WORKING MATERIAL

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 Second Research Coordination Meeting of an FAO/IAEA Coordinated Research Project, held in Bangkok, Thailand, from 6 to 10 May 2014

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TABLE OF CONTENTS

BACKGROUND ..................................................................................................................3
CO-ORDINATED RESEARCH PROJECT (CRP) .................................................................8
SECOND RESEARCH CO-ORDINATION MEETING (RCM) .............................................8

1 LARVAL DIETS AND RADIATION EFFECTS ............................................................10
   BACKGROUND SITUATION ANALYSIS ..................................................................10
   INDIVIDUAL PLANS ..............................................................................................13
       1.1. Cost and quality of larval diet ..................................................................13
       1.2. Preventing the growth of deleterious microorganisms .........................20
       1.3. Improving mass rearing efficiency and quality of the insects produced ....24

1 PROBIOTICS .............................................................................................................30
   BACKGROUND .......................................................................................................30
   INDIVIDUAL PLANS ..............................................................................................39
       2.1. Structure of microbiota ..........................................................................39
       2.3. Function of microbiota and fitness effects .............................................50
       2.4. Applications ...............................................................................................54

2 SYMBIONTS AND NOVEL CONTROL TOOLS .......................................................59
   BACKGROUND SITUATION ANALYSIS ..................................................................59
   INDIVIDUAL PLANS ..............................................................................................65
       3.1. Detection, molecular and phenotypic characterization ..........................65
       3.2. Interactions of reproductive symbionts and other microorganisms ...70
       3.3. Applications (CI and host fitness manipulations) ..................................72

LOGICAL FRAMEWORK .............................................................................................76

LIST OF REFERENCES ................................................................................................83
ANNEX 1 (LIST OF PARTICIPANTS) .............................................................................89
ANNEX 2 (AGENDA) ..................................................................................................93
ANNEX 3 (WORKING GROUPS) ..................................................................................97
ANNEX 4 (ABSTRACTS OF PRESENTATIONS) ..........................................................98
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 four 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. The fourth category includes symbionts that help insects to detoxify xenobiotics such as pesticides, plant related defensive molecules.
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 microorganismst, 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. Over the last 18 months, in the frameworks of the current Coordinated Research Project, *Wolbachia* as well as other microorganisms that affect the reproduction of tephritids have been characterized. *Wolbachia* infection was recorded in *Anastrepha striata*, *A. serpentina*, *A. obliqua* and *A. fraterculus*. Other studies have demonstrated the occurrence of *Wolbachia* in additional *Anastrepha* species of minor economic importance (REF). Regarding *Bactrocera* species, *Wolbachia* has been detected in *B. dorsalis*, *B. cucurbitae*, *B. zonata*, *B. correcta*, *B. tryoni* and *B. tau*. Detailed characterization of *Wolbachia* presence in these species should be conducted. A thorough screening of medfly populations from Brazil revealed no *Wolbachia* infection on the sampled populations. Additional studies from Turkey and Argentina confirm the absence of *Wolbachia* in wild medfly populations. Besides *Wolbachia* the occurrence of other symbionts, such as *Cardinium*, *Arsenophonus*, *Spiroplasma*, have been screened in *Bactrocera* spp. and *C. capitata*; however, confirmation of their occurrence is pending.

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. Field data from Brazil, Turkey and Argentina demonstrating no *Wolbachia* infection in wild medfly populations suggest that medfly is a good candidate for the application of IIT. Considering that wild populations of *A. ludens* are not infected with *Wolbachia*, current efforts have focused on evaluating the possibility of IIT for this species. First steps included establishing trans-infected mass reared *A. ludens* lines with *Wolbachia* strain using *A. striata* as donor. Possible application of IIT to control *Bactrocera* species (based on the current characterization of *Wolbachia* prevalence in different *Bactrocera* species) should be explored. Besides IIT other possible applications of the *Wolbachia*-tephritid association for controlling fruit flies should be explored.

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.

SECOND RESEARCH CO-ORDINATION MEETING (RCM)

Twenty-two scientists from 16 countries attended this first RCM, held in Bangkok, Thailand from 6-10 May 2014. The list of participants, which included CRP contract and agreement holders, as well as 3 additional observers, 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 second phase 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 second phase 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.
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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<th>3. Symbionts and novel control tools</th>
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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 labour 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. dorsalis (oriental fruit fly), B. curcubitae (melon fly), B. tryoni (Queensland fly), B. dorsalis, B. zonata, Anastrepha obliqua, A. ludens, A. fraterculus, A. serpentina, A. striata, 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, the protein source (different species of yeast), 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. curcubitae*, *B. tryoni*, *B. dorsalis*, *B. zonata*, *B. tau*, *B. correcta*, *Anastrepha obliqua*, *A. ludens*, *A. fraterculus*, *A. serpentina*, *A. striata*, *A. grandis*, *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. 16S rRNA characterization of the deleterious microorganisms is not adequate. Other genetic markers and/or biochemical methods are required for characterization and typing at species and strain level.
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, B. curcubitae, B. tryoni, B. dorsalis, B. zonata, A. ludens, Anastrepha obliqua, A. fraterculus, A. serentina, A. striata, Dirioxa pornia.

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

Current knowledge:

C1. General:
Despite the great progress achieved during the last 40 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).
4. Mass production of beneficial microorganisms in large scale should be provided in the early stages of the insect development (fermentors).
5. Develop robust and unified microbial preservation protocols
6. Develop robust and efficient inoculation methods for the beneficial microorganisms.
7. Technical and economic feasibility for the production and the inoculation of beneficial microorganisms.
8. 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. ludens*, *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: Hernán Donoso, Carolina Yáñez (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 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

Plans for the second 18 months
- Culture-dependent and independent identification and exploitation of microbial communities in larvae and pupae from natural populations of Peru, 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

Progress made up to 2nd RCM
- Molecular characterization using DGGE of the microbial communities from larvae, pupae, and the gut microbiota from adults of the mass-rearing facility has been completed.
- Isolation and characterization of bacterial strains from larvae, pupae and the gastrointestinal track of adults has been completed.

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

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.

• Development the formulation for probiotic applications at mature egg and/or the first/second larvae instars.

Plans for the second 18 months

• Development of protocols in order to introduce beneficial bacteria in the following magnification colonies: (a) released colony, (b) injection, (c) initiation, and (d) filter rearing system.

Progress made up to 2nd RCM

• Beneficial microorganisms have been isolated and characterized (*Klebsiella* sp., *Raoultella* sp.)

Participants: Jaime García 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.

• Development the formulation for probiotic applications at mature egg and/or the first/second larvae instars.

Plans for the second 18 months

• 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 gene sequencing).

