These results suggest that restoring key bacteria to mass-reared sterile flies prior to their release is a valid approach to improve the efficacy of the SIT. It is
worthwhile to validate this approach at an operational level and to extend it to other insects targeted by the SIT.

4. Symbiotic organisms and novel control tools

Certain symbiotic bacteria are known to manipulate mating behaviour and reproduction of their hosts. Identifying these organisms and introducing them to target populations can effectively reduce pest populations and their economic impact. For example, the interactions between hosts, parasitoid and symbiotic microflora affect host fitness and should be investigated. Furthermore, the incompatible insect technique (IIT) employs cytoplasmic incompatibility, induced by insect symbionts such as Wolbachia species. In a Wolbachia-based IIT strategy, female sterility is artificially sustained by repeated releases of cytoplasmically incompatible males. Since Wolbachia is not paternally transmitted, the infection type present in the release strain does not become established in the field. Similar to the SIT, the increasing ratio of incompatible matings over time can lead to population suppression. This strategy has been successfully tested under laboratory conditions for two major agricultural pests, the Mediterranean fruit fly and the olive fly. It is worthwhile for such an approach to be validated and extended, alone and/or in conjunction with the SIT, to other target insect pest species.

In conclusion, symbiosis, a powerful new field, increases our understanding of basic biological questions, and can have profound effects on applied fields, from medicine to agriculture. This CRP aims at the characterization and harnessing of endogenous symbiotic communities in order to: (a) reduce costs of mass-rearing, (b) determine the effects of radiation, (c) use symbionts as probiotics and (d) develop novel and SIT-compatible insect control tools. We believe that this initiative will lead to better and more cost-effective SIT programmes against fruit insect pests.
CO-ORDINATED RESEARCH PROJECT (CRP)

This Coordinated Research Project (CRP) is based on a Consultants Meeting that was held from 7-11 February 2011 in Vienna, Austria (report available) to assess the potential for conducting co-ordinated R&D in larval and adult insect for releases, and to formulate a proposal for a CRP on *Use of Symbiotic Bacteria to Reduce Mass-Rearing Costs and Increase Mating Success in Selected Fruit Pests in Support of SIT Application*.

The overall objective of this new CRP D4.10.24, approved for the period 2012-2017, is to ultimately reduce the cost and increase the effectiveness of SIT programmes by reducing costs of mass-rearing, determining the effects of radiation, using symbionts as probiotics and developing novel and SIT-compatible insect control tools.

FIRST RESEARCH CO-ORDINATION MEETING (RCM)

Twenty-five scientists from 16 countries attended this first RCM, held in Vienna, Austria from 21-25 May 2012. The list of participants, which included CRP contract and agreement holders, as well as 5 additional observers and 1 consultant, is given in Annex 1. The agenda for the meeting is attached in Annex 2.

During the first two days of the meeting RCM participants presented research relevant to the CRP, as well as their research plans for the first year of the CRP.

During the last three days of the meeting, general discussions were held to define and review the thematic areas of the CRP (Table 1), the review of the general and specific R&D objectives to be addressed during the 5 years of the CRP (Sections 1, 2, and 3), and the CRP Logical Framework, in order to agree on minimum outputs to be achieved at the end of the CRP. Furthermore, participants were divided into three working groups (Annex 3) to develop more detailed R&D plans to be conducted during the first 18 months of the CRP.

Abstracts of the presentations are presented in Annex 4 and a copy of all PowerPoint presentations was made available to all participants at the end of the RCM.

A protocol and procedures for the characterization of reproductive and gut symbionts was developed to harmonize methodologies among the CRP participants. This protocol and procedures was developed in a separate document.
Table 1. Thematic areas in relation to pest species being addressed by researchers (for main areas of work, names appear in bold).

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1. LARVAL DIETS AND RADIATION EFFECTS

Background Situation Analysis

Under natural conditions, fruits used by larvae have extremely low amino acid contents. Ovipositing females inject their eggs into fruit along with bacteria that most probably fix atmospheric nitrogen and others that break down the fruit to produce nutrients essential for larval growth. The mass-rearing process frequently disassociates the reared insects from their native microflora, allowing the proliferation in larval media of opportunistic microorganisms that may not be beneficial. Yeasts to provide nourishment, and chemicals to suppress opportunistic microorganisms, represent by far the largest cost of larval diets. Adding endogenous symbiotic bacteria to the artificial larval diet may significantly:

A. reduce mass-rearing costs by decreasing, replacing or eliminating the need for yeasts and chemicals
B. prevent the growth of competing microorganisms
C. improve mass-rearing efficiency and quality of the insects produced

Subtheme A: Cost and quality of larval diet

Current knowledge:

A1. General
Currently all mass rearing facilities of different fruit fly pest species use artificial larval diet which consists of bulking agents, yeasts, sugars, preservatives, acidifying agents and water. The ingredient which mainly affects the cost (other than the labor cost) and the quality of the larval diet is yeast. Yeast supplements can make up to 50% of the total larval diet cost. The presence of deleterious microorganisms is also an important issue and is mainly related to the type and the availability of bulking agents at the different facilities location.

A2. Species level background
Artificial larval diet is available for the following species: Ceratitis capitata (medfly), Bactrocera oleae (olive fly), B. curcubitae (melon fly), B. tryoni (Queensland fly), Anastrepha obliqua, A. ludens, A. fraterculus, A. serentina, A. striata, B. dorsalis, B. zonata, Dirioxa pornia.

Ceratitis capitata, Anastrepha species and Bactrocera species (other than B. oleae): all facilities use a diet of a very similar composition, the difference mainly being to the source of the bulking agents and the pH level which ranges from 3.5 to 4.5. Novel diets (liquid and gel diet) are under evaluation and may facilitate the incorporation of beneficial microorganisms.

Bactrocera oleae: Being a monophagous pest species on a fruit of an uncommon composition, olive fly larval diet requires specific ingredients including soy hydrolysate, which accounts for more than 50% of the total cost, and cellulose as bulking agent. The diet requires further quality improvement and cost reduction.

Gaps identified:

A1. General:
1. Replacement of the bulking agents
2. Replacement (reduction) of protein sources
3. Optimizing the use of preservatives
4. Egg/larval inoculation with beneficial microbes
5. Different facilities have access to different larval diet ingredients

A2. Species level background:
Each one of the five gaps identified needs to be considered for all fruit fly pest species targeted in this CRP: *Ceratitis capitata, Bactrocera oleae, B. curcubita, B. tryoni, Anastrepha obliqua, A. ludens, A. fraterculus, A. serpentina, A. striata, B. dorsalis, B. zonata, Dirioxa pornia*. Particular emphasis should be given to *B. oleae* being the species with the most inefficient larval diet. The need of a specialized scientist with good knowledge of food industry (animal nutrition) is recognized.

**Subtheme B: Preventing the growth of deleterious microorganisms**

**Current knowledge:**

B1. General:
The presence of deleterious microorganisms is a common problem for all mass rearing facilities. A monitoring protocol is in use in some facilities and is based on classical microbiological tests (API tests). Using this protocol, bacteria like *Lactobacillus* spp, *Pseudomonas* spp., *Morganella* spp., *Serratia* spp. and yeasts have been identified. However, these tests have limiting potential to identify microorganisms at the species level. Therefore, molecular approaches need to be incorporated. In addition, there is an urgent need to develop a monitoring protocol for the detection of deleterious microorganisms in all mass rearing facilities at three levels: (a) the ingredients, (b) the larval diet and (c) the environment (rearing equipment, air conditioning, HEPA filters, etc).

B2. Species level background:
The currently available monitoring protocol is only in use in the facilities of El Pino and Petapa, MoscaMed-USDA [Guatemala], MoscaFrut, MoscaMed [Mexico], Valencia [Spain], Mission Texas [USA] which rear the following species: *Ceratitis capitata, A. ludens, Anastrepha obliqua*.

**Gaps identified:**

B1. General:
1. There is an urgent need to combine microbiological and molecular methods for the accurate identification of deleterious microorganisms. Isolation and characterization of bacterial strains will also be required.
2. Uncover the conditions (pH, aeration, water activity, ingredients) which are conducive for the development of deleterious microorganisms in the larval diet, the rearing rooms and equipment.
3. Cost-effective preservatives to be tested in view of a complete list of deleterious microorganisms (see gap 1).
4. Beneficial microorganisms to be used as competitors of deleterious bacterial species (once gaps 1 and 3 are filled).
5. A revised monitoring protocol should be developed and implemented upon the filling of the above gaps.
6. An e-platform should be developed to integrate all data collected (particularly from gaps 1 and 2).

B2. Species level background:
All the above gaps need to be addressed for each one of the following species: *Ceratitis capitata*, *Bactrocera oleae*, *A. ludens*, *B. curcubitae*, *B. tryoni*, *Anastrepha obliqua*, *A. fraterculus*, *A. serpentina*, *A. striata*, *B. dorsalis*, *B. zonata*, *Dirioxa poneria*.

**Subtheme C: Improving mass rearing efficiency and quality of the insects produced**

**Current knowledge:**

C1. General:
Despite the great progress achieved during the last 30 years in the mass rearing protocols of major agricultural fruit fly pests, there is still plenty of room for improving the efficiency as well as the quality of the insects produced. Recent advancements in the field of insect symbiosis suggest the presence of a diverse group of symbiotic microorganisms which play a major role on the biology and the physiology of insects. Therefore, a deep knowledge on the composition, the dynamics and the functional role of insect symbiotic communities is required and this will certainly enhance the production of higher quality mass reared insects.

C2. Species level background:
The species currently under artificial rearing can be divided into three groups depending on the efficiency and the quality of insects produced: *Ceratitis capitata* and *A. ludens* are the only species on the top of this list of the most efficient rearing protocols, *B. oleae* is in the bottom while all the others are in the middle.

**Gaps identified:**

C1. General:
1. Monitoring (identification, dynamics and functional role) of the insect-associated microorganisms reared in different artificial larval diets by microbiological and/or molecular approaches.
2. Monitoring (identification, dynamics and functional role) of the symbionts associated with the fruit flies (larvae) under natural conditions (different host plants, geographic regions, seasons, etc.).
3. Define the beneficial microorganisms based on the outcome of (1) and (2).
5. Develop robust and efficient inoculation methods for the beneficial microorganisms.
6. Technical and economical feasibility for the production and the inoculation of beneficial microorganisms.
7. Gaps 4-6 can be addressed once cultivation protocols for insect beneficial microorganisms become available or developed.

C2. Species level background:
Intensive research efforts are currently being undertaken to characterize the symbiotic communities of several fruit fly pest species (for example, *Ceratitis capitata*, *Bactrocera oleae*). However, limited or scarce knowledge is available for the rest of the species considered in this CRP: *A. idens, B. curcubitae, B. tryoni, Anastrepha obliqua, A. fraterculus, A. serpentina, A. striata, B. dorsalis, B. zonata, Dirioxa pornia*.

**Individual plans**

1.1. **Cost and quality of larval diet**

**Species: Ceratitis capitata**
Participants: Hernan Donoso, Carolina Yañez (Chile)

*5 years plan*
- Identification of microbial communities in larvae and pupae of the laboratory colony (under mass rearing and small scale conditions) by molecular (DGGE, 16S rRNA gene sequencing and pyrosequencing, if possible) and classical culture-dependent approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.
- QC field tests using standard field cage in order to assess the real impact of the provision of probiotics during the period prior to their release, with special attention to the eventual increase of competitiveness, dispersal and survival.

*18 months plan*
- Identification of microbial communities in larvae, pupae and adults of the laboratory colony (under mass rearing and small scale conditions) by molecular (DGGE, 16S rRNA gene sequencing and pyrosequencing, if possible) and classical culture-dependent approaches.

Participants: Dina Melgar, Pedro Rendón, Felipe Jerónimo (Guatemala)

*5 years plan*
- Identification, comparative analysis and evaluation of the microflora associated with the mass reared insects and wild populations (at larval stage) by using molecular (16S rRNA gene-based approaches) and classical microbiological phenotyping approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

*18 months plan*
- Identification, comparative analysis and evaluation of the microflora associated with the mass reared insects and wild populations (at larval stage) by using molecular (16S rRNA gene-based approaches) and classical microbiological phenotyping approaches.

Participants: Jaime Garcia de Oteyza, Teresa Navarro (Spain)

*5 years plan*
- Identification of microbial communities in egg, larvae and pupae of wild and mass reared medfly (different strains and colonies), wild infested fruits and mass rearing larval diet by molecular and classical approaches (DGGE, 16S rRNA sequencing).
• Identify suitable beneficial microorganisms for probiotic applications at larval stage.

• Delelopement the formulation for probiotic applications at larval stage.

18 months plan

• Identification of microbial communities in egg, larvae and pupae of wild and mass reared medfly (different strains and colonies), wild infested fruits (from different seasons and regions) and mass rearing larval diet by molecular and classical approaches (DGGE, 16S rRNA sequencing).

Species: *Anastrepha ludens*  
Participants: Dina Melgar (Guatemala)

5 years plan

• Identification, comparative analysis and evaluation of the microflora associated with the mass reared insects and wild populations (at larval stage) by using molecular (16S rRNA gene-based approaches) and classical microbiological phenotyping approaches.  

• Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan

• Identification, comparative analysis and evaluation of the microflora associated with the mass reared insects and wild populations (at larval stage) by using molecular (16S rRNA gene-based approaches) and classical microbiological phenotyping approaches.

Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan

• Identification of microbial communities in wild and laboratory third instar-larvae by using molecular and classical microbiological phenotyping approaches.  

• Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan

• No activities are planned for this period.

Participants: Erin Schuenzel (USA)

5 years plan

• Identification of microbial communities in laboratory eggs and third instar-larvae by using molecular and classical microbiological phenotyping approaches.

• Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan

• Identification of microbial communities in laboratory eggs by molecular and classical microbiological phenotyping approaches.

Species: *Anastrepha obliqua*  
Participants: Emilio Hernandez, Pablo Liedo (Mexico)
5 years plan
- Identification of microbial communities in wild and laboratory third instar-larvae (irradiated and non-irradiated) by using molecular and classical microbiological phenotyping approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan
- Identification of microbial communities in wild and laboratory third instar-larvae by using molecular and classical microbiological phenotyping approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

**Species: Anastrepha serpentina**
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of microbial communities in wild and laboratory third instar-larvae by using molecular and classical microbiological phenotyping approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan
- No activities are planned for this period.

**Species: Anastrepha striata**
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of microbial communities in wild and laboratory third instar-larvae by using molecular and classical microbiological phenotyping approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan
- No activities are planned for this period.

**Species: Anastrepha fraterculus**
Participants: Fernando Consoli (Brazil)

5 years plan
- Identification of microbial communities in larvae of wild and lab reared fruit flies (different strains and colonies) as well as wild infested fruits by molecular approaches (16S rRNA pyrosequencing) and classical microbiological approaches only for the lab colony.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan
- Identification of microbial communities in larvae of wild and lab reared fruit flies (different strains and colonies) as well as wild infested fruits by molecular approaches (16S rRNA pyrosequencing) and classical microbiological approaches only for the lab colony.

- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

**Species: Bactrocera curcubitae**
Participants: Mahfuza Khan (Bangladesh)
Collaborators: Kostas Bourtzis, and George Tsiamis (Greece)

5 years plan
- Identification of gut microbial communities of larvae of laboratory reared (host and artificial larval diet) and from infested fruits, and artificial larval diets by classical microbiological/biochemical (API kits) approaches and molecular techniques (16s rDNA pyro sequencing).
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan
- Identification of gut microbial communities of third instar larvae of laboratory (host) reared *B. cucurbitae* by biochemical (API kits) approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

**Species: Bactrocera dorsalis complex**
Participants: Mahfuza Khan (Bangladesh)
Collaborators: Kostas Bourtzis, and George Tsiamis (Greece)

5 years plan
- Identification of gut microbial communities of larvae of laboratory reared (host and artificial larval diet) and from infested fruits, and artificial larval diets by classical microbiological/biochemical (API kits) approaches and molecular techniques (16s rDNA pyro sequencing).
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan
- Isolation and identification of gut microbial communities of third instar larvae of laboratory (host) reared *B. dorsalis* by biochemical (API kits) approaches.
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

Participants: Changying Niu (China)

5 years plan
- Identification of microbial communities in egg, larvae and pupae of wild and lab reared fruit flies (different strains and colonies), wild infested fruits and mass rearing larval diet by molecular approaches (16S rRNA pyrosequencing)
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.
18 months plan

- Identification of microbial communities in egg, larvae and pupae of wild and mass reared fruit flies (different strains and colonies), wild infested fruits (from different seasons and regions) and mass rearing larval diet by molecular approaches (16S rRNA pyrosequencing).

Participants: Kostas Bourtzis, George Tsiamis (Greece)

5 years plan

- Characterization of the gut associated bacterial communities by 16S rRNA pyrosequencing libraries and microarrays (PhyloChip)
- Quantitative analysis of the most dominant bacteria

18 months plan

- None planned.

**Species: Diorixa pornia**

Participants: Peter Crisp (Australia)

5 years plan

- Identification of microbial communities in egg, larvae and pupae of wild and lab reared fruit flies (different strains and colonies) by molecular approaches (16S rRNA pyrosequencing)
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

18 months plan

- None planned.

1.2. Preventing the growth of deleterious microorganisms

**Species: Ceratitis capitata**

Participants: Hernan Donoso, Carolina Yanez (Chile)

5 years plan

- Identification of deleterious microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan

- Since there is currently no routine testing in microbiology at the CPIE, during this period efforts will be made to train, design and implement a work plan in this area.

Participants: Dina Melgar, Pedro Rendón, Felipe Jerónimo (Guatemala)
5 years plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Participants: Jaime Garcia de Oteyza, Teresa Navarro (Spain)

5 years plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Species: *Anastrepha ludens*
Participants: Dina Melgar (Guatemala)

5 years plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Participants: Erin Schuenzel (USA)

5 years plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Species: *Anastrepha obliqua*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Species: Anastrepha serpentina
- No activities are planned

Species: Anastrepha striata
- No activities are planned

Species: Anastrepha fraterculus
Participants: Fernando Consoli (Brazil)

5 years plan
- Identification of deleterious microorganisms in laboratory larvae by molecular approaches (16S rRNA pyrosequencing).

18 months plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and pupae by molecular approaches (16S rRNA pyrosequencing).

Species: Bactrocera curcubitae
No activities are planned

Species: Bactrocera dorsalis complex
Participants: Changying Niu (China)

5 years plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and pupae by molecular approaches (16S rRNA pyrosequencing).
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and pupae by molecular approaches (16S rRNA pyrosequencing).

Participants: Kostas Bourtzis, George Tsiamis (Greece)
5 years plan
- Identification of (deleterious) microorganisms in laboratory larvae by 16S rRNA pyrosequencing libraries and microarrays (PhyloChip).

18 months plan
- None planned.

Species: Diorixa pornia
Participants: Peter Crisp (Australia)

5 years plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and pupae by molecular approaches (16S rRNA pyrosequencing).
- Development diagnostic tests for the early detection of deleterious microorganisms.

18 months plan
- Identification of deleterious microorganisms in laboratory eggs, larvae and pupae by molecular approaches (16S rRNA pyrosequencing).

1.3. Improving mass rearing efficiency and quality of the insects produced

Species: Ceratitis capitata
Participants: Hernan Donoso, Carolina Yañez (Chile)

5 years plan
- Identification of beneficial microorganisms in laboratory larvae by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory larvae by molecular and classical microbiological approaches.