• Development the formulation for probiotic applications at larval stage.

• Protocol development for introducing beneficial microorganisms in a mass-rearing facility.

Progress made up to 2nd RCM

• Bacterial strain identification for all isolates has been completed (*Klebsiella* sp., *Raoultella* sp.)

• DNA profiles for pure cultures has been produced

• 16S rRNA amplicon has been produced for the characterization of the gut microbial communities

Species: *Anastrepha ludens*

Participants: Dina Melgar (Guatemala)
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.
- Development the formulation for probiotic applications at mature egg and/or the first/second larvae instars.

Plans for the second 18 months
- Development of protocols in order to introduce beneficial bacteria in a mass-rearing facility.

Progress made up to 2\textsuperscript{nd} RCM
- Beneficial microorganisms have been isolated and characterized from wild insects (*Klebsiella* sp., *Enterobacter* sp., *Raoultella* sp.)

Participants: Emilio Hernández, Pablo Liedo (México)

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

Plans for the second 18th months
- Identification of microbial communities from wild and laboratory larvae (third instar) and adult populations (irradiated and non-irradiated) using molecular approaches (pyrosequencing).

Progress made up to the 2\textsuperscript{nd} RCM
- No activities were planned for this period.

Participants: Erin Schuenzel, Bacilio Salas, Hugh Conway, Don Vacek (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.

Plans for the second 18 months
- Identification of microbial communities in laboratory diseased and dead larvae by molecular and classical microbiological phenotyping approaches.

Progress made up to the 2\textsuperscript{nd} RCM
• Bacterial strains from eggs have been isolated and characterized molecularly.

**Species: Anastrepha obliqua**  
Participants: Emilio Hernández, Pablo Liedo (México)

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.
- Development of protocols for probiotic introduction into larval diet.

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

Progress made up to the 2nd RCM
- Isolation of bacterial strains from gastrointestinal track from wild and laboratory populations.
- Evaluation of the effect on the incorporation of the autogeneous culturable microorganisms in the larval diet is in progress.

**Species: Anastrepha serpentina**  
Participants: Emilio Hernández, Pablo Liedo (México)

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.
- Development of protocols for probiotic introduction into larval diet.

Plans for the second 18 months
- Identification of microbial communities in wild and laboratory third instar-larvae by using molecular and classical microbiological phenotyping approaches.
- Evaluation of the effect of the incorporation of the autogeneous culturable microorganisms in the larval diet

Progress made up to the 2nd RCM
- No activities were planned for this period.

**Species: Anastrepha striata**  
Participants: Emilio Hernández, Pablo Liedo (México)

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.

Plans for the second 18 months
• Isolation of bacterial strains from the gastrointestinal track using wild and laboratory populations

Progress made up to the 2\textsuperscript{nd} RCM
• No activities were planned for this period.

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

5 years plan
• Identification of microbial communities in larvae and adults of wild and lab reared fruit flies (different strains and colonies) by molecular approaches (16S rDNA library and/or 16S rDNA pyrosequencing).

• Identification of the culturable microbiota of larvae and adult of a lab colony.

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

Plan for second 18 months period
• Identification of microbial communities in larvae and adult of wild and lab reared fruit flies by molecular approaches (16S rDNA library and/or 16S rDNA pyrosequencing).

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

Progress made up to the 2\textsuperscript{nd} RCM
• Culturable microbiota associated with the larval and adult gut of a lab strain reared on papaya has been completed

**Species: *Bactrocera curcurbitae***
Participants: Mahfuza Khan (Bangladesh)
Collaborators: Kostas Bourtzis (IAEA), 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 approaches and molecular techniques (16S rDNA pyrosequencing).

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

• Assess the quality parameters of *B. cucurbitae* reared from bacteria incorporated larval diets.
Plans for the second 18 months
- Identification of gut microbial communities of third instar larvae/pupae from infested fruits of *B. cucurbitae* by molecular techniques (16S rDNA pyrosequencing).
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

Progress made up to the 2nd RCM
- Identified mid gut of third instar larvae of natural host reared *B. cucurbitae* using API kits.
- Determined bacteria enriched adult diets on mating performance, fecundity, and survival under laboratory and semi-field cage trials.

**Species: Bactrocera dorsalis complex**
**Participants:** Mahfuza Khan (Bangladesh)
**Collaborators:** Kostas Bourtzis (IAEA), 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 approaches and molecular techniques (16S rDNA pyro sequencing).
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.
- Assess the quality parameters of *B. dorsalis* reared from bacteria incorporated larval diets

Plans for the second 18 months
- Identification of gut microbial communities of third instars larvae from infested fruits of *B. dorsalis* by molecular techniques (16S rDNA pyro sequencing).
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

Progress made up to the 2nd RCM
Identified mid gut bacterial strains of third instar larvae reared on artificial liquid larval diet using API kits.

Participants: Changying Niu (China)

5 years plan
- Identification of microbial communities in egg, larvae and pupae of wild fruit flies by culture-dependent and molecular approaches (16S rRNA pyrosequencing)
- Identify suitable beneficial microorganisms for probiotic applications at larval stage.

Plans for the second 18 months
- Identification of microbial communities in egg, larvae and pupae of wild and mass reared fruit flies by molecular approaches (16S rRNA pyrosequencing).
- Monitor the population dynamics of some important bacteria species identified by culture-dependent approaches.

Progress made up to the 2nd RCM
• Analysed the molecular diversity of intestinal bacterial community of different developmental stages using 454 pyrosequencing technology. Bacterial strains have been isolated from wild fruit flies using culture-dependent approaches.

Participants: George Tsiamis (Greece)  
Collaborators: Kostas Bourtzis (IAEA)

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

Plans for the second 18 months
• Characterization of the gut associated bacterial communities by 16S rRNA pyrosequencing.

Progress made up to the 2nd RCM
• No work activities were planned

Species: Bactrocera minax
Participants: Changying Niu (China)

5 years plan
• Identification of microbial communities in egg, larvae, pupae and adults from the wild populations by culture-dependent and molecular approaches (16S rRNA pyrosequencing)
• Identify suitable beneficial microorganisms for probiotic applications at larval stage.
• Monitor the population dynamics of some important bacteria species identified by culture-dependent approaches

Plans for the second 18 months
• Identification of microbial communities in egg, larvae, pupae, adults from the wild population using culture-dependent and molecular approaches.
• Monitor the population dynamics of some important bacteria species identified by culture-dependent approaches.

Progress made up to the 2nd RCM
• The molecular diversity of intestinal bacterial communities of different developmental stages using 454 pyrosequencing technology has been completed
• Using 16S rRNA ARDRA analysis, colony morphology, physiological and biochemical characteristic tests, we identified the intestinal culturable bacteria of adults and second-instar larvae of B. minax.