Participants: Dina Melgar, Pedro Rendón, Felipe Jerónimo.

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
Participants: Jaime Garcia de Oteyza, Teresa Navarro (Spain)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Species: Anastrepha ludens
Participants: Dina Melgar (Guatemala)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Participants: Erin Schuenzel (USA)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
Species: *Anastrepha obliqua*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Species: *Anastrepha serpentina*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Species: *Anastrepha striata*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Species: *Anastrepha fraterculus*
Participants: Fernando Consoli (Brazil)

5 years plan
- Identification of beneficial microorganisms in laboratory larvae reared in natural fruits by molecular (16S rRNA gene pyrosequencing) and classical microbiological approaches.

18 months plan
• Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

**Species: Bactrocera curcubitae**  
**Participants:** Mahfuza Khan (Bangladesh)

5 years plan
• Identification of deleterious microorganisms in laboratory (host/artificial) reared larvae of *B. cucurbitae* and *B. dorsalis* by biochemical approaches (API kits).
• Evaluation of different preservatives to control the growth of deleterious microorganisms

18 months plan
• Identification of deleterious microorganisms in laboratory reared third instar larvae of *B. cucurbitae* and *B. dorsalis* by biochemical approaches (API kits).

**Species: Bactrocera dorsalis complex**  
**Participants:** Mahfuza Khan (Bangladesh)

5 years plan
• Identification of deleterious microorganisms in laboratory (host/artificial) reared larvae of *B. cucurbitae* and *B. dorsalis* by biochemical approaches (API kits).
• Evaluation of different preservatives to control the growth of deleterious microorganisms

18 months plan
• Identification of deleterious microorganisms in laboratory reared third instar larvae of *B. cucurbitae* and *B. dorsalis* by biochemical approaches (API kits).

**Participant: Changying Niu (China)**

5 years plan
• Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
• Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
• Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

**Participants: Kostas Bourtzis, George Tsiamis (Greece)**

5 years plan
• Identification of (beneficial) microorganisms in laboratory larvae by 16S rRNA pyrosequencing libraries and microarrays (PhyloChip).

18 months plan
• None planned.
**Species: Diorixa pornia**
**Participants: Peter Crisp (Australia)**

5 years plan:
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development diagnostic tests for the detection of beneficial microorganisms.

18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
2. Probiotics

Background

Symbiotic bacteria are ubiquitous in tephritid fruit flies. In the various genera of these flies similar microbial communities are generally found. A number of studies suggest that microorganisms contribute to the fitness of their insect host including the reproductive success of males.

Many studies have shown that mass-reared sterile males are clearly disadvantaged compared to wild males in both survival and sexual competitiveness. Apparently, the reasons for this low competitiveness stems from their colonization, mass-rearing conditions and the irradiation process. In order to compensate for their low competitiveness, sterile flies are released in large numbers relative to the number of wild flies present in the orchard (in ratios in excess of 50:1 or 100:1). This over flooding has economical and operational consequences. Therefore, there is a real need to improve the competitive ability of the released sterile males in SIT operations. Several approaches have been developed for this purpose. Chief amongst these have been manipulations of the olfactory environment of the sterile males (Papadopoulos et al., 2001; Shelly et al., 2005). Indeed, the exposure of adult flies to the aroma of ginger root oil improves sexual competitiveness and is being implemented in some SIT operations (Shelly et al., 2007). Other approaches, have been promising in laboratory or semi field conditions, but have yet to be applied on operational scales. These include manipulation of the pre-release diet (Yuval and Hendrichs 2000; Yuval et al., 2002) and juvenile hormone treatments (Teal et al., 2000; 2007).

The complement of bacteria present in released males following mass-rearing and irradiation may differ from their wild counterparts enough to impede their performance. There is evidence that inoculating symbiotic bacteria, prior to release, can significantly improve male sexual performance. Thus, restoring key bacteria to mass-reared sterile flies prior to their release is a valid approach to improve the efficacy of the SIT. It is worthwhile to validate this approach at an operational level and to extend it to other insects targeted by the SIT.

Subtheme A: Structure of microbiota

Current knowledge:

A1. General:
The association between fruit flies and bacteria was first recognized in the beginning of the twentieth century, when, based on microscopic observations, Petri described symbiotic relations between the olive fly Bactrocera oleae and a microorganism. He suggested that this symbiont might be Pseudomonas savastanoi, a bacterial pathogen causing the olive knot disease (Petri 1909).

Bacterial isolation from fruit flies and their oviposition sites were sporadically reported since 1934. However, beginning in the mid 1980s many studies were conducted on fruit fly – bacterial interactions (see reviews by Drew & Lloyd 1987, 1991; Lauzon 2003). These studies were based on traditional microbial methods such as viable plate counts and phenetic taxonomy and focused mainly on the bacteria inhabiting the digestive system of adult fruit flies (Table 2). Two species received quite a lot of attention – the Queensland fruit fly, Bactrocera tryoni, and the apple maggot fly Rhagoletis pomonella. The bacteria found to be
associated with *B. tryoni* were members of the family *Enterobacteriaceae*, mainly species of *Klebsiella* and *Enterobacter*, with *Klebsiella oxytoca* and *Enterobacter cloacae* as the most common species. These bacteria were also found in the different instars of the flies and in infested fruit (Fitt & O'Brien 1985; Drew & Lloyd 1987; Table 2).

Species of *Klebsiella* and *Enterobacter* are also commonly associated with *R. pomonella*. Lauzon described different associations, mainly with *Enterobacter agglomerans* and *Klebsiella pneumoniae* (Lauzon 2003). Howard et al. (1985) found that *Klebsiella oxytoca* is the most common species associated with this fly throughout its life and suggested that this bacterium might be the equivalent symbiont in *R. pomonella*, to *Pseudomonas savastanoi* in the olive fly (Howard et al. 1985) (Table 2). However, the use of the rRNA approach has redefined the microbial community associated with the olive fly. Capuzzo et al. (2005) showed that the olive fly's symbiont is not *Pseudomonas savastanoi* (as suggested by Petri (1909)), and propose a novel bacterial species: *Candidatus Erwinia dacicola*.

A2. Species level background:
The following table summarizes studies on the microbial communities of tephritid fruitflies. Classical methods of identification include biochemical, culture and/or microscopic techniques. Molecular techniques for identification include 16s rDNA sequencing, DGGE and/or RFLP analysis.

**Table 2. Summary of the studies on the microbial communities of tephritid fruit flies.**

<table>
<thead>
<tr>
<th>Tephritid species:</th>
<th>Source of sample:</th>
<th>Bacterial species found (most common species in bold):</th>
<th>Microbial Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anastrepha ludens</em></td>
<td>Adult gut</td>
<td><em>Enterobacter cloacae</em>, <em>Providencia</em> spp <em>Citrobacter koseri</em> <em>Enterobacter sakazakii</em> <em>Klebsiella pneumoniae</em> <em>Pseudomonas aeruginosa</em></td>
<td>Classical</td>
<td>Kuzina et al. 2001</td>
</tr>
<tr>
<td><strong>A. ludens</strong></td>
<td>Adult crop and gut</td>
<td><em>Citrobacter freundii</em> <em>Klebsiella oxytoca</em></td>
<td>Classical</td>
<td>Martinez et al. 1994</td>
</tr>
<tr>
<td><strong>A. ludens</strong></td>
<td>Adult crop</td>
<td><em>Enterobacter</em> spp, <em>Pseudomonas aeruginosa</em> <em>Pseudomonas</em> spp.</td>
<td>Classical</td>
<td>Martinez et al. 1994</td>
</tr>
<tr>
<td><strong>A. ludens</strong></td>
<td>Adult gut</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>Classical</td>
<td>Martinez et al. 1994</td>
</tr>
<tr>
<td><strong>A. ludens</strong></td>
<td>infested fruit</td>
<td><em>Citrobacter freundii</em> <em>Klebsiella oxytoca</em></td>
<td>Classical</td>
<td>Martinez et al. 1994</td>
</tr>
<tr>
<td><em>Bactrocera cacuminata</em></td>
<td>Adult gut, Pupae, Eggs, infested fruit</td>
<td><em>Citrobacter freundii</em> <em>Klebsiella pneumoniae</em> <em>Pseudomonas</em> spp.</td>
<td>Classical</td>
<td>Fitt &amp; Obrien 1985</td>
</tr>
<tr>
<td><strong>B. cacuminata</strong></td>
<td>Host Plant leaves and fruit surface Adults</td>
<td><em>Pantoea</em> spp, <em>Pantoea agglomerans</em> <em>Citrobacter</em> <em>Enterobacter</em> <em>Klebsiella</em> <em>Providencia</em> <em>Serratia</em></td>
<td>Classical</td>
<td>Raghu et al. 2002</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Insect</th>
<th>Life Stage</th>
<th>Normal Bacteria</th>
<th>Identification Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. dorsalis</em></td>
<td>Adult gut</td>
<td><em>Klebsiella</em>, <em>Citrobacter</em>, <em>Enterobacter</em>, <em>Pectobacterium</em>, <em>Serratia</em>,</td>
<td>Molecular</td>
<td>Wang et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Actinobacteria</em>, <em>Firmicutes</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. jarvisi</em></td>
<td>Adult gut, Pupae, Eggs, infested fruit</td>
<td><em>Enterobacter agglomerans</em>, <em>Enterobacter cloacae</em>, <em>Enterobacter spp.</em>, <em>Klebsiella pneumoniae</em>, <em>Providencia spp.</em></td>
<td>Classical</td>
<td>Fitt &amp; Obrien 1985</td>
</tr>
<tr>
<td><em>B. jarvisi</em></td>
<td>Adult gut, Pupae, Eggs, infested fruit</td>
<td><em>Pseudomonas spp.</em></td>
<td>Classical</td>
<td>Fitt &amp; Obrien 1985</td>
</tr>
<tr>
<td><em>B. neohumeralis</em></td>
<td>Adult gut, Pupae, Eggs, infested fruit</td>
<td><em>Enterobacter cloacae</em>, <em>Enterobacter spp.</em>, <em>Pseudomonas spp.</em></td>
<td>Classical</td>
<td>Fitt &amp; Obrien 1985</td>
</tr>
<tr>
<td><em>B. oleae</em></td>
<td>Adult oesophageal bulb, gut, ovipositor</td>
<td><em>Ca. Erwinia dacicola</em></td>
<td>Molecular (16S rRNA gene)</td>
<td>Capuzzo et al. 2005</td>
</tr>
<tr>
<td><em>B. oleae</em></td>
<td>Adult oesophageal bulb, gut, ovipositor</td>
<td><em>Ca. Erwinia dacicola</em></td>
<td>Molecular (16S rRNA gene)</td>
<td>Sacchetti et al., 2008</td>
</tr>
<tr>
<td><em>B. oleae</em></td>
<td>Adult oesophageal bulb</td>
<td><em>Pseudomonas savastani</em></td>
<td>Classical</td>
<td>Petri 1909</td>
</tr>
<tr>
<td><em>B. oleae</em></td>
<td>Adults</td>
<td><em>Acetobacter tropicalis</em></td>
<td>Molecular</td>
<td>Kounatidis et al., 2009</td>
</tr>
<tr>
<td><em>B. oleae</em></td>
<td>Adults</td>
<td><em>Ca. Erwinia dacicola</em></td>
<td>Molecular</td>
<td>Estes et al., 2012</td>
</tr>
<tr>
<td><em>B. tau</em></td>
<td>Adults</td>
<td><em>Enterobacteriaceae</em>, <em>Staphylococcus</em></td>
<td>Classical &amp; Molecular</td>
<td>Prabhakar et al., 2009</td>
</tr>
<tr>
<td><em>B. tryoni</em></td>
<td>Adult crop, gut, mouthparts, host plant</td>
<td><em>Enterobacter cloacae</em>, <em>Klebsiella oxytoca</em>, <em>Klebsiella ozaenae</em>, <em>Pantoea agglomerans</em>, <em>Providencia spp.</em></td>
<td>Classical</td>
<td>Drew &amp; Lloyd 1987</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td></td>
<td></td>
<td>Thaochan et al., 2010</td>
</tr>
<tr>
<td><em>B. tryoni</em></td>
<td>Adult gut, Pupae, Eggs, infested fruit</td>
<td><em>Enterobacter agglomerans</em>, <em>Enterobacter cloacae</em>, <em>Klebsiella pneumoniae</em>,</td>
<td>Classical</td>
<td>Fitt &amp; O'Brien 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Providencia spp.</em>, <em>Pseudomonas spp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. tryoni</em></td>
<td>Adult crop, gut</td>
<td><em>Klebsiella oxytoca</em>, <em>Enterobacter cloacae</em></td>
<td>Classical</td>
<td>Murphy et al. 1988, 1994</td>
</tr>
<tr>
<td><em>B. tryoni</em></td>
<td>Adults</td>
<td><em>Serratia</em></td>
<td>Molecular</td>
<td>Thaochan et al., 2010</td>
</tr>
<tr>
<td><em>Ceratitis capitata</em></td>
<td>Adult gut</td>
<td><em>Enterobacter spp.</em>, <em>Klebsiella spp.</em></td>
<td>Classical</td>
<td>Lauzon 2003</td>
</tr>
<tr>
<td>Species</td>
<td>Stage</td>
<td>Bacteria</td>
<td>Method</td>
<td>References</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>----------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>C. capitata</td>
<td>Adult oesophageal bulb</td>
<td><em>Enterobacter agglomerans</em> <em>Klebsiella oxytoca</em> <em>Enterobacter cloacae</em> <em>Pseudomonas putida</em> <em>Pseudomonas spp.</em></td>
<td>Classical</td>
<td>Marchini et al. 2002</td>
</tr>
<tr>
<td>C. capitata</td>
<td>Adult gut, larvae, pupae, eggs, host plant</td>
<td><em>Enterobacter</em> <em>Klebsiella</em> <em>Pseudomonas</em> <em>Pectobacter</em> <em>Pantoea</em></td>
<td>Molecular</td>
<td>Behar et al., 2005, 2008a,b</td>
</tr>
<tr>
<td>Rhagoletis alternata</td>
<td>Adult gut, larvae</td>
<td><em>Enterobacter</em> <em>spp.</em> <em>Erwinia</em> <em>spp.</em></td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
<tr>
<td>R. completa</td>
<td>Adult Oesophageal bulb</td>
<td><em>Klebsiella oxytoca</em> <em>Klebsiella ozaenae</em> <em>Klebsiella pneumoniae</em></td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>R. cornivora</td>
<td>Adult Oesophageal bulb</td>
<td><em>Klebsiella oxytoca</em> <em>Klebsiella pneumoniae</em></td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>R. electromorpha</td>
<td>Adult Oesophageal bulb</td>
<td><em>Klebsiella oxytoca</em> <em>Enterobacter cloacae</em> <em>Klebsiella ozaenae</em> <em>Klebsiella pneumoniae</em></td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>R. mendax</td>
<td>Adult Oesophageal bulb</td>
<td><em>Klebsiella oxytoca</em> <em>Enterobacter agglomerans</em> <em>Enterobacter cloacae</em> <em>Klebsiella ozaenae</em> <em>Klebsiella pneumoniae</em></td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>R. pomonella</td>
<td>Adult Oesophageal bulb</td>
<td><em>Klebsiella oxytoca</em> <em>Enterobacter agglomerans</em> <em>Enterobacter cloacae</em> <em>Klebsiella ozaenae</em> <em>Klebsiella pneumoniae</em> <em>Pseudomonas putida</em> <em>Pseudomonas spp.</em></td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>R. pomonella</td>
<td>Adult Crop, gut, oesophageal bulb</td>
<td><em>Enterobacter agglomerans</em></td>
<td>Classical</td>
<td>Lauzon 1998; 2002</td>
</tr>
<tr>
<td>R. pomonella</td>
<td>Adult gut</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>Classical</td>
<td>Lauzon 1998; 2002</td>
</tr>
<tr>
<td>R. pomonella</td>
<td>Adult Oesophageal bulb</td>
<td><em>Enterobacter cloacae</em></td>
<td>Classical</td>
<td>Rossiter et al. 1983</td>
</tr>
<tr>
<td>R. pomonella</td>
<td>Adult Oesophageal bulb, Eggs, Larvae, Pupae, infested fruit.</td>
<td><em>Klebsiella oxytoca</em></td>
<td>Classical</td>
<td>Rossiter et al. 1983</td>
</tr>
<tr>
<td>R. suavis</td>
<td>Adult Oesophageal</td>
<td><em>Klebsiella oxytoca</em> <em>Enterobacter</em></td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>Species</td>
<td>Part of Organism</td>
<td>Isolated Species</td>
<td>Identification Method</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
<td>-------------------------------------------------------</td>
<td>-----------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>R. tabellaria</td>
<td>Adult Oesophageal bulb</td>
<td><em>Klebsiella oxytoca</em>&lt;br&gt;<em>Enterobacter agglomerans</em>&lt;br&gt;<em>Enterobacter cloacae</em>&lt;br&gt;<em>Klebsiella ozaenae</em>&lt;br&gt;<em>Klebsiella pneumoniae</em></td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>Tephritis conura</td>
<td>Adult gut, Larvae</td>
<td><em>Erwinia spp.</em></td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
<tr>
<td>Tephritis dilacerata</td>
<td>Adult gut, Larvae</td>
<td><em>Enterobacter spp.</em></td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
<tr>
<td>Urophora cuspidata</td>
<td>Adult gut, Larvae</td>
<td><em>Erwinia spp.</em></td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
<tr>
<td>Urophora solstitialis</td>
<td>Adult gut, Larvae</td>
<td><em>Erwinia spp.</em></td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
</tbody>
</table>

In addition to these published reports, several unplished results have been reported in this meeting:

- **Anastrepha**: The microbial communities of laboratory populations of *A. ludens* and wild *A. serpentina* and *A. striata* have been isolated and identified using 16s rDNA sequencing (Schuenzel et al., unpublished; Martinez et al., unpublished). For *A. fraterculus*, communities from larvae and adult tissues have been characterized with classical and molecular tools (Muller et al., unpublished).

- **Bactrocera**: In field populations of *B. minax* RFLP analyses revealed the presence of *Klebsiella* and *Enterobacter* (Niu unpublished). Using biochemical techniques several species of Enterobacteriaceae were identified in *B. cucurbitae*, and *B. dorsalis* (Khan et al. unpublished).

- **Dirioxa**: From field populations of *D. pornia*, *Bacillus*, *Enterobacter*, *Citrobacter*, *Serratia*, *Pseudomonas* and *Pantoea* were isolated and identified with biochemical tests and 16s rDNA sequencing (Crisp et al. unpublished).

**Gaps identified:**

A1. General:

Many of the previous studies have relied on numerous biochemical and molecular techniques to identify the bacterial communities. Because of the variety of methods and collection strategies of the fruit fly populations, confounding results may be clouding the literature. Standard practices for identification of microbial communities and specific microbes need to be established. Additionally, many of the studies focused on biochemical methods of bacterial identification which are not as accurate as molecular techniques specifically 16s rDNA sequencing.

The fruit fly populations under study can also result in very different microbial communities. When evaluating the microbial communities, the identity of the flies will create a bias in the community. The following variables have been identified as important considerations in sampling design:

- Strain: laboratory versus wild-caught
- Age
- Sex
- Larval Environment – rearing media (lab) and host (wild)
- Nutritional Status
- Season and Environmental Conditions
- Fly tissue sampled

Although the current literature provides adequate coverage of bacteria communities, other domains of microorganisms need to be investigated. This includes Archaea, Eukaryotes such as fungi and protists as well as viruses.