Species: Bactrocera tryoni
Participants: Peter Crisp, Olivia Reynolds (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 culture-dependent and molecular approaches (16S rRNA pyrosequencing)

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

Plans for the second 18 months
• Assess beneficial bacterial strains previously isolated and identified from *D. pornia*

Progress made up to the 2\textsuperscript{nd} RCM
• No work activities were planned.

**Species: Diorixa pornia**  
Participants: Peter Crisp, Olivia Reynolds (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 culture dependent and molecular approaches (16S rRNA pyrosequencing)

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

• Develop protocols for the incorporation of beneficial bacterial into the larval diet

Plans for the second 18 months
• Assess beneficial bacterial strains previously isolated and identified from *D. pornia*

Progress made up to the 2\textsuperscript{nd} RCM
• No work activities were planned.

**1.2. Preventing the growth of deleterious microorganisms**

**Species: Ceratitis capitata**  
Participants: Hernán Donoso, Carolina Yánez (Chile)

5 years plan
• Identification of deleterious microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.

• Development of diagnostic tests for the early detection of deleterious microorganisms.

Plans for the second 18 months
• Implementation of aseptic protocols in the mass-rearing facility

Progress made up to the 2\textsuperscript{nd} RCM
• Preliminary screening for assessing microbiological contamination in the mass-rearing facility indicated the presence of *Morganella* sp., and *Stenotrophomonas* sp.

**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.

Plans for the second 18 months
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Progress made up to the 2nd RCM
- *Morganella* sp. and *Serratia* sp. have been identified as potential pathogenic microorganisms.

Participants: Jaime Garcia de Oteyza, Teresa Navarro (Spain)
Collaborators: George Tsiamis for the development of early detection protocol

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 of diagnostic tests for the early detection of deleterious microorganisms.

Plans for the second 18 months
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
- Development of diagnostic tests for the early detection of pathogenic microorganisms.

Progress made up to the 2nd RCM
- *Morganella* sp. and *Serratia* sp. have been identified as potential deleterious microorganisms.
Participants: Erin Schuenzel, Bacilio Salas, Hugh Conway, Don Vacek (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.

Plans for the second 18 months
- Identification of deleterious microorganisms in laboratory eggs, larval diet, dead/diseased larvae, dead/diseased pupae by molecular and classical microbiological approaches.
- Whole genome sequence of bacterial strains identified as deleterious.
- Development diagnostic tests for the early detection of deleterious microorganisms.

Progress made up to 2nd RCM

The effects of 20 bacterial strains have been tested, with four having deleterious effects on larval survivorship and adult emergence.

Species: Anastrepha obliqua
Participants: Emilio Hernández, Pablo Liedo (México)

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.

Plans for the second 18 months
- Identification of deleterious microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Progress made up to the 2nd RCM
- Identification of Serratia sp. as a potential deleterious microorganism

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/adult by culturable approaches
- Identification of deleterious microorganisms in laboratory larvae/adult by molecular approaches (16S rDNA library and 16S rDNA pyrosequencing).

Plans for the second 18 months
- Identification of deleterious microorganisms in laboratory larvae/adults by molecular approaches (16S rDNA library and 16S rRNA pyrosequencing).

Progress made up to 2\textsuperscript{nd} RCM
- Culturable microbiota associated with the gut of larvae and adults were identified in a laboratory strain reared on papaya.

**Species: Bactrocera curcubitae**

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

5 years plan
- Identification of deleterious microorganisms in laboratory (host/artificial) reared larvae of *B. cucurbitae* by culture-dependent and molecular approaches.
- Assess the quality parameters of *B. cucurbitae* reared from bacteria incorporated larval diets.

Plan for second 18 months
- Identification of deleterious microorganisms in laboratory (host/artificial) reared larvae of *B. cucurbitae* by molecular approaches.

Progress made since last RCM
- Identification of deleterious microorganisms in laboratory host reared larvae of *B. cucurbitae* by biochemical approaches (API kits) has been completed.

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

5 years plan
- Identification of deleterious microorganisms in laboratory (host/artificial) reared larvae of *B. dorsalis* by culture-dependent and molecular approaches.
- Assess the quality parameters of *B. dorsalis* reared from bacteria incorporated larval diets.

Plan for second 18 months
• Identification of deleterious microorganisms in laboratory (host/artificial) reared larvae of *B. dorsalis* by molecular approaches.

Progress made since last RCM
• Identification of deleterious microorganisms in artificial larval diet by biochemical approaches (API kits).

Participants: George Tsiamis (Greece)
Collaborators: Kostas Bourtzis (IAEA)

5 years plan
• Identification of (deleterious) microorganisms in laboratory larvae by 16S rRNA pyrosequencing libraries.

18 months plan
• Identification of deleterious microorganisms in laboratory larvae by 16S rRNA pyrosequencing.

Progress made up to the 2nd RCM
• No work was planned for the first 18 months

Species: *Diorixa pornia*
Participants: Peter Crisp, Olivia Reynolds (Australia)

5 years plan
• Identification of deleterious microorganisms in laboratory eggs, larvae and pupae by culture-dependent and molecular approaches (16S rRNA pyrosequencing).

• Development of diagnostic tests for the early detection of deleterious microorganisms.

Plans for the second 18 months
• Identification of deleterious microorganisms in laboratory eggs, larvae and pupae by culture-dependent approaches.

• Development of diagnostic tests (ELISA) for the early detection of deleterious microorganisms from a mass-rearing colony.

Progress made up to the 2nd RCM
• DNA has been extracted from adults, library construction of 454 amplicon pyrosequencing has been completed and candidate deleterious bacterial strains have been identified.

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

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

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

Plans for the second 18 months
• Molecular characterization (DGGE) of the bacterial communities in the larval gut
• Identification of beneficial microorganisms in laboratory larvae by classical microbiological approaches.
• Quality assessment of the bacterial fed larvae

Progress made up to the 2\textsuperscript{nd} RCM
• Molecular characterization of bacterial communities in larvae using DGGE has been completed.
• Isolation and characterization of bacterial strains has been completed

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.
• Monitoring beneficial microorganisms in egg suspension.
• Development of protocols for the incorporation of beneficial microorganisms in the egg suspension.

Plans for the second 18 months
• Monitoring beneficial microorganisms in egg suspension.

Progress made up to the 2\textsuperscript{nd} RCM
• Beneficial microorganisms have been identified

Participants: Jaime García 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 of diagnostic tests for the detection of beneficial microorganisms.
• Protocol development for introducing beneficial microorganisms in a mass-rearing facility.

Plans for the second 18 months
• Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
• Development of diagnostic tests for the detection of beneficial microorganisms.
• Protocol development for introducing beneficial microorganisms in a mass-rearing facility.

Progress made up to the 2\textsuperscript{nd} RCM
Potential beneficial microorganisms have been identified using molecular and culture-dependent 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.
- Monitoring beneficial microorganisms in egg suspension.
- Development of protocols for the incorporation of beneficial microorganisms in the egg suspension

Plans for the second 18 months
- Monitoring beneficial microorganisms in egg suspension.

Progress made up to the 2nd RCM
- Beneficial microorganisms have been identified

Participants: Emilio Hernández, Pablo Liedo (México)

5 years plan
- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development of protocols for the incorporation of beneficial microorganisms in the egg suspension

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

Progress made up to the 2nd RCM
- Identification of putative beneficial microorganisms is in progress.

Participants: Erin Schuenzel, Bacilio Salas, Hugh Conway, Don Vacek (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.

Plans for the second 18 months
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
- Whole genome sequence of the beneficial microorganisms.
Progress made up to the 2\textsuperscript{nd} RCM

- No beneficial effects have been found using twenty isolated bacterial strains

\textbf{Species: Anastrepha obliqua}
\textbf{Participants: Emilio Hernández, Pablo Liedo (México)}

5 years plan

- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development of protocols for the incorporation of beneficial microorganisms.

Plans for the second 18 months

- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
- Effect on the addition of the bacterial on the life history traits and quality parameters during the colonization and mass-rearing.

Progress made up to the 2\textsuperscript{nd} RCM

- Identification of putative beneficial microorganisms is in progress.

\textbf{Species: Anastrepha serpentina}
\textbf{Participants: Emilio Hernández, Pablo Liedo (Mexico)}

5 years plan

- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development of protocols for the incorporation of beneficial microorganisms

Plans for the second 18 months

- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
- Effect on the addition of the bacterial on the life history traits and quality parameters during the colonization and mass-rearing. (GROUP 2)

Progress made up to the 2\textsuperscript{nd} RCM

- Identification of putative beneficial microorganisms is in progress

\textbf{Species: Anastrepha striata}
\textbf{Participants: Emilio Hernández, Pablo Liedo (Mexico)}

5 years plan

- Identification of beneficial microorganisms in laboratory eggs, larvae and larval diet by molecular and classical microbiological approaches.
- Development of protocols for the incorporation of beneficial microorganisms
Plans for the second 18 months plan
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.

Progress made up to the 2nd RCM
- Identification of putative beneficial microorganisms is in progress.

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

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

Plans for the second 18 months period
- Identification of beneficial microorganisms in laboratory larvae and adult by molecular approaches.

Progress made up to 2nd RCM
- Culturable larval and adult gut microbiota of a laboratory population reared on papaya has been identified

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

5 years plan
- Identification of beneficial microorganisms in laboratory (host/artificial) reared larvae of *B. cucurbitae* by molecular/biochemical and culture-dependent approaches.
- Assess the quality parameters of *B. cucurbitae* reared from bacteria incorporated larval diets.

Plan for second 18 months
- Identification of beneficial microorganisms in laboratory (host/artificial) reared larvae of *B. cucurbitae* by molecular approaches.

Progress made since last RCM
- Identification of beneficial microorganisms in laboratory host reared larvae of *B. cucurbitae* by biochemical approaches (API kits).

**Species: Bactrocera dorsalis complex**
Participants: Mahfuza Khan (Bangladesh)
Collaborators: Kostas Bourtzis (IAEA) and George Tsiamis (Greece)
5 years plan
- Identification of deleterious microorganisms in laboratory (host/artificial) reared larvae of *B. dorsalis* by culture-dependent and molecular/biochemical approaches.
- Assess the quality parameters of *B. dorsalis* reared from bacteria incorporated larval diets.

Plan for second 18 months
- Identification of beneficial microorganisms in laboratory (host/artificial) reared larvae of *B. dorsalis* by culture-dependent and molecular/biochemical approaches.

Progress made since last RCM
- Identification of beneficial microorganisms in artificial larval diet by biochemical approaches (API kits).

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

5 years plan
- Identification of (beneficial) microorganisms in laboratory larvae by 16S rRNA pyrosequencing libraries.

18 months plan
- Identification of (beneficial) microorganisms in laboratory larvae by 16S rRNA pyrosequencing libraries.

Progress made up to the 2nd RCM
- No work activities were planned for the first 18 months.

**Species:** *Diorixa pornia*
**Participants:** Peter Crisp, Olivia Reynolds (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.

Plans for the second 18 months
- Identification of beneficial microorganisms in laboratory eggs and larval diet by molecular and classical microbiological approaches.
- Commence development of diagnostic tests (ELISA) for the detection of beneficial microorganisms