A2. Species level background

*Anastrepha*: Molecular data is lacking for the entire genus.

*Bactrocera*: Most species are lacking molecular data except *B. dorsalis, B. oleae, B. tau* and *B. tryoni*. For the relatively well-studied olive fly, the differences in community structure of wild and lab-reared flies need to be resolved.

*Ceratitis*: Only the medfly has been studied.

*Dacus*: Molecular data is lacking for the entire genus.

*Dirioxa*: Molecular data is lacking for the entire genus.

*Rhagoletis*: Molecular data is lacking for the entire genus.

**Subtheme B: Effects of radiation**

**Current knowledge**

B1 General

In addition to the changes induced by colonisation, irradiation (and probably sterilisation by other means) affects the structure of the microbial community (Ben-Ami et. al., 2010; Lauzon and Potter 2012).

B2. Species level background

*Anastrepha*: Irradiation affects the structure. Irradiation harms the epithelia of the gut and affects the ability of bacteria to colonize this region (Lauzon and Potter 2011).

*Ceratitis*: The structure of the microbial community in irradiated flies differs significantly from that of mass reared or wild flies. Specifically, the abundance of Enterobacteriaceae is reduced and that of pseudomonads increases.

**Gaps identified:**

B1. General

There is a general lack of understanding of this phenomenon. The main questions to be resolved are —why are some bacteria affected and others not, can the damage to the gut be remediated by probiotic bacteria (see subtheme D below).
B2. Species level background
The gaps mentioned above are relevant to all mass reared species and SIT programs that rely on the release of irradiated males.

Subtheme C: Function of microbiota and fitness effects

Current knowledge:

C1. General
The widespread and ubiquitous presence of discrete microbial communities, coupled with specific anatomical modifications in their hosts, suggests that these microorganisms contribute to the fitness of their insect host. In general, bacteria are an important food source for tephritids, but how this is mediated is unclear. To date several specific functions of resident bacteria have been identified. These include nitrogen fixation, pectinolysis, and utilization of urea as a nitrogen source. In addition the properties displayed by secondary symbionts of other insects, such as thermotolerance, insecticide resistance, immunity to pathogens and host range expansion may be present in tephritid microbial communities as well.

C2. Species level background
Anastrepha: No information available
Bactrocera: In the olive fly, the presence of symbionts is obligatory to the development of larvae in olives. Furthermore, symbionts enable adults to utilize nitrogen poor diets thus contributing to female fitness.
Ceratitis: Bacteria facilitate the utilization of the host plant by the medfly larvae. In the adult stage bacteria contribute to nutrition, longevity and male mating success.
Dacus: No information available
Dirioxa: In order to rear under laboratory conditions, D. pornia needs bacterial supplements.
Rhagoletis: A nutritional function has been assumed.

Gaps identified:

C1. General
Although the community structure of many species has been described, relatively little is known of their function and contribution to fly fitness, and this needs to be addressed in each fly species.
Novel tools can be brought to bear such as functional genomic assessment, metatranscriptomics and metagenomics.

C2. Species level background
Anastrepha: The function and fitness contributions of the microbial communities need to be determined.
**Bactrocera:** For the olive fly, determine the physiological basis of bacterial activity and the possibility to emulate or disrupt the activity artificially. The function and fitness contributions of the microbial communities need to be determined for other species of interest.

**Ceratitis:** Unravel how different members of the described community contribute alone and together to larval development and adult nutrition and reproduction. Determine the physiological basis of bacterial activity and the possibility to emulate the activity artificially.

**Dacus:** The function and fitness contributions of the microbial communities need to be determined.

**Dirioxa:** Determine the mechanism whereby the bacteria contribute to adult reproduction in *D. pornia*.

**Rhagoletis:** The function and fitness contributions of the microbial communities need to be determined.

**Subtheme D: Applications**

**Current knowledge:**

D1. General
The widespread presence of bacterial symbionts suggests that their presence can be manipulated to harm the insect host, or improve its condition. The latter option is relevant to the sterile insect technique, whereby mass reared males are sterilized and released in the field, where their mission is to copulate with wild females and disrupt their reproduction. Bacterial inoculations, provided to the larval or adult stage (or both) could improve the efficiency of mass rearing. Importantly, the performance of sterile males may be improved by providing a symbiotic inoculum prior to release in the field.

To date only a few studies have been carried out based on this rationale (detailed below). However their results suggest that this is a valid approach.

D2. Species level background

**Anastrepha:** For *A. obliqua* and *A. serpentina*, bacterial supplements to adults had a beneficial impact on matings (Gomez et al. unpublished).

**Bactrocera:** In the olive fly, feeding adults with *Pseudomonas putida* isolated from the oesophageal bulb of wild flies, showed improvement in subsequent egg production (Sacchetti et al., unpublished). In *B. dorsalis*, Khan et al. (unpublished) observed mating enhancement by adding *Klebsiella* and *Proteus* incorporated into a full diet and sugar diet of adults. For *B. tryoni* and *B. tau* bacteria added to female diet did not affect fecundity (Meats et al., 2009; Khan et al., unpublished).

**Ceratitis:** In the medfly, Niyazi et al., (2004) provided a mixture of *Enterobacter* and *Klebsiella* to irradiated males. Results obtained in the field were inconsistent, as no differences between treated and control males were found for any of the diets. Ben-Ami et al. (2010) provided sterile males with an oral inoculum of *K. oxytoca* derived from wild medflies. This strain became established in the flies gut, and preliminary results suggested improvement in sexual performance. Indeed, the latency to mating of sterile males was shorter following feeding on this bacterial strain (Ben-Ami et al., 2010). Recently, Gavriel et al (2011) found that enriching the sterile male diet with *K. oxytoca* significantly improved
mating competitiveness in the laboratory and in field cages. In addition, bacterially enriched sterile males inhibited female receptivity more efficiently than sugar fed males and survived longer duration of starvation.

Dirioxa: Bacteria provided to adults enable successful colonization. Specifically, the provision of bacteria increases activity levels and improves hatching rates (Crisp et al., unpublished).

**Gaps identified**

D1. General
This approach, based on community and function/fitness studies, needs to be brought to bear on additional species of economic importance.

As these studies evolve, several important standards should be observed:
- **Formulation** – determine that the formulation provided allows survival, and growth in the insect (growth stage of bacteria harvested, osmolarity of solution, PH, etc’).
- **Koch's postulates** - important to recover inoculated symbiont in order to be sure that it is providing the benefit. Suggested positive controls- gfp (OK for experiments, not release) antibiotic resistant bacteria or other genetic markers. Proper negative controls (e.g.- check that autoclaved bacteria do not provide the same benefits as live ones).

D2. Species level background

*Anastrepha*: For most species, no studies have been conducted. In *A. obliqua* and *A. serpentina* the nature of the supplemental bacteria as a food source or symbiont needs to be determined.

*Bactrocera*: For most species, no studies have been conducted. In olive flies, determine how combinations of bacteria can improve fitness. Explore the possibility of reintroducing *Ca. E. dacicola* to laboratory lines. In *B. dorsalis*, the nature of the supplemental bacteria as a food source or symbiont needs to be determined.

*Ceratitis*: In medflies, determine how combinations of bacteria can improve fitness. Optimal formulation of adult diets and preliminary field trials are needed.

*Dacus*: No studies have been conducted.

*Dirioxa*: In *D. pornia*, the nature of the supplemental bacteria as a food source or symbiont needs to be determined.

*Rhagoletis*: No studies have been conducted.

**Individual Plans**

2.1. **Structure of microbiota**

**Genus Anastrepha**

*Anastrepha ludens*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)
5 years plan
- Identification of microbial communities in wild and laboratory adult (irradiated and non-irradiated) by using molecular and classical microbiological phenotyping approaches.
- Identification of the beneficial bacteria and determine the role of the associated bacteria on the rearing (fecundity, fertility, life span) and behavior parameters.

18 months plan
- No activities.

*Anastrepha obliqua*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of microbial communities in wild and laboratory adult (irradiated and non-irradiated) by using molecular and classical microbiological phenotyping approaches.
- Identification of the beneficial and determine the role of the associated bacteria on the rearing (fecundity, fertility, life span) and behavior parameters.

18 months plan
- Identification of microbial communities in wild and laboratory adult (irradiated and non-irradiated) by using molecular and classical microbiological phenotyping approaches.
- Identification of the beneficial and determine the role of the associated bacteria on the rearing (fecundity, fertility, life span) and behavior parameters.

*Anastrepha serpentina*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of microbial communities in wild and laboratory non-irradiated adults by using molecular and classical microbiological phenotyping approaches.
- Determine the role of the associated bacteria on the rearing and behavior parameters.

18 months plan
- No activities.

*Anastrepha striata*
Participants: Emilio Hernandez, Pablo Liedo (Mexico)

5 years plan
- Identification of microbial communities in wild and laboratory non-irradiation adults by using molecular and classical microbiological phenotyping approaches.
- Determine the role of the associated bacteria on the rearing and behavior parameters.
18 months plan
- No activities.

*Anastrepha ludens, A. serpentina, A. obliqua and A. striata*
Participants: Mariana Mateos, Humberto Martinez-Montoya (USA)
Collaborators: Jorge Toledo, Emilio Hernandez (Mexico)

5 years plan
- Compare pyrosequencing results to culture-dependent results.
- Conduct further pyrosequencing analyses of irradiated (sterile) and non-irradiated males, to evaluate the effect of irradiation on microbiota

18 months plan
- Use of high-throughput DNA sequencing (16S Pyrosequencing) methods to characterize the additional bacterial composition of the microbiota (*A. ludens, A. serpentina, A. obliqua and A. striata*). Simultaneously, our colleagues in Mexico will conduct culture-dependent identification of bacteria in the same species

*Anastrepha fraterculus*
Participant: Diego Segura (Argentina)
Collaborator: Boaz Yuval (Israel)

5 years plan
- Description of the main bacteria inhabiting the gut of wild flies through the characterization of the 16S rDNA gene and its comparison with sequences published in databases using nucleotide Blastn.
- Evaluation of changes in microbiota due to adaptation to laboratory conditions following the methodology described above.
- Assessing differences in gut bacterial community among wild caught flies, wild flies that have emerged in the laboratory and laboratory flies following the methodology described above.
- Evaluation of less abundant bacteria

18 months plan
- Description of gut bacterial community in wild flies.
- Evaluation of changes in microbiota due to adaptation to laboratory conditions.

Participant: Fernando L. Consoli (Brazil)

5 year plan
- Isolate and characterize the microbiota of the adult gut of a population of *A. fraterculus* reared in a natural food source (completed)
- Assess the role of the food source (6 natural food sources) on the composition of the adult gut microbiota by 16S pyrosequencing in a FLX 454 platform (in progress)
- Check the effect of the natural and artificial diet on the prevalence of selected culturable symbionts associated with the esophageal bulb of *A. fraterculus* by qPCR analysis
• Test the occurrence of selected bacterial symbionts associated to adults of *A. fraterculus* from different locations and host fruits by using diagnostic PCR

• Check the occurrence of selected bacterial strains associated with the esophageal bulb of *A. fraterculus* in other species of *Anastrepha* from Brazil by using diagnostic PCR

• Check the molecular divergence of strains of selected symbionts associated with *A. fraterculus* with those strains occurring in other species of *Anastrepha*. Compare their molecular divergence with that of the species of *Anastrepha* studied by using existing or generating the ITS2 sequences available and/or use the molecular data produced by the CRP on cryptic species (if become available)

• Investigate the impact of interspecific larval competition in the microbiota community associated with the esophageal bulb of adults of *A. fraterculus*

18 month plan

• Isolate and characterize the microbiota of the adult gut of a population of *A. fraterculus* reared in a natural food source (completed)

• Assess the role of the food source (6 natural food sources) on the composition of the adult gut microbiota by 16S pyrosequencing in a FLX 454 platform (in progress)

**Genus Bactrocera**

*Bactrocera cucurbitae, Bactrocera dorsalis*

Participant: Mahfuza Khan (Bangladesh)
Collaborators: Kostas Bourtzis and George Tsiamis (Greece)

5 years plan

• Isolation and identification of the gut microbial community of laboratory (host) reared *B. cucurbitae* and *B. dorsalis* using classical microbial and biochemical tests.

• Collection four *Bactrocera* species (*B. cucurbitae, B. dorsalis, B. tau, and B. zonata*, from four different districts of Bangladesh using cue-lure and methyl-eugenol baited traps). Identification of gut microbial community using molecular techniques (16s rDNA pyro sequencing).

Plan for 18 months

• Isolation and identification of gut bacterial community of laboratory host reared adult *B. cucurbitae* and *B. dorsalis* using classical microbial and biochemical approaches (completed).

• Collection four *Bactrocera* species (*B. cucurbitae, B. dorsalis, B. tau, and B. zonata*, from four different districts of Bangladesh using cue-lure and methyl-eugenol baited traps) and identification of gut microbial communities using molecular techniques (16s rDNA pyrosequencing).

*Bactrocera cucurbitae*

Participant: Ramesh Hire (India)

5 years plan

• Isolation and characterization of gut microbiota of *B. cucurbitae* using molecular techniques.
- Phylogenetic analysis of gut microbiota.

18 months plan
- Isolation and characterization of gut microbiota of *B. cucurbitae*

**Bactrocera dorsalis, Bactrocera minax**  
Participant: Changying Niu (China)

5 years plan
- Collect specimens from the wild population and lab colony of *B. dorsalis* and *B. minax*;
- Dissect mid-gut and extract bacterial DNA;
- Use second-generation DNA sequencing technology to identify the bacteria in the midgut of *B. minax*, study the composition, diversity of gut symbiotic microbiota through the high-throughput sequencing of bacteria 16S rDNA V6 sequence
- Compare microbiota structure and diversity in *B. dorsalis* and *B. minax*.

18 months plan
- Isolate DNA and conduct high-throughput sequencing of bacteria 16S rDNA V6 sequence from *B. dorsalis* and *B. minax*.

**Bactrocera oleae**  
Participant Antonio Belcari

5 years plan
- Verify the presence of *Ca. Erwinia dacicola* in wild populations in different olive crop areas and during the season.
- Investigations on the gut microbiome of lab reared flies.

18 months plan
- Determine the presence of *Ca. Erwinia dacicola* in the olive fly populations

**Bactrocera cucurbitae, Bactrocera zonata**  
Participants: Sabrina Dyall, Preeaduth Sookar, Malini Alleck (Mauritius)

5 years plan
- Collection of wild *B. cucurbitae* and *B. zonata* adult males from 5 geographical locations in Mauritius and from laboratory-reared flies, at 3-monthly intervals
- Isolation of gut microbiota and cultivation of bacteria
- Extraction of DNA and PCR amplification of 16S rDNA fragments from cultivated bacteria followed by sequencing
- Extraction of community DNA and PCR amplification of bacterial-specific and archaeal-specific 16S rDNA fragments, and of eukaryotic-specific markers; production of libraries from the three domains.

18 months
- Collection of 6 X 5 batches of fruit flies from each species, isolation of microbiota and identification of microbes using molecular tools.
**Bactrocera tryoni**  
Participant Olivia Reynolds, Toni Chapman, Peter Crisp (Australia)

5 years plan
- Identification of microbial communities using molecular techniques (16S rDNA and sequencing). Compare microbial communities of wild *B. tryoni* (and other *Bactrocera* spp.) with both irradiated and non-irradiated communities of factory *B. tryoni*.
- Isolate and identify (through molecular profiling) probiotic candidate bacteria from sampled flies.

18 months
- Collection of *B. tryoni* from the Fruit Fly Production Facility (FFPF) before and after irradiation and other native and endemic *Bactrocera* spp. from different areas of Australia every 3 months for a 12 month period.
- Isolation and identification of intestinal microflora of all samples using molecular assays.

**Genus Ceratitis**

**Ceratitis capitata**  
Participant Peter Crisp (Australia)

5 years plan
- Commence assessment of bacteria from wild *C. capitata* from and identify volatile compounds

18 months
- Nothing planned for this time period

Participants: Hernán Donoso, Carolina Yáñez (Chile)

5 years plan
- Isolation of gut microbiota and cultivation of bacteria
- DNA extraction and PCR amplification of 16S rRNA genes for DGGE analysis
- Identification of microbial communities in adults of the laboratory colony under mass rearing and small scale conditions

18 months
- Isolation of gut microbiota and cultivation of bacteria
- DNA extraction and PCR amplification of 16S rRNA genes

Participants: Jaime Garcia de Oteyza and Teresa Navarro (Spain)

5 years plan
- Identification of microbial communities in adults of wild and mass reared medfly (different strains and colonies), by molecular and classical approaches (DGGE, 16S rRNA sequencing, pyrosequencing)
18 months plan

- Identification of microbial communities in adults of wild and mass reared medfly (different strains and colonies), by molecular and classical approaches (DGGE, 16S rRNA sequencing, pyrosequencing)

**Genus Dirioxa**

*Dirioxa pornia*

Participant Peter Crisp (Australia)

5 years plan

- Isolate bacteria from *D. pornia* for multiple populations and varied crops

18 months plan

- Isolate bacteria from *D. pornia* wild flies and identify using molecular techniques 16s rRNA.

2.2. Effects of radiation

**Genus Anastrepha**

*Anastrepha fraterculus*

Participant: Diego Segura (Argentina)

Collaborator: Boaz Yuval (Israel)

5 years plan

- Determine the effect of irradiation (gamma and X-rays, 70Gy) on the gut microbial community of laboratory flies through the characterization of the 16S rDNA gene and its comparison with sequences published in databases using nucleotide Blastn.

18 months plan

- Determine the effect of irradiation (gamma and X-rays) on the gut microbial community of laboratory flies.

*Anastrepha ludens, A. serpentina, A. obliqua and A. striata,*

Participants: Mariana Mateos, Humberto Martinez-Montoya (USA)

Collaborators: Jorge Toledo, Emilio Hernandez (Mexico)

5 years plan

- Conduct pyrosequencing analyses of irradiated (sterile) and non–irradiated males, to evaluate the effect of irradiation on microbiota.

18 months plan

- No planned activities.

**Genus Bactrocera**
**Bactrocera cucurbitae, Bactrocera zonata**  
Participant: Sabrina Dyall, Preeaduth Sookar, Malini Alleck (Mauritius)

5 years plan
- To study the effect of irradiation on laboratory-reared *B. cucurbitae* and *B. zonata* adult male flies: cultivate and identify, using molecular tools, bacteria from irradiated and non-irradiated flies

18 months plan
- To study the effect of radiation on the microflora of the alimentary tract of the fruit flies

**Bactrocera cucurbitae, Bactrocera dorsalis**  
Participant: Mahfuza Khan (Bangladesh)  
Collaborators: Kostas Bourtzis, and George Tsiamis (Greece)

5 years plan
- Observation on the possible change of gut microbial community of laboratory reared (host/artificial larval diet) irradiated (sterile) and control male *B. cucurbitae* and *B. dorsalis* using molecular techniques (16s rDNA pyrosequencing).

18 months plan
- Observation on the possible change of gut microbial community of laboratory reared (host/artificial larval diet) irradiated (sterile) and control male *B. cucurbitae* using molecular techniques (16s rDNA pyro sequencing).

**Bactrocera cucurbitae**  
Participant: Ramesh Hire (India)

5 years plan
- Study the effect of irradiation on gut microbiota of *B. cucurbitae*.  
- Comparative analysis of microflora before and after irradiation.