Progress made up to the 2nd RCM
- DNA has been extracted from adults, libraries for 454 amplicon pyrosequencing have been prepared and candidate beneficial bacterial strains have been identified.
1 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>
</table>
| *Anastrepha ludens* | Adult gut | *Enterobacter cloacae, Providencia spp*  
*Citrobacter koseri*  
*Enterobacter sakazakii*  
*Klebsiella pneumoniae*  
*Pseudomonas aeruginosa* | Classical | Kuzina et al. 2001 |
| *A. ludens* | Adult crop and gut | *Citrobacter freundii*  
*Klebsiella oxytoca* | Classical | Martinez et al. 1994 |
| *A. ludens* | Adult crop | *Enterobacter spp.*  
*Pseudomonas aeruginosa*  
*Pseudomonas spp.* | Classical | Martinez et al. 1994 |
| *A. ludens* | Adult gut | *Klebsiella pneumoniae* | Classical | Martinez et al. 1994 |
| *A. ludens* | infested fruit | *Citrobacter freundii*  
*Klebsiella oxytoca* | Classical | Martinez et al. 1994 |
| *Bactrocera cacuminata* | Adult gut, Pupae, Eggs, infested fruit | *Citrobacter freundii*  
*Klebsiella pneumoniae*  
*Pseudomonas spp* | Classical | Fitt & Obrien 1985 |
| *B. cacuminata* | Host Plant leaves and fruit surface Adults | *Pantoea spp*  
*Pantoea agglomerans*  
*Citrobacter*  
*Enterobacter*  
*Klebsiella*  
*Providencia*  
*Serratia* | Classical | Raghu et al. 2002 |
<p>| | | | | Thaochan et al. 2010 |</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Bacterial Species</th>
<th>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> &lt;br&gt; <em>Enterobacter</em> &lt;br&gt; <em>Pectobacterium</em> &lt;br&gt; <em>Serratia</em> &lt;br&gt; <em>Actinobacteria</em> &lt;br&gt; <em>Firmicutes</em></td>
<td>Molecular</td>
<td>Wang et al. 2011</td>
</tr>
<tr>
<td><em>B. jarvisi</em></td>
<td>Adult gut, Pupae, Eggs, infested fruit</td>
<td><em>Enterobacter</em> agglomerans &lt;br&gt; <em>Enterobacter cloacae</em> &lt;br&gt; <em>Enterobacter spp</em> &lt;br&gt; <em>Klebsiella pneumoniae</em> &lt;br&gt; <em>Providencia spp</em></td>
<td>Classical</td>
<td>Fitt &amp; O'Brien 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; O'Brien 1985</td>
</tr>
<tr>
<td><em>B. neohumeralis</em></td>
<td>Adult gut, Pupae, Eggs, infested fruit</td>
<td><em>Enterobacter cloacae</em> &lt;br&gt; <em>Enterobacter spp</em> &lt;br&gt; <em>Pseudomonas spp</em></td>
<td>Classical</td>
<td>Fitt &amp; O'Brien 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 savastanoi</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> &lt;br&gt; <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> &lt;br&gt; <em>Klebsiella oxytoca</em> &lt;br&gt; <em>Klebsiella ozaenae</em> &lt;br&gt; <em>Pantoea agglomerans</em> &lt;br&gt; <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> &lt;br&gt; <em>Enterobacter cloacae</em> &lt;br&gt; <em>Klebsiella pneumoniae</em> &lt;br&gt; <em>Providencia spp</em> &lt;br&gt; <em>Pseudomonas spp</em></td>
<td>Classical</td>
<td>Fitt &amp; O'Brien 1985</td>
</tr>
<tr>
<td><em>B. tryoni</em></td>
<td>Adult crop, gut</td>
<td><em>Klebsiella oxytoca</em> &lt;br&gt; <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> &lt;br&gt; <em>Klebsiella spp.</em></td>
<td>Classical</td>
<td>Lauzon 2003</td>
</tr>
</tbody>
</table>

**Notes:**
- *B. dorsalis* and *B. jarvisi* are species of bumblebees.
- *B. neohumeralis* and *B. oleae* are species of fruit flies.
- *B. tau* and *B. tryoni* are species of fruit flies.
- *Ceratitis capitata* is a species of fruit fly.
- The bacterial species listed are associated with the insect's gut microbiome.
- The methods used include classical and molecular techniques.
<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Pathogens</th>
<th>Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<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, 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>C. capitata</td>
<td>Adults, larvae</td>
<td><em>Kluyvera</em>, <em>Leuconostoc</em></td>
<td>Molecular</td>
<td>Aharon et al., 2013</td>
</tr>
<tr>
<td>Rhagoletis alternae</td>
<td>Adult gut, larvae</td>
<td><em>Enterobacter spp</em></td>
<td>Classical</td>
<td>Daser &amp; Brandl, 1992</td>
</tr>
<tr>
<td>R. complete</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>
</tbody>
</table>
In addition to these published reports, several unpublished 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., 2012). 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). Field and lab populations of *B. tryoni*, *B. neohumeralis*, *B. cacuminata* were analysed with 454 sequencing for bacterial communities (Morrow et al., unpublished). No major differences in the community structures were found within species, but fewer species found in lab populations. Between species, however, bacterial communities were more diverse for fruit flies with a larger host range (Morrow et al., unpublished).

- **Ceratitis**: Morrow et al (unpublished) sequenced bacterial communities for *C. capitata* and found fewer species in the lab populations compared to field populations.

- **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. 2013). Morrow et al (unpublished) sequenced bacterial communities for *D. pornia* and found fewer species in the lab populations compared to field populations.

### 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

<table>
<thead>
<tr>
<th>R. suavis</th>
<th>Adult Oesophageal bulb</th>
<th>Klebsiella oxytoca Enterobacter agglomerans Enterobacter cloacae Klebsiella ozaenae Klebsiella pneumoniae</th>
<th>Classical</th>
<th>Howard et al. 1985</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. tabellaria</td>
<td>Adult Oesophageal bulb</td>
<td>Klebsiella oxytoca Enterobacter agglomerans Enterobacter cloacae Klebsiella ozaenae Klebsiella pneumoniae</td>
<td>Classical</td>
<td>Howard et al. 1985</td>
</tr>
<tr>
<td>Tephritis conura</td>
<td>Adult gut, Larvae</td>
<td>Erwinia spp</td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
<tr>
<td>Tephritis dilacerate</td>
<td>Adult gut, Larvae</td>
<td>Enterobacter spp</td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
<tr>
<td>Urophora cuspidata</td>
<td>Adult gut, Larvae</td>
<td>Erwinia spp</td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
<tr>
<td>Urophora solstitialis</td>
<td>Adult gut, Larvae</td>
<td>Erwinia spp</td>
<td>Classical</td>
<td>Daser &amp; Brandl 1992</td>
</tr>
</tbody>
</table>
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 plant host (wild)
- Adult Environment – plant host (wild)
- Nutritional Status and Diet
- Season and Environmental Conditions
- Temperature – rearing conditions
- Fly tissue sampled
- Irradiation
- Exposure to Antibiotics and Preservatives

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: Reproductive endosymbionts for A. striata and A. ludens have been characterized. Community structures of lab-reared A. grandis, A. ludens, A. fraterculus (2 populations – Peru and Tucuman) have been studied.

Bactrocera: Most species are lacking molecular data except B. dorsalis, B. oleae, B. tau and B. tryoni, B. cucurbitae. 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: Several individual flies have been molecularly characterized for D. pornia. Molecular data is lacking for the rest of the 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:* For *A. fraterculus* there are preliminary data suggesting that removal of gut bacterial communities has an impact on male mating performance.

*Bactrocera:* In the olive fly, the presence of symbionts is obligatory to the development of larvae in olives probably due to the secondary plant metabolite oleuropein. 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*. In addition to female fitness effects, how adults use and acquire the bacteria need to be understood. The establishment and persistence of bacteria within the insect needs to be determined.

*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). For *A. fraterculus* there are preliminary data suggesting that removal of gut bacterial communities has an impact on male mating performance.

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., 2014). 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) but improved larval viability for *B. tryoni* (Crisp et al., 2013).

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). 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. Hamden et al (2013) enriched larval diets with *Klebsiella, Enterobacter* and *Citrobacter* and found increased male mating competitiveness.

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- green fluorescent protein (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).
- **Delivery of probiotics** – different methods of delivery of probiotics and entomopathogenic to adults can alter the production of the next generation adults. Guidelines should be developed to ensure consistency.
- **Consortium of bacteria** – more studies need to move beyond testing a single bacterium and begin to test consortium of bacteria in adult probiotic diets.
• Metabolic characterization of bacteria – the probiotic effect may be due to the metabolic capability of the bacterium and not the particular species. When possible, the biochemical abilities of the bacterium and the physiological effects on the host should be determined.

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, Marysol Aceituno (Mexico)

5 year 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 behaviour parameters.

Plan for the second 18 months
- Identification of the beneficial bacteria and determine the role of the associated bacteria on the rearing (fecundity, fertility, life span) and behaviour parameters.

Progress up to 2nd RCM
- Characterized microbiota of adult fruit flies.

*Anastrepha obliqua*

Participants: Emilio Hernández, Pablo Liedo, Marysol Aceituno (México)
5 year 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 behaviour parameters.

Plan for the second 18 months
- 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 behaviour parameters.

Progress up to 2nd RCM
- Characterized microbiota of adult fruit flies.
- Examined the role of bacteria on adults rearing and behaviour patterns

**Anastrepha serpentina**
*Participants: Emilio Hernández, Pablo Liedo, Marysol Aceituno (México)*

5 year 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 behaviour parameters.

Plan for the second 18 months
- 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 behaviour parameters.

Progress up to 2nd RCM
- Characterized microbiota of adult fruit flies.
- Examined the role of bacteria on adults rearing and behaviour patterns

**Anastrepha striata**
*Participants: Emilio Hernández, Pablo Liedo, Marysol Aceituno (México)*

5 year 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 behaviour parameters.

Plan for the second 18 months
• 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 behaviour parameters.

Progress up to 2nd RCM
• Characterized microbiota of adult fruit flies.
• Examined the role of bacteria on adults rearing and behaviour patterns

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

5 year 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

Plan for the second 18 months
• Use of high-throughput DNA sequencing (16S rRNA 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

Progress up to 2nd RCM
• Bacteria have been cultured from all four species.
• Samples collected for 16S rRNA sequencing

_A. fraterculus_
Participant: Diego Segura (Argentina)
Collaborator: Boaz Yuval (Israel)

5 year plan
• Description of the main bacteria inhabiting the gut of wild flies and laboratory strains through the characterization of the 16S rDNA gene.
• 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 changes in the gut microbiota due to adaptation to laboratory conditions following the methodology described above.

Plan for the second 18 months
• Continue the characterization of the gut bacterial community by incorporating adult wild flies obtained from different hosts as well as from traps, and also immature stages.
• Continue the preservation of individuals across generations (up to F10) to complete the analysis of bacterial community changes due to adaptation by means of analysis of the DGGE profiles.
Progress up to 2nd RCM

- The gut bacterial community was characterized in a laboratory strain by DGGE followed by sequencing of selected bands. Several OTUs were found. The majority of the bacteria were Enterobacteria from the order Gammaproteobacteria.
- Infested fruit was collected from the wild to obtain flies to initiate a laboratory strain under laboratory conditions. Individuals from the F0 and subsequent generations (F5) were preserved at different reproductive status (newly emerged and sexually mature) to assess changes in the gut bacterial community as a consequence of the adaptation process.

Participant: Fernando L. Consoli (Brazil)

5 year plan

- Isolate and characterize the microbiota of the larva and 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 larva and adult gut microbiota by 16S rRNA pyrosequencing in a Illumina platform and/or 16S rRNA library (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

Plan for the second 18 months

- Assess the role of the food source (6 natural food sources) on the composition of the larva and adult gut microbiota by 16S rDNA library screening and/or 16S pyrosequencing in a Illumina platform (in progress)

Progress up to 2nd RCM

- The cuturable microbiota of the larval and adult gut of a laboratory population of A. fraterculus reared on papaya has been completed.

Genus Bactrocera

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

5 year 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 pyrosequencing).

Plan for the second 18 months
- Characterization of the gut microbial community using 16S rRNA pyrosequencing

Progress up to 2nd RCM
- Isolated and identified with API strips the gut microbial community of laboratory (host) reared *B. cucurbitae* and *B. dorsalis* using classical microbial and biochemical tests.
- Collected microbiota samples for 16s rRNA community sequencing for all four species
- DNA has been extracted from bacterial communities and 16S rRNA libraries have been constructed.

*Bactrocera cucurbitae*
Participant: Ramesh Hire, Ashok Hadapad (India)

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

Plan for the second 18 months
- Isolation and characterization of gut microbiota of *B. cucurbitae*

Progress up to 2nd RCM
- The midgut bacteria were isolated from natural populations of adult melon fly. Based on 16s rRNA sequence analysis, several bacterial isolates were distributed in 9 genera.
- The dominant species in the gut of melon fly belonged to Enterobacteriaceae (75%), Bacillaceae (19%), Micrococcaceae (3%) and Staphylococcaceae (3%) families.
- These isolates were further characterized from growth patterns, effect of salt concentration and antibiotic sensitivity

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

5 year 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*.

Plan for the second 18 months
• Isolate DNA and conduct high-throughput sequencing of bacteria 16S rDNA V6 sequence from *B. dorsalis* and *B. minax*.
• Compare microbiota structure and diversity in *B. dorsalis* and *B. minax*.

Progress up to 2nd RCM
• Analysed the molecular diversity of intestinal bacterial community of different developmental stages using 454 pyrosequencing.

*Bactrocera oleae*
Participant Antonio Belcari

5 year plan
• Determine the presence of *Ca. Erwinia dacicola* in the olive fly populations
• 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.

Plan for the second 18 months
• Probiotic diets will be further evaluated.

Progress up to 2nd RCM
• Evaluated *Pseudomonas putida, Acetobacter tropicalis* as probiotic for lab-reared adults.

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

5 year 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.
• Test isolated bacteria on adult populations to improve longevity, male mate competitiveness

Plan for the second 18 months
• Collection of 6 X 5 batches of fruit flies from each species, isolation of microbiota with classical culture methods.

Progress up to 2nd RCM
• Isolated bacteria with classic culture techniques, including *K. oxytoca*, *K. pneumonia*, *Citrobacter freundii* and *Enterobacter cloacae* for *B. cucurbitae* and *Serratia marcescens* and *E. coli* for *B. zonata*

*Bactrocera tryoni, Bactrocera neohumeralis, Bactrocera cacuminata*
Participant Olivia Reynolds, Toni Chapman, Peter Crisp, Markus Riegler, Deane Woruba, Jen Morrow (Australia)

5 year plan
• 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 and different vegetation every 3 months for a 12 month period.
• 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 FFPF *B. tryoni*.
• Isolate and identify (through molecular profiling) probiotic candidate bacteria from sampled flies.