**Bactrocera dorsalis**  
Participant: Changying Niu (China)

5 years plan
- Irrariate the GSS of laboratory colony for *B. dorsalis* male with X-ray;  
- Supplement probiotics in the adults diets and determine the bacterial difference before and after irradiation;  
- Conduct cage experiments to investigate whether probiotics will improve the mating competitiveness and survival ability for *B. dorsalis* sterile males.

18 months plan
- Irritate the GSS of *B. dorsalis* male with X-ray.

**Bactrocera oleae**
Participant Antonio Belcari (Italy)

5 year plan
- Evaluation of presence of Ca. Erwinia dacicola and/or other bacteria in the olive fly sterilized males

18 months plan
- No planned activities

Genus Ceratitis

Ceratitis capitata
Participants: Hernán Donoso, Carolina Yáñez (Chile)

5 years plan
- Isolation of gut microbiota and cultivation of bacteria in irradiated adults
- DNA extraction and PCR amplification of 16S rRNA genes for DGGE analysis
- Identification of microbial communities in irradiated adults of the laboratory colony under mass rearing and small scale conditions

18 months plan
- Isolation of gut microbiota and cultivation of bacteria in irradiated adults
- DNA extraction and PCR amplification of 16S rRNA genes

2.3. Function of microbiota and fitness effects

Genus Anastrepha

Anastrepha fraterculus
Participant: Diego Segura (Argentina)
Collaborator Boaz Yuval (Israel)

5 year plan
- Evaluation of the effect of gut bacteria on mating competitiveness, by assessing the mating choice of A. fraterculus females facing males treated with antibiotics and untreated males under laboratory conditions. Fly origin (laboratory and wild) and nutritional status (protein-fed and protein-deprived) dependent responses will be assessed.

- Evaluation of the effect of gut bacteria on starvation resistance by comparing (under laboratory conditions) survival rates of males treated with antibiotics and untreated males. Fly origin (laboratory and wild) and nutritional status (protein-fed and protein-deprived) dependent responses will be assessed.

- Evaluation of the effect of gut bacteria on male sex pheromone and cuticle hydrocarbons (CHC). Differences in sex pheromone and CHC between antibiotic treated males and non-treated males will be assessed by GC-FID/GC-MS
18 months plan
- Evaluation of the effect of gut bacteria on mating competitiveness.
- Evaluation of the effect of gut bacteria on starvation resistance.

Participant Fernando L. Consoli (Brazil)

5 years plan
- Test the requirement of selected bacterial strains for adult reproduction (in progress)

18 months plan
- Test the requirement of selected bacterial strains for adult reproduction (in progress)

**Genus Bactrocera**

*Bactrocera cucurbitae, Bactrocera zonata*
Participant Sabrina Dyall, Preaduth Sookar, Malini Alleck (Mauritius)

5 years plan
- To investigate the effect of isolated bacterial symbionts of *B. cucurbitae* and *B. zonata* during pre-release period on sterile insect performance.

18 months plan
- To isolate and cultivate potential bacterial candidates for probiotic development

*Bactrocera oleae*
Participants: Boaz Yuval, Edouard Jurkevitch, Inbar Shuster-Dagan (Israel)

5 years plan
- For *Bactrocera oleae*, determine the bacterial contribution to olive fly fitness in relation to its nutritional ecology.
- Determine how bacteria enable the olive fly to develop in olives.

18 months plan
- Examine the ability of wild and mass reared flies, with and without bacteria, to develop in olives of different varieties at varying stages of maturity.
- Decipher the mechanism whereby bacteria enable larvae to overcome the activity of oleorupin.

*Bactrocera tryoni*
Participant Peter Crisp, Olivia Reynolds (Australia)

5 years plan
- Conduct laboratory and field cage trials assessing the effects of a probiotic supplemented diet on life history parameters including emergence, longevity (including under starvation), fecundity, female remating & flight of sterile (irradiated) male *B. tryoni*.
18 months plan
  • Sampling fruit fly populations as above.

**Bactrocera dorsalis and Bactrocera minax**
Participant: Changying Niu (China)

5 years plan
  • Supplement antibiotics in the diets of larvae for *B. dorsalis* and *B. minax*;
  • Compare the survival, weight, pupation rate of larvae with control;
  • Calculate and compare the cost of different larval diets for *B. dorsalis* and *B. minax*.

18 months plan
  • Supplement antibiotics in the diets of larvae for *B. dorsalis* and *B. minax*.

**Genus Ceratitis**

*Ceratitis capitata*
Participant Peter Crisp (Australia)

5 years plan
  • Commence feeding studies using individual bacteria and assess probiotics effect on longevity and quality of irradiated male *C. capitata*
  • Elucidate the behavioral changes in *C. capitata* induced by exposure to volatile compounds identified from the bacteria

18 months plan
  • The bacteria species isolated from *D. pornia* and associated will be assessed for probiotic and behavioral changes for improvement of performance of sterile male *C. capitata* used in SIT eradication programs.

Participants: Jaime Garcia de Oteyza, Teresa Navarro (Spain)

5 years plan
  • Identify suitable beneficial bacteria and quality control after their inoculation (longevity under stress, flight capacity, eggs/female…)

18 months plan
  • Identify suitable beneficial bacteria and quality control after their inoculation (longevity under stress, flight capacity, eggs/female…)

Participant Nikos Papadopoulos (Greece)
Collaborators: K. Bourtzis and G. Tsiamis (Greece)

5 years plan
  • Effects of probiotic provision of symbionts on the sexual behaviour of Vienna 8 males.
• Define interactions of aromatherapy and provision of probiotic inoculum to Vienna 8 males.

18 months plan
• No planned activities

**Genus Dirioxa**

*Dirioxa pornia*

Participant Peter Crisp (Australia)

5 years plan
• Feeding studies to assess longevity, fecundity and quality of *D. pornia* continued
• Continue lure and kill trials with *D. pornia*

18 months plan
• Commence feeding studies using individual bacteria and assess effect on longevity, fecundity and quality of adult *D. pornia*.
• DNA analysis of eggs, larvae and pupae of *D. pornia* from laboratory culture for bacteria.
• Identity volatile compounds associated with the bacteria.
• Elucidate which volatile compounds are attractive to *D. pornia*.

2.4. Applications

**Genus Anastrepha**

*Anastrepha ludens*

Participant Erin Schuenzel (USA)

5 years plan
• Track presence of probiotics given to *Anastrepha ludens* larvae in the adult midgut using marked bacteria and selective media
• Geneotype probiotic strains surviving in *A. ludens* adults using MLST or newly developed genetic markers
• Assess ability to produce probiotic strain for large scale application
• Make strains available for adult fitness tests

18 months plan
• Identify probiotic symbionts in *A. ludens* larval populations from mass-rearing facility using Koch’s postulates

Participants: Dina Melgar, Pablo Rendón, Felipe Jerónimo (Guatemala)

5 years plan
• Isolation and identification through molecular techniques RT- PCR (16S rDNA) of entomopathogenic and endosymbiotic bacteria for both wild and mass reared insects
• Evaluation of the effect of endosymbiotic bacteria inoculation in the adult diets in mass rearing facilities
• Endosymbionts inoculation in agar diets and pellum (cellulose matrix + sugar water) in release center and measure impact on insect fitness

18 months plan
• Isolation and identification through molecular techniques RT- PCR (16S rDNA) of entomopathogenic and endosymbiotic bacteria for both wild and mass reared insects

Anastrepha fraterculus
Participant: Diego Segura (Argentina)
Collaborator Boaz Yuval (Israel)

5 year plan
• For A. fraterculus - Evaluation of methods to restoring bacteria identified in the wild flies in the sterile laboratory male gut.
• Evaluation of the effect of restoring key bacteria on sterile male sexual competitiveness, male sex pheromone and CHC, and survival.
• Evaluation of the effect of restoring key components of the male sex pheromone and/or CHC on sterile male sexual competitiveness.

18 months plan
• No activities planned for this time period.

Genus Bactrocera

Bactrocera cucurbitae, Bactrocera zonata
Participants: Sabrina Dyall, Preeaduth Sookar, Malini Alleck (Mauritius)

5 years plan
• To design probiotic diets that will improve fitness of B. cucurbitae and B. zonata irradiated adult males
• To determine the effects of the probiotic diets on the following quality control parameters: egg hatch, pupal weight, calling and mating behavior

18 months plan
• To isolate and characterize microbial candidates for probiotic diet development

Bactrocera cucurbitae, Bactrocera dorsalis
Participant Mahfuza Khan (Bangladesh)

5 years plan
• Semi-field cage experiments on the effect of probiotic adult diets as pre-release supplement to enhance the mating competitiveness of control and sterile B. cucurbitae and B. dorsalis.
• Determine the survival of control and sterile \textit{B. cucurbitae} and \textit{B. dorsalis} fed on probiotic adult diets under semi-field cage trials.

18 months plan
• Determine the survival of adult \textit{B. cucurbitae} fed on probiotic adult diets under semi-field cage trials.

\textbf{Bactrocera oleae}
Participant Antonio Belcari (Italy)

5 year plan
• Development of bacterial symbiosis transfer (\textit{Ca.} Erwinia dacicola from wild flies to lab reared flies)
• Development of a probiotic diet for the adult

18 months plan
• Preliminary lab trials aimed at development of symbiosis transfer

\textbf{Bactrocera dorsalis}
Participant: Changying Niu (China)

5 years plan
• Clone the full-length of HMG-R gene in \textit{B. dorsalis}
• Study the function of HMG-R gene by RNAi in \textit{B. dorsalis}
• Express dsRNA of HMG-R gene in \textit{E. coli} strain HT-115
• Feed these bacteria to the larvae of \textit{B. dorsalis} in the diets
• Conduct lab and cage experiments to examine mating, oviposition of females, and hatching rate of the offspring

18 months plan
• Clone the full-length of HMG-R gene in \textit{B. dorsalis} and study the function of HMG-R gene by RNAi.

\textbf{Bactrocera tryoni}
Participant Olivia Reynolds, Toni Chapman, Peter Crisp (Australia)

5 years plan
• Field cage trials assessing longevity and mating success of probiotic supplemented flies (subject to funding)
• Field releases of of probiotic supplemented sterile \textit{B. tryoni} (subject to funding)

18 months plan
• Collection of \textit{B. tryoni} from the Fruit Fly Production Facility (FFPF) before and after irradiation and other native and endemic \textit{Bactrocera} spp. from different areas of Australia every 3 months for a 12 month period.
• Isolation and identification of intestinal microflora of all samples using molecular assays.
**Genus Ceratitis**

*Ceratitis capitata*

Participants: Dina Melgar, Pablo Rendón, Felipe Jerónimo (Guatemala)

5 years plan
- Isolation and identification through molecular techniques RT-PCR (16S rDNA) of entomopathogenic and endosymbiotic bacteria for both wild and mass reared insects
- Evaluation of the effect of endosymbiotic bacteria inoculation in the adult diets in mass rearing facilities
- Endosymbionts inoculation in agar diets and pellum (cellulose matrix + sugar water) in release center and measure impact on insect fitness

18 months plan
- Isolation and identification through molecular techniques RT-PCR (16S rDNA) of entomopathogenic and endosymbiotic bacteria for both wild and mass reared insects

Participants: Peter Crisp (Australia)

5 years plan
- Establish trials to assess effect of exposure to bacterial volatiles for SIT *C. capitata*
- Establish field trials to assess effect of bacterial feed at emergence for SIT *C. capitata*
- Establish field trials to assess effect of exposure to bacterial volatiles for SIT *C. capitata*

18 months plan
- No activities planned for this time period.

Participants: Jamie Garcia de Oteiza Teresa Navarro (Spain)

Five years plan
- Development of probiotic culture and supply/inoculation methodology for mass rearing programmes

18 months plan
- No activities planned for this time period.
3. SYMBIONTS AND NOVEL CONTROL TOOLS

Background situation analysis

Certain symbiotic bacteria are known to manipulate the mating behaviour and reproduction of their hosts. These include Wolbachia and other symbiotic bacteria such as Cardinium, Arsenophonus, Spiroplasma and Rickettsia. Most widespread is Wolbachia with about 60% of infected insects - yet Arsenophonus and Cardinium have also been found to be relatively widespread in arthropods. There is a need to characterize these symbionts, to determine their phenotypes and their fitness effects on hosts, and their interactions with other microorganisms. This characterization is essential to develop approaches that will allow introduction of symbionts such as Wolbachia and others into target populations with potential to effectively reduce pest populations and their economic impact.

There are two potential approaches. First, the incompatible insect technique (IIT) employs cytoplasmic incompatibility, which is induced by insect symbionts such as Wolbachia. In a Wolbachia-based IIT strategy, female sterility is artificially sustained in pest populations by repeated releases of cytoplasmically incompatible mass-reared males. Since Wolbachia is not paternally transmitted, the infection type present in the release strain does not become established in the field. For this reason, IIT requires the release of males only, thus the availability of an efficient sexing technique. Similar to the conventional SIT, the increasing ratio of incompatible matings over time can lead to population suppression.

Secondly, Wolbachia and other reproductive symbionts could be used to manipulate host population fitness (a) to reduce/block the capacity to transmit pathogens such viruses through life span reduction and interference with pathogens, (b) to modulate the behaviour (feeding behaviour, mating behaviour) and (c) to impact abiotic stress resistance (thermotolerance, dessiccation resistance, dormancy). Some of these approaches have already been tested in the laboratory and in the field, e.g. pathogen transmitting mosquitoes. It is worthwhile to explore their potential application against tephritid pests.

Subtheme A: Detection, molecular and phenotypic characterization

Current knowledge:

A1. General:
A range of reproductive symbionts has been found in Tephritids. So far, Wolbachia seems the most dominant, with detection in several species of Rhagoletis, Bactrocera, Anastrepha, Dacus and Ceratitis. Furthermore, other reproductive symbionts have been detected.

The largest knowledge so far is available about Wolbachia, with a wide diversity of strains of mostly A supergroup and some B supergroup. Wolbachia infections either occur as single infections, but also as multiple infections in individuals, with dominant and less dominant strains.

The starting point of detection for reproductive parasites is the conserved bacterial 16S rDNA sequence analysis; however this approach is not sufficient enough for discriminating strains that can induce different phenotypes and/or have different origins. The molecular characterization of these symbionts is made available through Multi Locus Sequence Typing (MLST) systems that have recently been developed for Wolbachia while it is only partially developed for Arsenophonus.
So far the only characterized phenotypes of *Wolbachia* in tephritids is induction of cytoplasmic incompatibility (CI).

*Wolbachia* infections have been reported to have either positive or negative effects on fitness of host populations. Artificially *Wolbachia* infected medfly lines suffer from reduced fitness such as survival and reproduction.

A2 Genus level background:

**Anastrepha:** *Wolbachia* detected in some populations of *A. serpentina, A. striata, A. obliqua, A. fraterculus, A. amita, A. sororcula, A. pickelli*. With exception of *A. striata* (carries both A and B supergroups) all supergroup A. There is an indication of CI in *A. fraterculus*.

**Bactrocera:** *Wolbachia* detected in some populations of *B. dorsalis, B. philippinensis, B. carambolae*, and several Australian *Bactrocera* (e.g. *B. neohumeralis*).

**Ceratitis:** *Wolbachia* detected in one Brazilian and one French population of *C. capitata*.

**Dacus:** *Wolbachia* detected in *D. destillatoria* from Thailand and also in some Australian *Dacus* species.

**Rhagoletis:** *Wolbachia* detected in all populations of *R. cerasi*, in some populations of *R. pomonella*, and *R. cingulata*. There are several *Wolbachia* strains present in field populations of *R. cerasi*. CI expressed in field population of *R. cerasi*.

**Gaps identified**

A1. General:
(i) molecular detection
- Lack of universal system to detect reproductive parasites
- So far limited sampling and screening of species and populations

(ii) molecular characterization
- Lack of MLST systems for other reproductive parasites other than *Wolbachia*
- So far limited characterisation of symbionts in different species and populations

(iii) phenotypic characterization of
- Most interactions have not yet been characterized phenotypically
- Limited knowledge about the effects of reproductive symbionts on behavioural traits
- Lack of understanding of the molecular mechanism causing full or partial CI.
- Lack of understanding of host symbiont interaction leading to sustainable artificial infected insect pest lines.

A2. Genus level background:

**Anastrepha:**
- CI in *Anastrepha* species is not fully understood.
- Lack of knowledge of infection frequencies and types in field populations.
- No understanding of fitness effects other than CI.

**Bactrocera:**
- CI in *Bactrocera* species is not fully understood.
- Lack of knowledge of infection frequencies and types in field populations.
- No understanding of fitness effects other than CI.

**Ceratitis:**
- More data need to be gathered regarding infection frequencies and types in field populations.
- No understanding of fitness effects or phenotypes in naturally *Wolbachia* infected populations.
- Additional studies required to understand fitness effects in artificially infected medfly lines.

**Dacus:**
- Lack of knowledge of infection frequencies and types in field populations.
- No understanding of fitness effects or phenotypes in naturally *Wolbachia* infected populations

**Rhagoletis:**
- Lack of knowledge of infection frequencies and types in field populations of *Rhagoletis* species other than *R. cerasi*.
- Need to continue monitoring population dynamics of *Wolbachia* infected *R. cerasi* populations.
- No understanding of fitness effects or phenotypes in naturally *Wolbachia* infected populations

**Subtheme B: Interactions of reproductive symbionts and other microorganisms**

**Current knowledge:**

**B1. General:**
The interactions between reproductive symbionts and other microorganisms are largely unknown. A good understanding of the host microbiome is required to be able to determine interactions with reproductive microorganisms. Preliminary data indicate that in artificially *Wolbachia* infected *C. capitata* bacterial community is largely suppressed. In other insect groups such as *Drosophila* and mosquitoes, *Wolbachia* suppresses viral infection of the host, and pathogen transmission (bacteria, plasmodia, filarial nematodes, viruses).

**B2. Genus level background:**
*Anastrepha:* Interactions have not been studied.
*Bactrocera:* An artificially *Wolbachia* infected line has been established for the olive fly only. Interactions have not been studied.
*Ceratitis:* Preliminary data indicate that in two artificially *Wolbachia* infected *C. capitata* lines bacterial community is largely suppressed.
*Dacus:* Interactions have not been studied.
*Rhagoletis:* Interactions have not been studied.

**Gaps identified:**
B1. General:
Characterization of the host microbiome is prerequisite to be able to determine effects of reproductive parasites. There is need to establish artificially reproductive symbiont infected lines and/or to identify infected and uninfected individuals of the same wild population in order to be able to determine interactions.

B2. Genus level background:
Anastrepha:
- No artificially infected lines have been established
- Status of naturally infected populations by reproductive symbionts has not been fully characterized.
Bactrocera:
- No artificially infected lines have been established (except B. oleae),
- Status of naturally infected populations by reproductive symbionts has not been fully characterized.
Ceratitis:
- The status of naturally infected populations by reproductive symbionts has not been fully characterized.
Dacus:
- No artificially infected lines have been established
- Status of naturally infected populations by reproductive symbionts has not been fully characterized.
Rhagoletis:
- No artificially infected lines have been established
- Status of naturally infected populations by reproductive symbionts has not been fully characterized.