Plan for the second 18 months
• Isolation and identification of intestinal microflora of lab and field samples using molecular assays.

Progress up to 2nd RCM
• Field and lab populations of *B. tryoni, B. neohumeralis, B. cacuminata* were analysed with 454 sequencing for bacterial communities (Morrow et al., unpublished). No major differences in the community structures were found within species, but fewer species found in lab populations. Between species, however, bacterial communities were more diverse for fruit flies with a larger host range (Morrow et al., unpublished).

*Genus Ceratitis*

*Ceratitis capitata*
Participant Peter Crisp (Australia)

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

Plan for the second 18 months
• Commence assessment of bacteria from wild *C. capitata* from and identify volatile compounds

Progress up to 2nd RCM
• No activities
Participants: Hernán Donoso, Carolina Yáñez (Chile)

5 year 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

Plan for the second 18 months
- Continue isolation of gut microbiota and cultivation of bacteria
- Continue DNA extraction and PCR amplification of 16S rRNA genes

Progress up to 2nd RCM
- Molecular characterization by DGGE of the gut bacterial community.
- Isolated gut microbiota using classic cultivation techniques

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

5 year 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)

Plan for the second 18 months
- 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)

Progress up to 2nd RCM
- Characterized adult microbiota from mass reared and wild flies.

*Genus Dirioxa*

*Dirioxa pornia*
Participant Peter Crisp (Australia)

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

Plan for the second 18 months
- Isolate bacteria from *D. pornia* wild flies and identify using molecular techniques 16s rRNA.

Progress up to 2nd RCM
- Identified bacterial communities using next generation sequencing in a single wild *D. pornia* population

2.2. Effects of radiation
*Genus Anastrepha*

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

5 year plan
- Determine the effect of irradiation (gamma and X-rays) on the gut microbial community of laboratory flies through the characterization of the 16S rDNA gene.

Plan for the second 18 months
- Continue the characterization of the gut bacterial community of irradiated flies by means of DGGE followed by sequencing of selected bands and analysis of DGGE profiles.

Progress up to 2nd RCM
- Pupae from a laboratory strain were irradiated at three different doses and emerged adults were preserved for gut bacterial characterization to determine the effect of irradiation (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 year plan
- Conduct pyrosequencing analyses of irradiated (sterile) and non-irradiated males, to evaluate the effect of irradiation on microbiota.

Plan for the second 18 months
- Will conduct 16s rRNA community sequencing on irradiated and non-irradiated males.

Progress up to 2nd RCM
- Collected microbiota samples from irradiated and non-irradiated males

*Genus Bactrocera*

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

5 year plan
- To study the effect of radiation on the microflora of the alimentary tract of the fruit flies
- 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

Plan for the second 18 months
To study the effect of radiation on the microflora of the alimentary tract of the fruit flies

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

Progress up to 2nd RCM

- No activities

**Bactrocera cucurbitae, Bactrocera dorsalis**

Participant: Mahfuza Khan (Bangladesh)

Collaborators: Kostas Bourtzis (IAEA), and George Tsiamis (Greece)

5 year 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).

Plan for the second 18 months

- Characterization of the gut microbial communities from irradiated and control male *B. cucurbitae* and *B. dorsalis*.

Progress up to 2nd RCM

- Collected samples of irradiated and control males for both *B. cucurbitae* and *B. dorsalis*
- DNA extraction and 16S rRNA libraries constructed for the microbiota of irradiated and control males

**Bactrocera cucurbitae**

Participant: Ramesh Hire, Ashok Hadapad (India)

5 year plan

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

Plan for the second 18 months

- Study the effect of irradiation on gut microflora of *B. cucurbitae*

Progress up to 2nd RCM

- Melon fly cultures were established under laboratory conditions
- Mass produced melon fly pupa were used for the effect of 8 doses of gamma radiation on gut microflora
- Identification of microflora by 16S rRNA sequencing is ongoing

**Bactrocera dorsalis**

Participant: Changying Niu (China)

5 year plan
• Irradiate the GSS of laboratory colony for *B. dorsalis* male with gamma radiation.
• 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.

Plan for the second 18 months
• Irradiate the GSS of *B. dorsalis* male with gamma radiation.

Progress up to 2nd RCM
• No activities

*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

Plan for the second 18 months
• Evaluation of presence of *Ca. Erwinia dacicola* and/or other bacteria in the olive fly sterilized males

Progress up to 2nd RCM
• No activities

*Genus Ceratitis*

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

5 year 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

Plan for the second 18 months
• Isolation of gut microbiota and cultivation of bacteria in irradiated adults
• DNA extraction and PCR amplification of 16S rRNA genes
• Evaluate effects of irradiation with temperature controlled larvae and pupae

Progress up to 2nd RCM
• Molecular characterization by DGGE of the gut bacterial community
• Isolated gut microbiota using classic cultivation techniques
• Found effect of irradiation on microbiota communities
• Found effect of temperature on microbiota communities
Participants: Jamie Garcia de Oteiza Teresa Navarro (Spain)

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

Plan for the second 18 months
- Isolation of gut microbiota and cultivation of bacteria in irradiated adults
- DNA extraction and PCR amplification of 16S rRNA genes for DGGE analysis and next generation sequencing
- Test effects of probiotic supplementation in irradiated males

Progress up to 2nd RCM
- No activities

2.3. Function of microbiota and fitness effects

Genus *Anastrepha*

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

5 year plan
- Evaluate 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.
- Evaluate 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.
- Evaluate 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.
- Evaluate the impact of antibiotics on flies’ ingestion rate, nutritional reserves and adult dry weight.
- Evaluate the effect of adding bacteria to the adult diet on male mating competitiveness and on female fecundity.

Plan for the second 18 months
- Continue the evaluation of the effect of gut bacteria on mating competitiveness by assessing the impact on sexual signalling (quality and quantity of the pheromone released).
• Collect pheromones and extract CHC from antibiotic treated and non-treated males.
• Evaluate the effect of gut bacteria on starvation resistance.
• Evaluate the impact of antibiotics on flies’ ingestion rate, nutritional reserves and adult dry weight.
• Continue with the molecular characterization of gut microbiota.

Progress up to 2nd RCM
• The effect of antibiotic treatment on male mating competitiveness was assessed under laboratory conditions. Two origins (laboratory and wild males) and two different diets (sugar and sugar + protein) were evaluated.
• The gut bacterial community of the different males used in the behavioural tests was characterized with DGGE approach.