Subtheme C: Applications (CI and host fitness manipulations)

Current knowledge:
C1. General:
The intracellular symbiont Wolbachia manipulates the reproductive performance of its insect hosts and among others causes cytoplasmic incompatibility in crosses between infected males and non-infected females (Uni-directional CI), and between individuals infected with incompatible Wolbachia strains (Bi- direction CI). Wolbachia infection may be manipulated to induce CI into wild insect population directly suppressing pest populations in an environmental friendly manner. This method, “Incompatible Insect Technique” (IIT), is similar to SIT, the only difference being the sterilization mode – IIT does not use γ-rays – and can be used as complementary to SIT. IIT has been recently proposed to suppress wild populations of the Mediterranean fruit fly. This method has been successfully tested under laboratory conditions for two major agricultural pests, the Mediterranean fruit fly and the olive fly. It is worthwhile for such an approach to be validated and extended, alone and/or in conjunction with the SIT, to other target insect pest species.

In artificially infected mosquitoes and Drosophila, Wolbachia infections have been found to affect adult life span, reduce resistance to abiotic conditions, suppress responses of the immune system, and interfere with pathogen transmission. These studies led to the application of
*Wolbachia* to reduce fitness of mosquito populations. Similar approaches could be exploited for Tephritid pests. In Tephritid pests without available sexing strains, *Wolbachia* could potentially be used in IIT approach if female fitness in the field is suppressed, while it is conditionally not expressed in the mass-rearing.

C2. Genus level background

**Anastrepha:** There are no tools for the application of IIT or for exploiting fitness effects of reproductive symbionts to control pest populations.

**Bactrocera:** There are no tools for the application of IIT for most species or for exploiting fitness effects of reproductive symbionts to control pest populations. An artificially *Wolbachia* infected olive fly line can be used as a tool for implementing IIT approach.

**Ceratitis:** In *C. capitata* there are potential tools for the application of IIT approach. There are artificially *Wolbachia* infected *C. capitata* lines including a genetic sexing line that can be used as tools for implementing IIT approach. However, there are no tools available for exploiting fitness effects of reproductive symbionts to control field populations.

**Dacus:** There are no tools for the application of IIT or for exploiting fitness effects of reproductive symbionts to control pest populations.

**Rhagoletis:** Although IIT has been tested in the past using *R. cerasi* populations that are naturally infected with different *Wolbachia* strains, there is no mass rearing system and no genetic sexing lines available for the *R. cerasi*. There are no tools for the application of IIT or for exploiting fitness effects of reproductive symbionts to control pest populations.

**Gaps identified:**

C1. General:
- There are no genetic sexing strains for the majority of Tephritid pest species.
- Lack of mass rearing system for *Wolbachia* infected genetic sexing lines.
- Lack of understanding of all effects of *Wolbachia* on life history and behavioral traits as well on the field performance of the released – infected males.
- Lack of understanding of effects of irradiation on *Wolbachia* in case irradiation is used to guarantee sterility in females that are accidentally released.
- Lack of technology to transfer reproductive symbionts from a donor host to a target pest that can be mass reared.

C2. Genus level background

**Anastrepha:**
- Lack of tools for the application of IIT
- Lack of tools for exploiting fitness effects of reproductive symbionts to control field populations.

**Bactrocera:**
- Lack of characterization of the *B. oleae Wolbachia* infected line under mass rearing conditions.
- Lack of semi-field and field assessment of the performance of the *B. oleae Wolbachia* infected line.
- Lack of knowledge regarding fitness effects of *Wolbachia* in the artificially infected *B. oleae* line.
There are no IIT tools for other *Bactrocera* species than *B. oleae*.

Lack of tools for exploiting fitness effects of reproductive symbionts to control field populations.

*Ceratitis*:
- Lack of characterization of *C. capitata* Wolbachia infected lines under mass rearing conditions.
- Lack of semi-field and field assessment of the performance of *C. capitata* Wolbachia infected lines.
- Lack of complete understanding of the fitness effects of Wolbachia in artificially infected lines.
- Lack of tools for exploiting fitness effects of reproductive symbionts to control field populations.

*Dacus*:
- Lack of tools for the application of IIT
- Lack of tools for exploiting fitness effects of reproductive symbionts to control field populations.

*Rhagoletis*:
- Lack of mass rearing system and genetic sexing lines for *R. cerasi*.
- There are no IIT tools for other *Rhagoletis* species than *R. cerasi*.
- Lack of tools for exploiting fitness effects of reproductive symbionts to control populations of *Rhagoletis* species.

**Individual plans**

**3.1. Detection, molecular and phenotypic characterization**

**Genus: Anastrepha**
Participants: Mariana Mateos, Humberto Martinez-Montoya (USA)
Collaborators: Emilio Hernandez, Jorge Toledo (Mexico)

5 years plan
- Obtain specimens from four species of *Anastrepha* (*A. ludens, A. striata, A. serpentina, and A. obliqua*) from different regions of Mexico. These will be provided by our collaborators at ECOSUR-Tapachula, Chiapas, Mexico.
- PCR screening in wild populations of *A. ludens, A. obliqua, A. serpentina* and *A. striata* from different geographical origins in Mexico to determine presence/absence of heritable endosymbionts (including Wolbachia and Spiroplasma).
- Phenotypic characterization of different Wolbachia strains comparing infected and non infected populations that share the same genetic background.
- Characterization of Wolbachia strains and other heritable endosymbionts.

18 months plan
- Obtain specimens from four species of *Anastrepha* (*A. ludens, A. striata, A. serpentina, and A. obliqua*) from different regions of Mexico. These will be provided by our collaborators at ECOSUR-Tapachula, Chiapas, Mexico.
• PCR screening in wild populations of *A. ludens*, *A. obliqua*, *A. serpentina* and *A. striata* from different geographical origins in Mexico to determine presence/absence of heritable endosymbionts (including *Wolbachia* and *Spiroplasma*).

• Characterization of *Wolbachia* strains and other heritable endosymbionts.

Participants: Diego Segura (Argentina)

5 years plan

• Phenotypic characterization of different *Wolbachia* strains in a laboratory colony of *A. fraterculus* comparing infected and non infected populations that share the same genetic background.

• *Wolbachia* strains will be characterized by MLST.

18 months plan

• *Wolbachia* strains will be characterized by MLST.

Genus: *Bactrocera*

Participants: Kostas Bourtzis, G. Tsiamis (Greece)

5 years plan

• Detect the prevalence of *Wolbachia*, *Cardinium*, *Rickettsia*, *Spiroplasma* and *Arsenophonus* strains in populations of the *Bactrocera dorsalis* complex (*B. dorsalis* sensu stricto, *B. carambolae*, *B. papayae*, *B. philippinensis* and *B. invadens*)

• Genotype the *Wolbachia* strains (time permitting genotyping of *Arsenophonus* strains)

18 months

• Detect the prevalence of *Wolbachia*, *Cardinium*, *Rickettsia*, *Spiroplasma* and *Arsenophonus* strains in populations of the *Bactrocera dorsalis* complex (*B. dorsalis* sensu stricto, *B. carambolae*, *B. papayae*, *B. philippinensis* and *B. invadens*)

• Genotype the *Wolbachia* strains (time permitting genotyping of *Arsenophonus* strains)

Participants: Ramesh Hire (India)

Collaborators K. Bourtzis, G. Tsiamis (Greece)

5 years plan

• Collect *B. cucurbitae* adults from different agro-climatic conditions and regions for screening *Wolbachia* presence.

• Screening of *B. cucurbitae* cultures collected from different agro-climatic conditions for *Wolbachia* infection using wsp, ftsz and 16 S rDNA specific primers.

• Genotype the *Wolbachia* strains found.

18 months plan

• Collect *B. cucurbitae* adults from different agro-climatic conditions and regions for screening *Wolbachia* presence.

Participant: Markus Riegler, Olivia Reynolds, Toni Chapman Peter Crisp (Australia)

5 years plan
- Detection of *Wolbachia* and other reproductive symbionts in *Bactrocera tryoni* and other Australian tephritids (e.g. *D. pornia*)
- Molecular characterization (MLST) of *Wolbachia* and other reproductive symbionts in *B. tryoni* and other Australian tephritids
- Characterization of *Wolbachia* (and other reproductive symbionts) induced phenotypes in naturally infected Australian tephritid species, in particular *B. neohumeralis*.

18 months plan
- Continuation of ongoing screening work of previously collected field population samples in *Bactrocera tryoni* and other Australian tephritids (e.g. *D. pornia*)
- Obtain more field samples (in particular of adult females, and also larval instars) of the above species.
- Establishment of naturally *Wolbachia* infected laboratory populations of *Bactrocera neohumeralis*.

**Genus: Ceratitis**

**Participant:** Fernando L. Consoli (Brazil)
**Collaborators:** Kostas Bourtzis (Greece), Jair Virginio, Julio Walder (Brazil)

5 year plan
- Sample populations of *C. capitata* in several states of Brazil, particularly those representing the major fruit production areas in Brazil
- Detect the presence of *Wolbachia* in each population by diagnostic PCR using the 16S rRNA and *wsp* gene as targets, testing anywhere from 10 to 20 specimens/population
- Characterize *Wolbachia* in wild *C. capitata* infected populations by MLST analysis
- Assess the molecular diversity of each *C. capitata* population sampled by identification of the barcode region of the COI
- Molecular diversity of W+ and W- *C. capitata* will be compared at the population (interpopulation) and individual level (intrapopulation)
- W+ *C. capitata* populations will be selected based on previous data and frequency of *Wolbachia* infection will be determined
- Compatibility crossing assays between W+ and W- individuals will be carried out within a single population or among populations depending on the results obtained

18 month work plan
- Sample populations of *C. capitata* in several states of Brazil, particularly those representing the major fruit production areas in Brazil
- Detect the presence of *Wolbachia* in each population by diagnostic PCR using the 16S rRNA and *wsp* gene as targets, testing anywhere from 10 to 20 specimens/population
- Characterize *Wolbachia* in wild *C. capitata* infected populations by MLST analysis
- Assess the molecular diversity of each *C. capitata* population sampled by identification of the barcode region of the COI

**Participant:** Nikos Papadopoulos (Greece)
**Collaborators:** Kostas Bourtzis, George Tsiamis (Greece)
5 years plan
- Reproductive behavior of *Wolbachia* infected medfly lines.
- Determine the progress of sexual maturation of both males and females of the *Wolbachia*-infected and non-infected cohorts.
- Study the effect of *Wolbachia* infection on the mating behaviour of both males and females.
- Determine remating rates of females mated with *Wolbachia*-infected males.
- Assess the mating competitiveness of *Wolbachia*-infected males against wild males to mate with wild females.

18 months plan
- Reproductive behaviour of *Wolbachia* infected medfly lines.
- Determine the progress of sexual maturation of both males and females of the *Wolbachia*-infected and non-infected cohorts.
- Study the effect of *Wolbachia* infection on the mating behaviour of both males and females.
- Determine remating rates of females mated with *Wolbachia*-infected males.
- Assess the mating competitiveness of *Wolbachia*-infected males against wild males to mate with wild females.

Participant: Aydin Tuncbilek (Turkey)
Collaborators: Kostas Bourtzis and George Tsiamis (Greece)

5 years plan
- Collect wild *Ceratitis capitata* populations from different region of Turkey and over different seasons.
- Detect presence of *Wolbachia* strain and other reproductive symbionts.
- Genotype *Wolbachia* strains.
- Evaluate potential cytoplasmic incompatibility induced by *Wolbachia* under laboratory condition.

18 months plan
- Collect wild *Ceratitis capitata* populations from different region of Turkey and over different seasons.
- Detect presence of *Wolbachia* strain and other reproductive symbionts.

**Genus Dacus**
- No plans.

**Genus Rhagoletis**
Participant: Nikos Papadopoulos (Greece)
Collaborators: Kostas Bourtzis, George Tsiamis (Greece)

5 years plan
- Determine the demographic and behavioral profile of *Wolbachia* infected Greek *R. cerasi* populations.
- Define the genetic structure of Greek populations of *R. cerasi*.
- Genotype the *Wolbachia* strains in Greek populations of *R. cerasi*.
• Determine Wolbachia infection status in larvae, pupae and different ages of adult R. cerasi.

18 months plan
• Determine the demographic and behavioral profile of Wolbachia infected R. cerasi populations.

Participants: Markus Riegler (Australia), Wolfgang Miller, Christian Stauffer (Austria)

5 years plan
• Monitoring of Wolbachia population dynamics in R. cerasi
• Detection and molecular characterization of reproductive symbionts other than Wolbachia in Rhagoletis cerasi
• Detection and molecular characterization of reproductive symbionts in other Rhagoletis species
• Genome analysis of Wolbachia strains of R. cerasi

18 months plan
• Apply next generation sequencing approaches to establish genome sequence of Wolbachia strains of R. cerasi.

3.2. Interactions of reproductive symbionts and other microorganisms

Genus: Anastrepha

Participants: Diego Segura (Argentina)

5 years plan
• Evaluation of the role of Wolbachia on the interactions between A. fraterculus larvae and Diachasmimorpha longicaudata.

18 months plan
• Evaluation of the role of Wolbachia on the interaction between A. fraterculus larvae and Diachasmimorpha longicaudata.

Genus: Bactrocera

Participant: Kostas Bourtzis, George Tsiamis (Greece)

5 years
• Characterization of the gut symbiotic bacteria using 16S rRNA pyrosequencing libraries and microarrays (PhyloChip) in populations of the Bactrocera dorsalis complex (B. dorsalis sensu stricto, B. carambolae, B. papayae, B. philipinnensis and B. invadens) that are infected by Wolbachia.
• Quantitative analysis of the most dominant bacteria of the above species
• Characterization of the irradiation impact on the host microbiome (reproductive and gut symbionts)
18 months
  - None planned

Participants: Markus Riegler, Olivia Reynolds, Toni Chapman, Peter Crisp (Australia)

5 years plan
  - Characterise the microbiome of *B. tryoni* and other Australian tephritids
  - Test for interactions of *Wolbachia* and microbiome in *B. neohumeralis* infected with *Wolbachia*
  - Test for interactions of microbiome in *B. tryoni* and artificially introduced *Wolbachia* (if successful with transfer)
  - Run 454 tagged pyrosequencing of samples of field collected individuals, laboratory individuals (irradiated and not irradiated) of *B. tryoni* and Australian tephritid species.
  - Perform metagenomic analysis of field collected and laboratory reared individuals of *B. tryoni*

18 months plan
  - Run 454 tagged pyrosequencing of samples of field collected individuals, laboratory individuals (irradiated and not irradiated) of *B. tryoni* and Australian tephritid species
  - Perform metagenomic analysis of field collected and laboratory reared individuals of *B. tryoni*

**Genus: Ceratitis**
Participants: Aydin Tuncbilek (Turkey)
Collaborators: Kostas Bourtzis, George Tsiamis (Greece)

5 years plan
  - Characterize the effects of irradiation on the interaction between reproductive and gut microorganisms of *C. capitata*

18 months plan
  - No activities planned.

**Genus: Dacus**
  - No plans

**Genus: Rhagoletis**
Participants: Markus Riegler (Australia), Christian Stauffer, Wolfgang Miller (Austria)

5 years plan
  - Characterize the microbiome of *R. cerasi* and that of other *Rhagoletis* species to be able to determine possible interactions with *Wolbachia*
  - Characterize interactions between microbiome and *Wolbachia in R. cerasi*.
  - Run 454 tagged pyrosequencing of samples of field collected individuals, laboratory individuals of *R. cerasi*
  - Run a metagenomic analysis of field collected *R. cerasi*

18 months plan
• Run 454 tagged pyrosequencing of samples of field collected individuals, laboratory individuals of *R. cerasi*
• Run a metagenomic analysis of field collected *R. cerasi*

### 3.3. Applications (CI and host fitness manipulations)

**Genus *Anastrepha***

Participants: Mariana Mateos, Humberto Martinez-Montoya (USA)
Collaborators: Emilio Hernandez, Jorge Toledo (Mexico)

5 years plan
• Evaluate possible CI phenotypes induced by *Wolbachia* in *A. ludens, A. obliqua, A. serpentina* and *A. striata* in laboratory populations.

18 months plan
• No plans

Participants: Diego Segura (Argentina)

5 years plan
• Evaluate possible CI phenotypes induced by *Wolbachia* in *A. fraterculus* in laboratory populations.
• Evaluate possible phenotypes induced by *Wolbachia* in *A. fraterculus* larvae in terms of its impact on the mass production of *D. longicaudata*.

18 months plan
• No plans

**Genus: Bactrocera**

Participants: Ramesh Hire (India)

5 years plan
• Determine different doses of radiation on *B. cucurbitae* that assures female sterility in a possible IIT approach.
• Performance of *B. cucurbitae* sterile males with or without *Wolbachia* infection.

18 months plan
• No activity planned for this period

Participants: Markus Riegler Olivia Reynolds, Toni Chapman (Australia)

5 years plan
• Optimization of microinjection techniques
• Identification of *Wolbachia* strains of Australian tephritids that can be used as donor for *Wolbachia* infection experiments either through (1) introgression or (2) microinjection.
• Establishment of *Wolbachia* infected *B. tryoni* under laboratory conditions
• Establishment of *Wolbachia* infected laboratory populations of Australian tephritid species that are naturally infected by *Wolbachia*

• Test under laboratory conditions control approaches that are based on *Wolbachia* IIT and fitness manipulations

18 months plan

• Optimization of microinjection techniques

• Identification of *Wolbachia* strains of Australian tephritids that can be used as donor for *Wolbachia* infection experiments either through (1) introgression or (2) microinjection.

• Establishment of *Wolbachia* infected laboratory populations of Australian tephritid species that are naturally infected by *Wolbachia*.

**Genus: Ceratitis**

**Participants:** Fernando Consoli (Brazil)

**Collaborators:** Kostas Bourtzis (Greece), Jair Virgilio (Brazil)

5 years plan

• Establish in the laboratory a colony of the *C. capitata* ViennaE8EE (artificially infected with *Wolbachia*).

• Perform under laboratory conditions mating compatibility assays between the *C. capitata* ViennaE8EE and the naturally occurring *Wolbachia*-infected and uninfected populations that will be selected from Brazil;

• Perform field cage assays to test the efficiency of *C. capitata* ViennaE8EE to suppress selected populations from Brazil.

18 months plan

• No activities are planned for this period.

**Participants:** Nikos Papadopoulos (Greece)

**Collaborators:** Kostas Bourtzis, George Tsiamis (Greece)

5 years plan

• Determine the population increase parameters of *Wolbachia* infected medfly lines.

• Population model

• Quantify the performance of the *Wolbachia* infected lines under small scale mass rearing.

• Determine the quality properties and performance of mass reared (under small scale mass rearing) *Wolbachia* infected lines

• Determine changes in the demographic and behavioral profile of artificially *Wolbachia* infected medfly lines over time to analyze the evolution of the symbiotic relationships

• Evaluate under semi field conditions an IIT approach using the two available *Wolbachia* infected lines

18 months plan
• Determine the population increase parameters of *Wolbachia* infected medfly lines.

**Genus: Dacus**
• No plans

**Genus: Rhagoletis**
• No plans
**Overall Objective:**
The objective of the project is to understand and harness the role that microorganisms play in the larval and adult biology of selected key fruit and vegetable pests to improve the SIT and related emerging techniques as part of area-wide integrated pest management programmes applied against such pests.

<table>
<thead>
<tr>
<th>Project Design Elements</th>
<th>Verifiable Indicators</th>
<th>Means of Verification</th>
<th>Important Assumptions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall Objective:</strong></td>
<td>N/A</td>
<td>N/A</td>
<td>Fruit and vegetable production in Member States continue to suffer major losses to endemic and introduced pests. International trade in fruit and vegetable commodities will continue to increase and be disrupted by pests requiring expensive post-harvest and quarantine measures. The increasing demand for area-wide integrated pest management approaches to control fruit and vegetable pests, including where appropriate the SIT and related emerging techniques as non-polluting suppression/eradication component, mandates improvement of the cost-effectiveness of this environment-friendly sustainable approach.</td>
</tr>
<tr>
<td>Specific Objectives:</td>
<td>N/A</td>
<td>N/A</td>
<td>1. Microorganisms contribute to the nutrition and health of their insect hosts and can be used to reduce the costs and increase efficiency of mass-rearing.</td>
</tr>
</tbody>
</table>

1. To develop methods of using beneficial bacteria to replace costly ingredients in larval and adult diets in insect mass rearing facilities.
2. To determine the effect of radiation on the symbiotic associations in target species.

3. To explore the use of symbionts as probiotics provided to adult sterile males before their release to significantly improve sterile male performance.

4. To harness symbiotic associations towards the reproductive manipulation and suppression of target populations.

**Outcomes:**

<table>
<thead>
<tr>
<th>1. Costs of mass-rearing of target fruit pests reduced by incorporating microorganisms in larval and adult diets.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. The impact of radiation on symbiotic associations of mass-reared insects better understood.</td>
</tr>
<tr>
<td>1. A protocol developed and validated under mass-rearing conditions.</td>
</tr>
<tr>
<td>2. A monitoring protocol for the characterization of post-radiation symbiotic communities developed.</td>
</tr>
<tr>
<td>3. A protocol developed and validated under operational conditions.</td>
</tr>
<tr>
<td>1. Mass rearing data and cost-benefit analysis.</td>
</tr>
<tr>
<td>2. Monitoring protocol and data collected on its implementation.</td>
</tr>
<tr>
<td>3. Data collected and cost-benefit analysis.</td>
</tr>
</tbody>
</table>

2. Radiation affects symbiotic associations.

3. Probiotic supplements to the pre-release adult diet of sterile males can improve their subsequent sexual performance.

4. Symbiotic associations can be harnessed to manipulate host reproduction and reduce target populations.

<table>
<thead>
<tr>
<th>1. A protocol developed and validated under mass-rearing conditions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. A monitoring protocol for the characterization of post-radiation symbiotic communities developed.</td>
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<tr>
<td>3. A protocol developed and validated under operational conditions.</td>
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<td>1. Mass rearing data and cost-benefit analysis.</td>
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<td>2. Monitoring protocol and data collected on its implementation.</td>
</tr>
<tr>
<td>3. Data collected and cost-benefit analysis.</td>
</tr>
</tbody>
</table>

1. Facilities are eager to reduce mass-rearing costs. The protocol developed is effective and can help reduce these costs.

2. Irradiation detrimentally affects the mass-reared insects’ symbiotic associations. There is a need to understand and minimize these effects.

3. Performance of sterile males is not optimal and can be improved. Symbiont-based probiotic supplements to pre-release adult diet can improve sterile male performance.
4. Additional control tool that is based on symbiotic associations and complementary to the SIT available.

4. Additional control tool designed and validated.

4. Data collected and feasibility analysis.

4. Symbiont-based population control strategies can be developed and these novel control tools will interact complementarily with the SIT.

**Outputs:**

<table>
<thead>
<tr>
<th>1.a. Egg and larval microflora of pests (natural and mass-reared) characterized.</th>
<th>1.a. Characterized in at least 4 target species.</th>
<th>1.a. Reports and peer reviewed publications.</th>
<th>1.a. The microbial assemblages of mass-reared insects are species specific and amenable to characterization by classical and molecular microbiological approaches.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.b. The ability of beneficial bacteria to reduce production costs of mass-reared larval and adult diets explored.</td>
<td>1.b. Explored in the mass-reared larval diets of at least 2 target species.</td>
<td>1.b. Reports and peer reviewed publications.</td>
<td>1.b. Beneficial bacteria can be cultured and harnessed to replace costly ingredients in larval and adult diets, such that high quality mass-reared insects are produced. Gel-based and liquid diets will allow the elimination of bulking agents that introduce and perpetuate deleterious microorganisms in mass-rearing facilities. The beneficial bacteria can also serve as barriers against deleterious microorganisms.</td>
</tr>
<tr>
<td>3.a. Adult associated microflora of pests (natural and mass-reared) characterized.</td>
<td>3.a. Characterized in at least 4 target species.</td>
<td>3.a. Reports and peer reviewed publications.</td>
<td></td>
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</tr>
<tr>
<td>3.b The impact of symbiont-based probiotic supplements to adult pre-release diet on sterile male sexual performance determined.</td>
<td>3.b Determined for at least 3 target species.</td>
<td>3.b. Reports and peer reviewed publications.</td>
<td></td>
</tr>
<tr>
<td>4.a. Microorganisms that affect host reproduction characterized.</td>
<td>4.a. Characterized in at least 2 target species.</td>
<td>4.a. Reports and peer reviewed publications.</td>
<td></td>
</tr>
<tr>
<td>4.b. The tripartite interactions of host-parasitoid-symbionts characterized.</td>
<td>4.b. Characterized in at least 1 target species.</td>
<td>4.b. Reports and peer reviewed publications.</td>
<td></td>
</tr>
<tr>
<td>4.c. Population suppression by microorganisms that manipulate host reproduction explored.</td>
<td>4.c. Explored in at least 1 target species.</td>
<td>4.c. Reports and peer reviewed publications.</td>
<td></td>
</tr>
</tbody>
</table>

3.a. The microbial assemblages of adult insects are species-specific and amenable to characterization by classical and molecular microbiological approaches.

3.b. Symbionts can be used as probiotic supplements in diets provided to sterile males before their release to improve their performance.

4.a. Microorganisms that are potential reproductive manipulators are amenable to characterization by molecular microbiological approaches.

4.b. The tripartite interactions of host-parasitoid-symbionts affect the host fitness and are amenable to manipulation.

4.c. Symbiont-based population control strategies can be developed against target pest species.
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>1. Hold Consultants Meeting.</td>
<td>2. CRP announced, and research contracts and agreements submitted, evaluated and forwarded to IAEA committee.</td>
<td>2. Issued contracts and agreements.</td>
<td>2. Proposals submitted and approved by IAEA committee.</td>
</tr>
<tr>
<td>2. Announce project amongst established entomologists and symbiologists researchers and fruit and vegetable pest control programmes and establish CRP.</td>
<td>3. 1st RCM held in mid-2012.</td>
<td>3. Working material printed and distributed for 1st RCM.</td>
<td>3. Research activities commence. Reports published and distributed following each RCM.</td>
</tr>
<tr>
<td>3. Organize first RCM to plan, coordinate and review research activities (2nd quarter 2012).</td>
<td>4. Research carried out by contract and agreement holders.</td>
<td>4. Reports and publications.</td>
<td>4. Renewal requests and continued funding of RCM’s and CRP.</td>
</tr>
<tr>
<td>4. Carry out R&amp;D and draft technical protocols.</td>
<td>5. 2nd RCM held in 2014.</td>
<td>5. Working material printed and distributed for 2nd RCM; Research published in scientific literature and disseminated to member states and scientific community.</td>
<td>5. Research activities continue, progress satisfactory.</td>
</tr>
<tr>
<td>5. Second RCM to present data and coordinate future research as required (early 2014).</td>
<td>6. Workshop held 2014. Harmonized procedures and trainees capable of implementing novel techniques.</td>
<td>6. Workshop report.</td>
<td>6. There is need for training; techniques and instructors are available.</td>
</tr>
<tr>
<td>6. In conjunction with second RCM, hold workshop on &quot;Novel tools for the characterization and bioinformatic analysis of insect symbiotic communities&quot;.</td>
<td>7. Research carried out by contract and agreement holders.</td>
<td>7. Reports and publications.</td>
<td>7. Renewal requests and continued funding of RCM’s and CRP.</td>
</tr>
<tr>
<td>7. Continue R&amp;D.</td>
<td></td>
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</table>

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<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>8.</td>
<td>Review the CRP after its third year.</td>
<td>8.</td>
<td>Mid-CRP review carried out.</td>
</tr>
<tr>
<td>9.</td>
<td>Third RCM to present data and coordinate future research as required (late 2015).</td>
<td>9.</td>
<td>3rd RCM held in 2015.</td>
</tr>
<tr>
<td>10.</td>
<td>In conjunction with third RCM, hold workshop on &quot;Incorporating bacteria to larval and pre-release adult diets&quot;.</td>
<td>10.</td>
<td>Workshop held 2015. Harmonized procedures and trainees with novel techniques.</td>
</tr>
<tr>
<td>13.</td>
<td>Evaluate the CRP and submit evaluation report.</td>
<td>13.</td>
<td>CRP evaluation carried out.</td>
</tr>
<tr>
<td>9.</td>
<td>3rd RCM held in 2015.</td>
<td>9.</td>
<td>Working material printed and distributed for 3rd RCM; Research published in scientific literature and disseminated to member states and scientific community.</td>
</tr>
<tr>
<td>11.</td>
<td>Research carried out by contract and agreement holders.</td>
<td>11.</td>
<td>Reports and publications.</td>
</tr>
<tr>
<td>13.</td>
<td>CRP evaluation carried out.</td>
<td>13.</td>
<td>CRP evaluation report.</td>
</tr>
<tr>
<td>8.</td>
<td>Mid-CRP review by Agency committee is positive.</td>
<td>9.</td>
<td>Mid-CRP review approved by IAEA committee. Research activities continue, progress satisfactory.</td>
</tr>
<tr>
<td>10.</td>
<td>There is need for training; techniques, equipment and instructors are available.</td>
<td>11.</td>
<td>Renewal requests and continued funding of RCM’s and CRP.</td>
</tr>
<tr>
<td>12.</td>
<td>Research and dissemination activities concluded.</td>
<td>13.</td>
<td>CRP evaluation by Agency committee is positive.</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES


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ANNEX 1 (List of participants)

First RCM on “Use of Symbiotic Bacteria to Reduce Mass-rearing Costs and Increase Mating Success in Selected Fruit Pests in Support of SIT Application”
21-25 May 2012, Vienna, Austria

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MONDAY, 21 MAY 2012

08:00 – 09:00  **Identification and registration at VIC Gate (next to subway station U1)**

09:00 – 09:15  **Jorge Hendrichs:** Welcome statement and goals of the CRP and the meeting

09:15 – 09:30  **Rui Cardoso Pereira:** Agenda and administrative issues

**SESSION I: Larval diets to improve mass-rearing efficiency** (Chairperson: Boaz Yuval)

09:30 – 10:00  **Peter Crisp:** The physiological importance of bacteria isolated from the gut of  *Dirioxa pornia* (Tephritidae) and the behavioural influence of associated volatile compounds.

**COFFEE BREAK**

10:30 – 11:00  **Changying Niu:** The effects of symbiotic bacteria on *Bactrocera dorsalis* and *Bactrocera minax*.

11:00 – 11:30  **Antonio Belcari:** The olive fly and bacteria: baselines for artificial rearing improvements.

11:30 – 12:00  **Inbar Dagan Shouster:** Mass production of Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and Olive fly *Bactrocera oleae* (Rossi) at Bio-Fly and the potential of symbiotic bacteria supplements.

**LUNCH**

**SESSION I (cont.): Larval diets to improve mass-rearing efficiency** (Chairperson: Diego Segura)

13:00 – 13:30  **Emilio Hernandez:** The role of the associated bacteria on the biology, rearing and behavior of *Anastrepha obliqua*, *A. serpentina* and *A. striata*.

13:30 – 14:00  **Humberto Martinez Montoya:** Survey of heritable endosymbionts in Southern Mexican populations of the genus *Anastrepha*.

14:00 – 14:30  **Dina Melgar:** Comparative identification of microorganisms associated to wild and mass reared Mediterranean fruit fly “Medfly” *Ceratitis capitata* (Wied) and Mexican fruit fly “Mexfly” *Anastrepha ludens* (Loew) in sterile insect production and release facilities.
Carlos Cáceres: Improving mass rearing technology for the medfly temperature sensitive lethal strain (tsl): Gel larval diet development and validation.

COFFEE BREAK

SESSION I (cont.): Larval diets to improve mass-rearing efficiency (Chairperson: Edouard Jurkevitch)

Erin Schuenzel: Identification of bacteria with detrimental and beneficial effect on the larval survivorship of the Mexican fruit fly (Anastrepha ludens).

Jaime García de Oteyza: Identification and inoculation of potential beneficial bacteria for SIT application: larval diet modification and adult foraging system.

Carolina Yañez Prieto: Characterization of intestinal microbiotic organisms associated to nutrition processes in Ceratitis capitata (Wied.) mass rearing and study of symbionts enhancers of its development and capacity in field work in SIT programs.

TUESDAY, 22 MAY 2012

SESSION II: Probiotics and adult diets (Chairperson: Nikos Papadopoulos)

Edouard Jurkevitch: Are all fruit fly symbionts equal?

Boaz Yuval: Bacterial symbionts and the nutritional ecology of the olive fly, Bactrocera oleae.

Ramesh Hire: Integration of sterile insect technique and symbiotic bacteria for the control of Bactrocera cucurbitae.

COFFEE BREAK

SESSION II (cont.): Probiotics and adult diets (Chairperson: Kostas Bourtzis)

Sabrina Dyall: Use of symbiotic bacteria in adult diet to increase mating success of Bactrocera zonata and Bactrocera cucurbitae.

Mahfuza Khan: Mating success of melon fly, Bactrocera cucurbitae (Coq.) and the oriental fruit fly, Bactrocera dorsalis (Hendel) in support of SIT application in Bangladesh.

Diego Segura: Bacteria associated to Anastrepha fraterculus (Diptera: Tephritidae): characterization and effect on sterile males sexual competitiveness.

LUNCH

SESSION III: Wolbachia and molecular control tools (Chairperson: Antonio Belcari)
13:00 – 13:30  **Markus Riegler**: Wolbachia in drosophilid and tephritid fruit flies - same but different?

13:30 – 14:00  **Kostas Bourtzis**: Harnessing medfly symbiotic associations for novel and effective pest control strategies.

14:00 – 14:30  **Nikos Papadopoulos**: Effect of *Wolbachia* on life history and behavioral traits of the Mediterranean fruit fly.

14:30 – 15:00  **Ayidin Suzu Tuncbilek**: Wolbachia-based Strategies to Increase Sterile *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) Quality in Support of SIT.

**COFFEE BREAK**

**SESSION III (cont.): Wolbachia and molecular control tools**  
(Chairperson: Carlos Cáceres)

15:30 – 16:00  **George Tsiamis**: Towards the characterization of SymBioKosmos of the *Bactrocera dorsalis* complex.

15:30 – 16:00  **Fernando Consoli**: Incompatible Insect Technique (IIT) in Brazil for Medfly Control.

**SESSION IV: General discussion (Chairperson: Rui C. Pereira)**

16:30 – 17:30  General discussion

18:30  **GROUP DINNER**

**WEDNESDAY, 23 MAY 2012**

**SESSION V: Review of the individual proposals**  
(Chairperson: Rui Cardoso Pereira and Group Leaders)

08:30 – 10:00  Working Groups. Background situation analysis, baseline knowledge at start of CRP and review of research gaps that need to be addressed  
(Rooms available A2311, A2172, A2243)

**COFFEE BREAK**

10:30 – 12:00  Working Groups: Continued review of research gaps that need to be addressed

**LUNCH**

13:00 – 15:00  Working Groups: Continued review of research gaps that need to be addressed

**COFFEE BREAK**

15:30 – 17:30  General Discussion: Review of baseline knowledge at start of CRP and review of research gaps that need to be addressed
THURSDAY, 24 MAY 2012

08:30 – 10:00  Working Groups: Review of individual research proposals for the different working areas

COFFEE BREAK

10:30 – 12:00  Working Groups: Continued review of individual research proposals for the different working areas

LUNCH

13:00 – 15:00  Working Groups: Continued review of individual research proposals for the different working areas

COFFEE BREAK

15:30 – 17:30  General Discussion: Review of individual research proposals for the different working areas

FRIDAY, 25 MAY 2012

SESSION VI: RCM report (Chairperson: Rui Cardoso Pereira)

08:30 – 10:00  Review and adjustment of the logical framework

COFFEE BREAK

10:30 – 12:00  Agreement on content of RCM report, and drafting and compiling of RCM report

LUNCH

13:00 – 15:00  Finalization of draft RCM report

COFFEE BREAK

15:30 – 17:30  Agreement on information exchange mechanisms, on location of 2nd RCM, and closure of the RCM
### ANNEX 3 (Working groups)

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The physiological importance of bacteria isolated from the gut of *Dirioxa pornia* (Tephritidae) and the behavioural influence of associated volatile compounds

Peter Crisp, Ahmed Al-Hashimi Kala Bhandari, Nilesh Chand and Greg Baker

South Australian Research and Development Institute, Adelaide, Australia

*Dirioxa pornia* is endemic in irrigated fruit production areas of Australia, and while not a pest species, populations have increased in abundance in recent years and management strategies need to be developed. Until recently attempts to rear *D. pornia* had been unsuccessful and not progressed past a third generation of adult flies. The abundance of each generation established in the laboratory from wild flies steadily decreased and the adult flies appeared smaller than the wild captured flies. However, the addition of symbiotic bacteria isolated from the crop of wild flies to the adult diet of the laboratory colony has resulted in a viable culture. Feeding the bacteria to *D. pornia* adults has led to increased size, activity, fecundity and fertility in the laboratory reared flies and a significant increase in the size of the culture with subsequent generations.

The purpose of the proposed research is to identify the species of bacteria that are associated with the gut of *D. pornia*, and their effect on the survival, development time and fecundity of *D. pornia*. The identification will involve molecular diagnosis and will be conducted on species isolated from the gut and on whole of gut contents. The bacterial community of *D. pornia* specimens collected in several regions of Australia will be identified for comparison with gut bacteria from *Bactrocera tryoni* and *Ceratitis capitata*. *D. pornia* will be collected from different horticultural crops and their gut bacterial fauna compared. Of particular interest will be a comparison of the enzyme profile of these bacteria, as it is possible that some of these bacteria may be relatively specific to the nutrient profile of the host fruit e.g. sugar, starch or oil. Knowledge gained in these experiments could potentially be used to improve the rearing of economic species such as *B. tryoni* and *C. capitata* and also improve the health of irradiated flies. Trials will also be established to test whether the gut bacterial species which had beneficial effects on *D. pornia* growth and reproduction have any similar effects on cultured *B. tryoni* and *C. capitata*, and if so, to assess whether they effect the survival, flight ability and competitiveness of sterile flies released as part of pest management and eradication programs.

Some species of the bacteria are highly attractive to adult *D. pornia* and volatile compounds have been observed to induce significant behavioural changes such as increased feeding, motor activity and excitation. Volatile compounds will be identified using GC-MS head-space analysis and individual compounds will be evaluated for attraction to *D. pornia* and any behavioural changes induced. Compounds that are found to be attractive to or induce behavioural changes in *D. pornia* will also be evaluated with *B. tryoni* and *C. capitata* and will be trialled as potential lures for monitoring or lure and kill technologies.
Insects live in close association with symbiotic bacteria. Until recently, the increasing number of studies addressing the bacteria-insect interaction reveal that bacteria influence the nutritional status and reproduction of hosts. In addition, there is preliminary evidence that bacterially enriched adult diet improves mating competitiveness of sterile fruit flies significantly when the sterile insect technique (SIT) is used against some major pests. However, to date we still know little about the diversity and function of the symbiotic bacteria within insect body. In this study, we will use *Bactrocera dorsalis* and *Bactrocera minax*, two economically important fruit flies in China as insect models, to investigate the bacteria-insect interface in target pests. The preliminary study conducted with PCR-RFLP method in our laboratory, shows that wild populations of *B. minax* and *B. dorsalis* share common representatives of *Enterobacter* and *Klebsiella*. Further, our immediate goals are to: (1) identify the microbial diversity in *B. minax* and *B. dorsalis* by a high-throughput sequencing approach; (2) study the effects of symbiotic bacteria in larval or adult diets on *B. dorsalis*; (3) assess the effects of symbiotic bacteria in larval or adult diets on *B. minax*; (4) investigate the effects of symbiotic bacteria on sterile male flies in *B. dorsalis*; and (5) develop novel pest control method via feeding engineered bacteria in adult *B. dorsalis*. The results from this study will improve our understanding regarding the relationship between insects and symbiotic bacteria, also contribute the sustainable implementation of the SIT against target pests in the long run.
The olive fly and bacteria: baselines for artificial rearing improvements

Antonio Belcari

*Dipartimento di Biotecnologie Agrarie (DIBA), Universita degli Studi die Firenze. Italy*

The olive fly, *Bactrocera oleae* is the key pest of the olive crops in all cultivated areas of the world. The microbiome of the fly has been studied from the beginning of the last century; Petri was the first person who discovered in the olive fly both in adults and larvae two specialized organs hosting bacteria, the oesophageal bulb and midgut caeca. Since then much research has been conducted and nowadays, thanks to the discovery of new investigation techniques, the relationships between the olive fly and bacteria are more clear and might allow to improve the artificial rearing techniques required for future SIT applications.

The olive fly establishes two types of relationship with bacteria: the first one with epiphytic species which are acquired from the olive phylloplane and used as food source, the second one with an unculturable bacterial species, detectable by molecular techniques, *Candidatus Erwinia dacicola* which has been proposed as coevolved endosymbiont of *B. oleae*. Our investigations carried out in field on wild olive fly population, showed the primary importance of several groups of acquired bacteria belonging to the *Enterobacteriaceae* and *Pseudonadaceae* families. Among others, *Pseudomonas putida* has been detected several times both in olive leaves and fruits and in the midgut of wild and lab populations; for that reason this species might play an important role for the adult fitness. Preliminary data on a probiotic diet based on *P. putida* on survival and female fecundity are discussed. Concerning, the real role played by this symbiont has to be demonstrated, but there are some evidences which could speak in favour for an effective activity as endosymbiont. *Ca. Erwinia dacicola* is transmitted vertically and elicits the protein hydrolysis in young larvae feeding on green olives. As a matter of fact previous work carried out in field demonstrated how copper products (bactericidal activity) produced high level of mortality in young larvae. Our research on the olive fly by molecular tools showed in the wild population (Tuscany) a high presence of this species both in males and females; on the contrary *Ca. Erwinia dacicola* was never detected in the lab strain.
Mass production of Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and Olive fly *Bactrocera oleae* (Rossi) at Bio-Fly and the potential of symbiotic bacteria supplement

Inbar Shouster Dagan and Shimon Steinberg

*Bio-Fly Ltd., Kibbutz Sde Eliyahu, Israel*

Bio-Fly was established in 2004 as a daughter company of Bio-Bee Ltd. Initially, production of the Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) aimed at 15 million sterile males per week. This capacity has amplified gradually through increasing rearing efficiency and technical improvements at the production floor. To date, Bio-Fly produces more than 70 millions sterile males per week. In addition, Bio-Fly runs two emergence and release centers and fully operates the majority of the SIT projects in Israel. Recently, Bio-fly has been engaged in development of SIT for the Olive fly (OLF), *Bactrocera oleae* (Rossi).

A major threat to Medfly's production stability is microbial contamination, especially in the artificial diet during larval development. Larval diet encompasses various microbial populations that may originate from: row materials, egg seeding solution, rearing equipment etc. Supplement of symbiotic bacteria to the rearing process may assist in obtaining a proper balance between deleterious and beneficial bacteria. This may lead to an increase in production efficiency, fly's quality and the stability of both.

The introduction of symbiotic bacteria to irradiated Medfly males, under lab conditions has demonstrated a positive impact on mating competitiveness under both laboratory conditions and field-cages. However, the effect of symbiont's supplement under mass emergence conditions should be further studied.

SIT for OLF relies, to a large extent, on the ability to scale-up the production from small to economic and robust commercial scale. The development of an effective larval diet for the OLF proved to be most challenging. The rate of pupal recovery from the artificial diet is poor and varies substantially, probably due to occasional contaminations. The monophagous OLF is expected to possess significant microbial associations. Hence it is expected that introduction of symbiotic bacteria to OLF's laboratory culture will generate a breakthrough in rearing efficiency as well as in fly's quality.

Implementation of symbiotic bacteria to fruit fly's mass rearing process may result in major improvements to fruit fly's SIT, through reduction of production costs and better performance of sterile fly's. A thorough insight into the microbiota of both fruit fly's mass rearing process and the wild fly's populations, together with the understanding of their dynamics and functions, is fundamental to achieve the above mentioned goals.
The role of the associated bacteria on the biology, rearing and behavior of *Anastrepha obliqua*, *A. serpentina* and *A. striata*

I.S. Gómez¹, I.B. Puon¹, J.P. Rivera², J. Toledo³ and E. Hernández²

¹ Centro de Biociencias, UNACH, Mexico
² Programa Moscafrut (SAGARPA-IICA), Mexico
³ El Colegio de la Frontera Sur, Mexico

**Bacteria in larvae**

*Anastrepha striata*

The goal was evaluate the effect of the addition of the bacteria in the larval diet on the attributes of *Anastrepha striata*. Four species of bacteria were isolated from the gut of wild flies of *A. striata*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Enterococcus faecalis*. Tests of sensitivity to methyl paraben preservatives and sodium benzoate at different pH were performed for each species of bacteria in order to determine its effect on their viability. Bacteria were inoculated into a starter diet using 5x10⁵ and 5x10⁷ concentrations UFC/g. The results indicated that the addition of *Enterobacter cloacae* allows larva-pupa survival of 33%, while *Klebsiella spp.* inoculation without metabolites can increase the weight of the pupa 19.27 mg and 83.07% in fliers.

**Bacteria in adult flies**

*Anastrepha obliqua*

Eight bacterial species were isolated from adult flies of *A. obliqua*, which were added to the adult food for release, sugar and Mb food type. The results indicated that the highest percentages of mating were obtained when food was supplemented with Mb type food with the strains with the codes AOBWTAP201105 and AOBWTAP201103. While, with sugar only the highest mating percentages were obtained with the strains with the codes AOBWTAP201104 and AOBWTAP201105.

*Anastrepha serpentina*

Eight bacterial strains were isolated from adult flies of *A. serpentina*, which were added to adult food for release, sugar and Mb food type. The results indicated that the highest percentages of matings were obtained when the Mb food type was supplemented with the strains with codes ASEWTAP201102 and ASEWTAP201107. While, with sugar only the highest mating percentages were obtained with the strains with the codes ASEWTAP201104 and ASEWTAP201107.
Survey of heritable endosymbionts in Southern Mexico populations of genus *Anastrepha*

Humberto Martinez-Montoya\(^1\), Jorge Toledo\(^2\), Pablo Liedo\(^2\) and Mariana Mateos\(^1\)

\(^1\) Texas A\&M University, TX, USA  
\(^2\) El Colegio de la Frontera Sur, Mexico

Heritable endosymbiotic bacteria associated with insects are ubiquitous and taxonomically diverse. Many of these endosymbionts influence the fitness of their hosts and/or manipulate their host reproduction. Exploiting the effects of endosymbionts on hosts for pest control is a growing research area, but requires knowledge of endosymbionts associated with the target pest population. In this study, we used molecular methods to screen southern Mexico populations of two species of tephritid fruit fly pests, *Anastrepha ludens* and *A. striata*, for heritable bacteria. The only heritable endosymbiont found was *Wolbachia* in *A. striata*. Based on Multi-Locus Sequence Typing and phylogenetic analyses, this *Wolbachia* strain is new and belongs to the *Wolbachia* supergroup B. *Wolbachia* strains previously reported in members of the genus *Anastrepha* in South America belong to supergroup A. We discuss the potential implications for pest control of the presence of a different *Wolbachia* strain in southern Mexico.
Comparative identification of microorganisms associated to wild and mass reared Mediterranean Fruit fly “Medfly” *Ceratitis capitata* (Wied) and Mexican fruit fly “Mexfly” *Anastrepha ludens* (Loew) in sterile insect production and release facilities

Dyna Melgar¹, F. Jerónimo¹, C. Cáceres² and P. Rendón²

¹ El Pino and San Miguel Petapa Mass Rearing Plants/Moscamed Program, Guatemala
² USDA APHIS PPQ CPHST, Guatemala

During the past years, additional understanding of the relationship between microorganisms and their hosts has been published. Studies on insects have revealed several contributions of microorganisms to their nutrition, health and reproductive success. Furthermore, there is evidence that during colonization and mass rearing production process for SIT application the presence of endosymbionts is modified fact that could affect sterile insect fitness at the field. Thus, for the sterile insect technique (SIT) where billions of insects are produced artificially, it is relevant to determine the endosymbiont’s role and their possible optimization presence to optimize their competitiveness and compatibility in the field.

In order to establish the differences between the egg and larvae microflora associated to wild insects in *Ceratitis capitata* and *Anastrepha ludens* and those produced at mass rearing systems at El Pino - "Medfly" and San Miguel Petapa “Mexfly” rearing facilities. Molecular techniques RT-PCR (16S RDNA) will be utilized to identify in real time the bacterial community present within the different insect’s origen. The assays will be carried out collecting wild insects from fruit host plants. Oviposition of wild material inside fruit host material, under controlled conditions, will used to isolate microorganism transferred to the fruit and those present in eggs and larvae as well. Isolated microorganisms will identified using PCR techniques and later compared to those that are present in the insect colonies that are maintained at both "Medfly" and "Mexfly" mass rearing facilities.
Improving mass rearing technology for the medfly temperaure sensitive lethal strain (tsl): gel larval diet development and validation

Carlos Cáceres

USDA APHIS PPQ CPHST, San Miguel Petapa Fruit Flies Mass Rearing Facility and Research Center, San Miguel Petapa, Guatemala

Mass rearing technology is crucial element to establish a functional SIT program for any species. Insect rearing represent around 50% of the SIT cost. Any improvement that could significantly reduce costs could become SIT more feasible. A Gel larval diet and its rearing system for Medfly (Ceratitis capitata) production were developed at San Miguel Petapa Facility in Guatemala. The diet was composed of Torula yeast, sugar, antifungal agents (sodium benzoate), hydrochloric acid, tap water and organic gel agent. Larval rearing of Medfly on this diet resulted in 30% more pupal production while in the colony production line resulted in 47% more female pupal production than from the control diet. Pupal weight, adult emergence, adult fliers, and egg hatch and egg production equal trend to insects rearing in a conventional corncob diet. Benefits from a Gel diet technology include reduction in waste diet; no need of bulking agent; reduction in diet ingredient storage and labor, and reduction of diet’s preservatives fact that could allow the inoculation of desirable microorganisms to allow better larva nutrition therefore produces improved sterile insects for SIT insect’s release. The state of the art of Gel technology allows its utilization at mass rearing at any time. However more research will be necessary in the future to design the best equipment and facility configuration for this new technology. Diet formulation need to be revised so that more savings could be achieved without compromise the quality of the insects.
Identification of bacteria with detrimental and beneficial effect on the larval survivorship of the Mexican fruit fly \textit{(Anastrepha ludens)}

Erin Schuenzel, Norman Barr, Bacilio Salas, Don Vacek, Hugh Conway

\textit{University of Texas-Pan American, \& USDA-APHIS, Mission, Texas USA.}

The Mexican fruit fly, \textit{Anastrepha ludens} (Loew) is a major pest that attacks the fruit groves in the southwest region of the United States and Mexico. Female Mexican fruit flies can lay up to 1500 eggs during their lifetime representing a major threat to citrus and other fruit industries in these regions. One method to control the population of Mexican fruit flies is through biocontrol, specifically sterile insect technique (SIT). Key to this method is raising millions of sterile males per week for release. Because of the sheer number of sterile males released each week, the reproductive success of female fruit flies is severely reduced. For SIT to be successful, sterile males must be released constantly and bacteria are now being considered as a resource to decrease the cost of SIT by increasing larval-adult survivorship of the sterile males. Bacteria can play a dual role in the success of a fruit fly colony. As a probiotic, bacteria have been found to increase adult survivorship and mating success. This work will identify possible candidate bacteria that increase the survivorship of larvae to pupa and adult. Conversely bacteria can also act as pathogens that, if introduced, can wipe out the larvae in a colony or severely reduce the number of larvae that pupate.

To assess the potential of a bacterium as a probiotic or pathogen, the identity of the bacterium must be known. Bacteria were isolated from the eggs and larvae of \textit{A. ludens}. The universal bacterial primers were used to amplify and sequence the 16s rDNA region. The sequences were aligned against the GenBank nucleotide database using the megaBLASTn algorithm. Of the 115 bacterial sequences analyzed, only seven were not identified using BLASTn or BLASTx. The identified bacteria included representatives from the phyla Proteobacteria (98), Bacilli (3), Actinobacteria (5) and Bacteroidetes (2). The gamma subdivision of Proteobacteria included 90 of the identified isolates with the vast majority, 60 isolates, belonging to Pseudomonadeles. Another 10 belonged to Enterobacteriaceae, a group with known pathogens and probiotics such as \textit{Klebsiella} sp. The analysis found 13 strains of \textit{Pseudomonas aeruginosa}, a known pathogen of \textit{Drosophila melanogaster}. Additionally, the two Bacteroidetes are similar to probiotics used for humans.

Preliminary larval survivorship trials are underway to test the effects the identified bacteria have. Initially eggs are sterilized using a hydrogen peroxide solution. A single bacterial inoculum is mixed into a sterilized larval diet. The hatched larvae are placed onto the supplemented diet and a control diet. Larval survivorship is then quantified at each instar until pupation. The number of emerged adults is then counted. Two pathogenic bacteria have been identified with additional replicates needed to verify the results.

Future work will include isolating and identifying bacteria from late stage instars and adults, completing the larval survivorship trials, creating diagnostics to monitor for pathogenic bacteria, and optimizing a larval diet with probiotics.
Identification and inoculation of potential beneficial bacteria for SIT application: larval diet modification and adult foraging system

Teresa Navarro and Jaime García de Oteyza

TRAGSA, Valencia, Spain

The total or partial replacement of yeast and preservatives in the larval diet composition, without a decrease on insect quality parameters, would mean an important economic saving for SIT programmes.

As a first approach, isolation and identification of microorganisms found in different infested hosts will be done, as well as in larvae found in this fruits. In the other side, microorganisms found in mass reared larvae will be identified in order to compare both populations. Selection of promising microorganisms will be done in order to develop an optimized culture system and start with preliminary tests of larval diet inoculation.

In relation to probiotics, isolation and culture of the already identified beneficial bacteria will be carried out prior to its inoculation to the adult insects. Isolation and identification of bacteria found in wild captured flies in different seasons will be done for its comparison with the described in bibliography.

Bacteria inoculation procedure will be developed to ensure the bacteria assimilation by the adults, the permanence in absence of the bacteria foraging source, and the effect on quality parameters.
Caracterization of intestinal microbiotic organisms associated to nutrition processes in *Ceratitis capitata* (Wied.) mass rearing and study of symbionts enhancers of its development and capacity in field work in SIT programs

Carolina Yáñez¹, Hernán Donoso², Constanza Rojas¹ and Carlos Sarabia²

¹ Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile.
² Centro de Producción de Insectos Estériles (CPIE), Servicio Agrícola y Ganadero, Región Arica y Parinacota, Chile.

The understanding of the relationship between the bacterial microbiota inhabiting insects and their physiological significance is an important factor for developing novel pest control tools. Studies conducted in different regions of the world have addressed the analysis of the gut bacterial community in insects, showing that bacteria-diet interactions affect longevity. However, these results cannot be globally extended. In mass rearing, control of opportunistic pathogens adds another factor that threatens the natural expression of symbiotic bacteria, which function is to help the insect development.

In order to efficiently compete with other pest control strategies, the sterile insect technique (SIT) today focuses on reducing operating costs, by minimizing expenses on nutritional supplements to increase mass-reared sterile insect quality and the worldwide market acceptance and establishment of this control.

This project aims to identify and characterize the microbiota associated with the digestive tract of *Ceratitis capitata* (Wied.) in various stages of development in populations under mass rearing conditions maintained at the CPIE facilities. Analyses of both diversity and relationship between species in the bacterial community will be carried in larval stage, as well as in adults, in pre and post irradiation conditions. In parallel, the microbiological conditions of the larval diet will be assessed at different times during the development cycle of insect (at seedling time, 1st, 2nd and 3rd instars larvae).

Since the conditions of mass rearing on artificial diet are far from natural conditions provided by the host, attempts to develop a breeding line by natural infestation in cages and field conditions for at least three generations of the insect will be made. Then, the colonizing microbial fauna will be studied and compared.

In a second phase, the differences between the symbiotic species found will be determined. A feasibility study involving the introduction of nutrition collaborating agents of the insect into the production process, especially in its larval stage, will be also carried out.
Are all fruit fly symbionts equal?

Edouard Jurkevitch¹, Yael Aharon¹,², Michael Ben Yosef¹,², and Boaz Yuval²

¹ Department of Plant Pathology and Microbiology, Faculty of Agriculture, Food and Environment, Rehovot. The Hebrew University of Jerusalem, Israel
² Department of Entomology, Faculty of Agriculture, Food and Environment, Rehovot. The Hebrew University of Jerusalem, Israel

Insects are hugely diverse. Yet, similar bacterial species can be found forming the bulk of the microbiota communities associated with many different insects. As an example, Tephritids appear to often bear the same bacterial species. We examined Ceratitis capitata, the Mediterranean fruit fly in detail using culture dependent, as well as culture independent, including massive parallel sequencing approaches. Our results show that at the larval stage, a high titer of pectin-degrading Enterobacteriaceae produces an environment with high-sugar availability. After pupation, the young adult gut contains a few foci of bacteria. These expand as the insect matures to form a contiguous mixed species biofilm-like community composed almost exclusively of Enterobacteriaceae that fills the entire lumen, contained by the peritrophic membrane, many of which perform nitrogen fixation.

Although functions are conserved, sequence analysis reveals a large microdiversity at the strain level. This aspect will discussed in the light of SIT applications as we recently showed that provisioning male flies after sterilization with one of their gut symbionts significantly improved mating performance and competitiveness. Yet, we do not know the reasons underlying this effect: is it strain specific, what bacterial functions are involved in it, and which traits in the fly are affected. A comparative genome analysis of the symbiont and related species was performed and will be presented along with a strategy for assessing the potential of microdiversity and implementation of this symbiont-based approach.
Bacterial symbionts and the nutritional ecology of the olive fly, *Bactrocera oleae*

Michael Ben-Yosef, Edouard Jurkevitch and Boaz Yuval

*Department of Entomology, Hebrew University of Jerusalem, Israel*

The olive fruit fly (*Bactrocera oleae*), one of the most important pests of worldwide olive horticulture, is intimately associated with a symbiotic gut microbiota throughout its life cycle. Adult flies accommodate a large population of extracellular bacteria in a specialized diverticulum of the oesophagus and in the midgut. This association extends via the egg and continues in the larvae where bacteria populate the four midgut caeca during the entire larval development within the olive. Despite the evidence of a highly developed association the significance of the intestinal microbiota to the fly’s fitness is poorly understood.

We tested the hypothesis that adult flies gain nutritional advantages from their bacterial companions. Specifically we postulated that bacteria contribute to adult fitness by complementing for amino-acid deficiencies in the diet. We show that when flies are maintained on a diet containing the non-essential amino-acids as the sole nitrogen source bacteria contribute significantly to fecundity. Conversely, on a basal diet of sucrose, or when all amino-acids are included in the diet the effect of bacteria on female fecundity is negligible. These results suggest that the intestinal bacteria of olive flies contribute to the nitrogen budget of their host, probably by supplementing the diet with protein or amino-acids. Although wild olive flies have the potential for acquiring the nitrogen needed for reproduction by ingesting a varied diet in the field, by means of their intestinal microbiota they gain the ability to subsist on low quality food sources without suffering from protein deficiencies.

We hope that a better understanding of the symbiotic aspects of olive fly biology will provide a foundation for the use of bacteria to improve existing control measures and for developing new control strategies based on the manipulation of the gut microbiota.
Integration of sterile insect technique and symbiotic bacteria for the control of 
*Bactrocera cucurbitae*

Ramesh S. Hire, Ashok B. Hadapad, and Stanislaus F. D’Souza

*Bhabha Atomic Research Centre, Trombay, Mumba, India*

Around 325 different species of fruit fly occur in Indian subcontinent, of which 205 are in India alone. Majority of these species belong to genus *Bactrocera* and *B. cucurbitae*, *B. dorsalis* and *B. zonata* are major fruit fly insect pests in India. The melon fly, *B. cucurbitae* (Diptera: Tephritidae) is a major pest of cucurbits and also causes heavy damage to fruits and vegetables. The extent of losses due to fruit fly is around 30-100% depending on cucurbit species and season. Since the maggots damage the fruits internally, it is difficult to control this pest with insecticides. Sterile insect technique (SIT) is widely used for fruit fly control worldwide. Several microorganisms are residing inside the insect body either intracellularly or extracellularly. Many of such associations probably started as transient encounters or infectious parasitic relationships and evolved into more benign or beneficial relationships. During the sterilization process gut microbial community of fruit fly gets adversely affected and thus significantly influences the mating behaviour of the sterile fruit flies. Hence understanding the microbial community structure in *B. cucurbitae* is important for the successful use of SIT. In view of this, we proposed the study on microbiota associated with *B. cucurbitae*. Melon fruit fly adults were collected from vegetable and fruit growing area in Southern India. The experiments were initiated to study the gut microflora of male and female fruit fly. The microflora will be identified using microbial, biochemical and molecular methods. Further, fruit fly samples will be collected from different agro-climatic zones in India and gut microflora will be studied at pre- and post irradiation.
Use of symbiotic bacteria in adult diet to increase mating success of *Bactrocera zonata* and *Bactrocera cucurbitae*

Sabrina D. Dyall¹, Preaduth Sookar², Malini Alleck².

¹ Department of Biosciences, University of Mauritius, Réduit, Republic of Mauritius
² Entomology Division, Agricultural Services, Ministry of Agro Industry and Food Security, Réduit, Republic of Mauritius

In Mauritius, the melon fly, *Bactrocera cucurbitae*, and the peach fruit fly, *Bactrocera zonata*, are major pests of vegetables and fruits respectively. Both fruit fly species are current targets of the sterile insect technique (SIT). Fruit flies maintain stable and diverse communities of microorganisms in their digestive tract. These symbiotic microorganisms are important for nutrition, health and reproduction in fruit flies. There is evidence that during mass-rearing and radiation processes, the native microflora is disrupted and its contribution to the host is reduced.

We propose to carry out a seasonal and spatial survey to identify and characterise the symbiotic microorganisms found in the gut of both the melon and the peach fruit flies. We shall compare the community of microorganisms found in wild fruit flies versus that found in laboratory-reared flies. The specific objectives are to:

(i) identify the microbial communities in the gut of the melon fly and the peach fruit fly;
(ii) determine whether the different surroundings and diets influence the bacteria composition;
(iii) study the effect of radiation on the microflora of the alimentary tract of the fruit flies;
(iv) investigate the effect of probiotics during pre-release period on the sterile insect quality.

Wild *B. cucurbitae* and *B. zonata* fruit flies will be collected from selected cucurbit fields and fruit-growing areas using appropriate traps at regular intervals and from different agroclimatic regions. These samples will be analysed for spatial and seasonal variation in microbiota. The fly gut microbiota will be collected following dissection of isolated insects, and community genomic DNA (gDNA) will be extracted from the isolated microorganisms using commercial kits. Laboratory-reared insects will also be studied. To identify the microorganisms present in the fly guts, we shall amplify specific gene fragments from bacterial, archaeal and eukaryotic small subunit ribosomal RNA genes, and clone these fragments to create three separate and comprehensive DNA libraries representing the fly gut microbiota. These gene fragments will be analysed by molecular methods such as sequencing in order to identify the corresponding species represented by the gDNA. In parallel to these molecular analyses, we plan to isolate and cultivate the fly gut microbiota in liquid or solid media for both identification purposes and for use as insect feed. Here, pure cultures of the isolated bacteria will be fed to the fruit flies. Quality control parameters such as egg hatch, pupal weight, calling and mating behaviour will be monitored.

The studies to be undertaken are expected to yield information on the temporal and spatial variations of bacterial communities that reside in the digestive system of the melon and the peach fruit flies. Studies will reveal which microorganisms are dominant, constant and stable in the populations. Additionally, we shall generate data on the influence of probiotic adult diets on the nutritional state and reproductive ability of the melon and peach fruit flies respectively.
Mating success of melon fly, *Bactrocera cucurbitae* (Coq.) and the oriental fruit fly, *Bactrocera dorsalis* (Hendel) in support of SIT application

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Attempts were made towards the utilization of symbiotic bacteria to enhance the mating success and to improve the quality of artificial rearing diets of melon fly, *Bactrocera cucurbitae* (Coq.) and the oriental fruit fly, *Bactrocera dorsalis* (Hendel). Efforts were made to isolate and identify the gut bacterial community of laboratory host reared *B. cucurbitae* and *B. dorsalis* using cultural, morphological, and different bio-chemical tests. Isolated gut bacterial species viz., *Klebsiella oxytoca*, and *Proteus rettgeri* was examined by incorporated into protein (casein:yeast extract:sugar, 1:1:2) and sugar diets to study the fitness parameters of *B. cucurbitae* and *B. dorsalis*. Commercially available probiotic micro-agents, ‘Protenix® Balance’ capsule, ‘Probiotic Rose Fiber’, and the gut bacteria, *K. oxytoca* and *P. rettgeri* was evaluated to improve the quality of laboratory established artificial liquid larval diet for mass rearing of *B. dorsalis*. Based on different colony morphology total forty and thirty-five predominant isolates were selected from *B. cucurbitae* and *B. dorsalis*, respectively. The bacterial species viz., *Aeromonas hydrophila*-1, *Aeromonas hydrophila*-2, *Alcaligenes faecalis*-1, *Alcaligenes faecalis*-2, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Serratia mercescens*, *Serratia liquefaciens* and *Streptobacillus moniliformis* was identified from the gut of *B. cucurbitae*. Whereas *Aeromonas hydrophila*-1, *Aeromonas hydrophila*-2, *Aeromonas hydrophila*-3, *Bacillus alvei*, *Erwinia herbicola*, *Moraxella lacunata*, *Serratia liquefaciens*, *Serratia rubidaea*, *Streptobacillus moniliformis*, *Proteus inconstses*, *Proteus rettgeri*, and *Proteus vulgaris* was identified from *B. dorsalis*. *Enterobacteriaceae* was the dominating group among the identified bacterial species. Some pathogenic bacteria viz, *Salmonella arizonae*-1, *Salmonella aerizonae*-2, *Salmonella salamae*, *Pseudomonas* sp-1, and *Salmonella houtenae* and *Vibrio alginolyticus* was found in the gut of *B. cucurbitae* and *B. dorsalis*, respectively. The preliminary study on the mating performance of *B. dorsalis* fed on *K. oxytoca* and *P. rettgeri* added protein and sugar diets in the present study appeared promising, but not for *B. cucurbitae*. *B. cucurbitae* and *B. dorsalis* started mating within 8-10 days, and 7-9 days, respectively while fed on bacteria incorporated protein diets, and also only protein diet under controlled laboratory condition. Conversely, bacteria added sugar fed *B. cucurbitae* did not initiate mating even before 12 days of adult emergence. The longevity of bacteria added sugar fed *B. cucurbitae* was higher than those fed on bacteria added protein diets. No effect on the fecundity of *B. cucurbitae* and *B. dorsalis* was recorded while fed on bacteria added protein or sugar diets. Addition of commercially available probiotic bacteria did not exert much variation in the quality parameters of mass-reared *B. dorsalis*. Further research related towards the isolation and identification of gut bacteria of wild flies and evaluation of mating success of sterile *B. cucurbitae* and *B. dorsalis* is in progress.
Bacteria associated to *Anastrepha fraterculus* (Diptera: Tephritidae): characterization and effect on sterile males sexual competitiveness

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Research scientists in Argentina are devoting efforts to develop the sterile insect technique against the South American fruit fly, *Anastrepha fraterculus*, a key fruit pest in America. The experience gained in other tephritid fruit fly species and transferred to *A. fraterculus* will surely result in a better performance of the sterile male in the field, particularly in topics such as sexual competitiveness. Increased mating performance of sterile males would allow a higher overall efficacy of the SIT. In addition, sexual competitiveness has been tightly linked to nutritional status in fruit flies, which in turn has been shown to be affected by symbiotic associations with gut bacteria. Preliminary results from our laboratory indicate that in *A. fraterculus* this may also be the case. The main objective of the present project is to describe the gut bacteria associated to *A. fraterculus* and explore the importance of this association on the reproductive success of the males. We will also focus on the effect of colonization and irradiation of adult insects on bacteria diversity. Males’ reproductive success will be assessed by standard mating competitiveness tests, where female choice between males treated with antibiotics and un-treated males will be registered. Changes in the sex pheromone and cuticle hydrocarbons due to the presence of specific bacteria will also be tested. The species richness of the bacteria in the gut will be determined by means of PCR amplification of 16S rRNA gene and sequencing. Effect of irradiation on bacterial diversity and mating competitiveness will be further included in this project.

In addition, bacterial symbionts have also been found to influence the ability of certain dipterans to cope with the attack of parasitoids, through an enhancement of the immune response of the host. Tephritid fruit flies are known to carry the symbiotic bacteria *Wolbachia*, mainly located in the gonads, which in other insects strengthen their immune system. If this is also the case in tephritid fruit flies, then the presence of *Wolbachia* will increase the production cost of parasitoids, as some of the parasitized, *Wolbachia*-carrying, larva would be able to stop parasitoid development. The present project will contribute to better understand the relationship between bacterial symbionts and *A. fraterculus* in two aspects that will surely contribute to the management of this pest in South America.
Wolbachia is a ubiquitous maternally inherited intracellular $\alpha$-Proteobacterium associated with up to 65% of insect species, many arthropods and filarial nematodes, and displays all possible arrays of symbiotic interactions extending from parasitism to mutualism. Drosophila fruit fly species (Diptera: Drosophilidae) are well-studied hosts of Wolbachia symbiosis – many are ideal laboratory models with an extensive repertoire of genetic tools and available information. There is an emerging body of work about Wolbachia in true fruit flies (Diptera: Tephritidae), including pest species of economic importance. Wolbachia associations of tephritids confirm many aspects of Wolbachia biology but also challenge others, with common multiple infections in individual hosts, a diversity of high and low bacterial titres as well as titre changes throughout a flies’ life span, both contributing to difficulties in detection. In comparison with drosophilids, tephritids have developed different strategies to exploit resources, they are longer lived and, thus, may have extended opportunities for multiple species interactions. I will summarise and compare Wolbachia infections of tephritids from different biogeographic regions and present new findings of Wolbachia in the Australian species of the diverse genus of Bactrocera. I will particularly focus on the two sibling species of Queensland fruit fly, Bactrocera tryoni and Bactrocera neohumeralis. Newly detected Wolbachia infections in the latter may provide additional reinforcement for speciation of these two genetically very similar sympatric species.
Harnessing medfly symbiotic associations for novel and effective pest control strategies

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The Mediterranean fruit fly (Medfly), *Ceratitis capitata*, is one of the world’s most destructive fruit pests. In most countries, insecticide application remains the predominant method of control despite major concerns about product quality, environment protection, human health and insecticide resistance. There is an increased interest for the development and application of environment-friendly such as the sterile insect technique (SIT) and other biological control strategies. The effectiveness of these methods depends on the quality of mass-reared insects produced for SIT and other related applications.

Insects commonly establish symbiotic associations with a variety of microorganisms, which affect many aspects of host biology and physiology including nutrition, immunity, mating behaviour and reproduction. In the present study, we will report on: (a) how Wolbachia, the most ubiquitous insect endosymbiont described so far, could be used for the development of novel pest control tools, complementary to the SIT and (b) the activities of a recently funded Greek research project “Symbiotic bacteria and –omics technologies towards the development of novel and environment-friendly of insect pest control methods: the case of the Mediterranean fruit fly (Symbiomics)” which employs a systems biology approach, towards the development of novel and environment-friendly control methods of this major insect pest. In the frame of “Symbiomics”, metagenomics is being used to characterize in depth the symbiotic communities of Medfly reared under different conditions.
Effect of *Wolbachia* on life history and behavioral traits of the Mediterranean fruit fly

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*Wolbachia pipientis* is a widespread endosymbiont of insects exerting a wide range of biological effects on their hosts. Recent studies have documented the influence of *Wolbachia* on reproduction and life span of insect host species. However, there is a limited number of studies on the effects *Wolbachia* may have on the demographic traits and/or fitness components (including behavioral aspects) of different populations of insect host species. We used two laboratory lines of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), that have been transinfected with two *Wolbachia* strains to determine effects on *Wolbachia* infection on demographic traits and some aspects of adult behavior. All experiments were conducted under constant laboratory conditions. In an initial approach, we demonstrated that *Wolbachia* infection (a) shortens the egg-to-adult developmental duration of both *C. capitata* lines, albeit prolongs embryonic development, (b) shortens adult life span (to a different extent in males and females), and (c) reduces female fecundity in both *C. capitata* lines. Interestingly, different *Wolbachia* strains differentially affect both immature and adult demographic traits. Experiments in progress address effects of *Wolbachia* on components of the sexual behavior of male Mediterranean fruit flies. The importance of our findings for establishing mass rearing for *Wolbachia*-infected *C. capitata* lines, the application of the Incompatible Insect Technique and/or the enhancement of Sterile Insect Technique is discussed.
Wolbachia-based Strategies to Increase Sterile *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) Quality in Support of SIT

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The Mediterranean fruit fly (medfly), *Ceratitis capitata* Wiedemann is one of the most important and most researched pests in the world in the past as well as in the present. It becomes more important for its polyphagous species, distribution in all tropic and subtropic areas in world, and inclusion in outer quarantine lists. It exists in all the costal areas from North Aegean towards East Mediterranean in citrus orchards in Turkey. Various control strategies have been developed but are ineffective for the management of the pest. These strategies include chemical, biological and microbial control, sterile insect technique, and incompatible insect technique. The strategies for area wide integrated management of this invasive pest increasingly use the environmentally friendly approach of the sterile insect technique (SIT) as their central component. Although SIT is widely used to control Mediterranean fruit fly, the sterilizing irradiation procedure affects the gut bacterial community structure of the fly and the mass-rearing procedures inherent to the SIT often lead to a reduction in the mating ability of the released males. The bacteria are important partners in the fly’s life cycle, but the function of these microorganisms is not completely understood. In this study we will focus on the *Wolbachia*-based integrated control methods for improving effectiveness of SIT. *Wolbachia* and other bacteria have significant contributions to various components of fitness and it is likely that the mass rearing and irradiation processes disrupt the bacteria–medfly partnership. The general aim of this project is to increase quality of sterile *C. capitata* male combining with symbiont-based strategies to suppress or modify natural medfly populations. In light of this idea, there is a need to improve the competitive ability of the released sterile medfly males to enhance the effectiveness of SIT control programs.
Towards the characterization of SymBioKosmos of the *Bactrocera dorsalis* complex

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The Tephritidae family encompasses more than 4000 species, most of which are polyphagous. The *Bactrocera dorsalis* complex, member of the Tephritidae family (subfamily Dacinae), has 75 described species, which are mainly endemic to Southeast Asia. This complex, also known as the Oriental fruit fly complex, is considered one of the most harmful insect pests. Significant research has been performed on the *Bactrocera dorsalis* complex regarding: (a) taxonomy, systematics and diagnostics, (b) ecology and (c) pest status and invasion biology. Despite these extensive research activities, the species limit of the major *B. dorsalis* complex pest species (*B. dorsalis* sensu stricto, *B. carambolae*, *B. papayae* and *B. philippinensis* and *B. invadens*) has not yet been clarified. The lack of resolution for the *B. dorsalis* complex and unambiguous identification of these five major pest species prevent the development and implementation of the Sterile Insect Technique in Area-Wide Integrated Pest Management programmes.

Despite the important role insect symbionts can have in the biology, physiology, ecology and evolution of their hosts, only a limited number of studies dealt with the *B. dorsalis* complex. In the present study we will report: (a) on the detection and genotyping of *Wolbachia*, *Arsenophonus* and other reproductive parasites in laboratory and natural populations of *B. dorsalis* sensu stricto, *B. carambolae*, *B. papayae*, *B. philippinensis* and *B. invadens*; (b) on the use of *Wolbachia* as a tool for the population control of a major *Bactrocera* pest species, the olive fly *B. oleae* and (c) the activities for sequencing the genome of *Acetobacter tropicalis*, a major symbiont of *B. oleae* using new sequencing technologies.
Incompatible Insect Technique (IIT) in Brazil for medfly control.

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The alpha-proteobacterium *Wolbachia pipientis* is the most common and widespread secondary bacterial symbiont associated with invertebrates known to date. *Wolbachia* is largely recognized by the changes induced in the sex determination of the host, but the body of evidence that this symbiont may have acquired functions other than affecting the host sex determination is increasing. *Wolbachia* can induce four different host reproductive morphotypes depending on the strain of *Wolbachia* and on its interactions with the genetic background of each particular host. *Wolbachia* has been intensively studied as a tool to develop alternative strategies to control insect pests, vectors or the diseases they transmit due to one of the several changes it may induce in the host, such as cytoplasmic incompatibility, high fitness cost or increased resistance to pathogens. However, the induction of incompatible crosses due to cytoplasmic unidirectional or bidirectional incompatibility is certainly the most studied and the most promising to generate *Wolbachia*-based alternatives for pest control. I will then discuss the possibilities of exploiting the capabilities of *Wolbachia*-inducing cytoplasmic incompatibility strains as an additional strategy for medfly control in Brazil through the recent proposed “Insect Incompatible Technique” (IIT).