Participant Fernando L. Consoli (Brazil)

5 year plan

• Test the requirement of selected bacterial strains for adult reproduction (in progress)

Plan for the second 18 months

• Test the requirement of selected bacterial strains for adult reproduction (in progress)

Progress up to 2nd RCM
• No activities

Genus Bactrocera

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

5 year plan

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

Plan for the second 18 months

• To isolate and cultivate potential bacterial candidates for probiotic development
• To investigate the effect of isolated bacterial symbionts of B. cucurbitae and B. zonata during pre-release period on sterile insect performance.

Progress up to 2nd RCM
• Isolated bacteria with classic culture techniques and identified with API strips, including K. oxytoca, K. pneumonia, Citrobacter freundii and Enterobacter cloacae for B. cucurbitae and Serratia marcescens and E. coli for B. zonata.

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

5 year 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.

Plan for the second 18 months
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.

Progress up to 2nd RCM
Bacteria counter-acted the effects of oleuropein on larvae.
Determined activity of oleuropein in olives as reducing lysine

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

5 year 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*.

Plan for the second 18 months
Improve delivery method of probiotics to adults
Develop optimal feeding protocol

Progress up to 2nd RCM
Completed preliminary selection and assessment of probiotic material isolated from *D. pornia* with adult *B. tryoni* flies.
- Fecundity
- Adult longevity
- Egg eclosion
- Larval survivorship
- Next generation emergence

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

5 year plan
Supplement probiotics in the diets for *B. dorsalis* and *B. minax*;
Compare the survival, weight, pupation rate of larvae with control;
Study the fitness effects for *B. dorsalis*

Plan for the second 18 months
Supplement probiotics in the diets of larvae for *B. dorsalis* and *B. minax*.
Supplement probiotics from green citrus fruits in the diets of larvae for *B. minax*

Progress up to 2nd RCM
- Finished preliminary results on longevity, copulation, and adult dry weight for *B. dorsalis*

**Genus Ceratitis**

*Ceratitis capitata*

Participant Peter Crisp (Australia)

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

Plan for the second 18 months
- The bacteria species isolated from *D. pornia* and associated will be assessed for probiotic and behavioural changes for improvement of performance of sterile male *C. capitata* used in SIT eradication programs.

Progress up to 2nd RCM
- No activities

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

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

Plan for the second 18 months
- Identify suitable beneficial bacteria and quality control after their inoculation (longevity under stress, flight capacity, eggs/female)
- Develop procedures for probiotic delivery under mass rearing conditions.

Progress up to 2nd RCM
- Identified bacteria with 16s rRNA sequence analysis.

Participant Nikos Papadopoulos (Greece)

Collaborators: K. Bourtzis (IAEA) and G. Tsiamis (Greece)

5 year 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.

Plan for the second 18 months
- Define interactions of aromatherapy and provision of probiotic inoculum to Vienna 8 males.
Progress up to 2nd RCM

- Effects of probiotic symbionts on demographic and quality traits of the Vienna 8 strain has been determined in Effects of probiotic provision of symbionts on the sexual behaviour of Vienna 8 males.
- Effects of probiotic provision of symbionts on the sexual behaviour of Vienna 8 males has been determined in the laboratory of IAEA in Siebersdorf.

Genus *Dirioxa*

*Dirioxa pornia*
Participant Peter Crisp (Australia)

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

Plan for the second 18 months
- DNA analysis of eggs, larvae and pupae of *D. pornia* from laboratory culture for bacteria.
- Identify volatile compounds associated with the bacteria in collaboration with Plant and Food New Zealand.
- Elucidate which volatile compounds are attractive to *D. pornia*.
- Elucidate the alimentary tract with CT imaging, electron microscopy and light microscopy to understand where bacteria colonization occurs.

Progress up to 2nd RCM
- Commenced feeding studies using individual bacteria and assessed effect on longevity, fecundity and quality of adult *D. pornia*.
- Identified some volatile compounds associated with the bacteria.

2.4. Applications

Genus *Anastrepha*

*Anastrepha ludens*
Participants: Erin Schuenzel, Hugh Conway, Basilio Salas, Don Vacek (USA)

5 year plan
- Track presence of probiotics given to *Anastrepha ludens* larvae in the adult midgut using marked bacteria and selective media
- Genotype 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
Plan for the second 18 months
- Continue testing probiotic symbionts in *A. ludens* larval populations from mass-rearing facility using Koch's postulates

Progress up to 2nd RCM
- Koch’s postulates test on possible symbionts performed.
- Twenty bacteria tested so far and no probiotics identified.

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

5 year 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 in release centre and measure impact on insect fitness

Plan for the second 18 months
- No activities planned

Progress up to 2nd RCM
- No activities

*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.

Plan for the second 18 months
- No activities planned for this time period.

Progress up to 2nd RCM
- No activities done.

*Genus Bactrocera*

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

5 year 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 behaviour

Plan for the second 18 months
• To isolate and characterize microbial candidates for probiotic diet development

Progress up to 2nd RCM
• Isolated bacteria with classic culture techniques and identified with API strips, including *K. oxytoca, K. pneumonia, Citrobacter freundii* and *Enterobacter cloacae* for *B. cucurbitae* and *Serratia marcescens* and *E. coli* for *B. zonata*.

*Bactrocera cucurbitae, Bactrocera dorsalis*
Participant Mahfuza Khan (Bangladesh)

5 year 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 *B. cucurbitae* and *B. dorsalis* fed on probiotic adult diets under semi-field cage trials.

Plan for the second 18 months
• 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 *B. cucurbitae* and *B. dorsalis* fed on probiotic adult diets under semi-field cage trials.

Progress up to 2nd RCM
• Determined the effect of bacteria enriched diets on mating, fecundity and survival under laboratory and semi-field conditions

*Bactrocera oleae*
Participant Antonio Belcari (Italy)

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

Plan for the second 18 months
• Preliminary lab trials aimed at development of symbiosis transfer

Progress up to 2nd RCM
• Developed and tested probiotic diets based on *P. putida* and *A. tropicalis*. 
Two different concentrations tested with live bacteria
Two different concentrations tested with heat-killed bacteria
- Preliminary lab trial on symbiont transfer initiated

**Bactrocera dorsalis**

Participant: Changying Niu (China)

5 year plan
- Clone the full-length genes related to spermatogenesis of male *B. dorsalis*
- Study the function of the selected genes by RNAi in *B. dorsalis*
- Express dsRNA of genes in *E. coli* strain HT-115
- Feed these bacteria to the larvae of *B. dorsalis* in the diets
- Conduct lab and cage experiments to examine mating, oviposition of females, and hatching rate of the offspring

Plan for the second 18 months
- Clone the full-length of genes related to spermatogenesis in male *B. dorsalis* and study the function of HMG-R gene by RNAi.

Progress up to 2nd RCM
- Finished cloning the full-length of genes related to spermatogenesis in male *B. dorsalis*
- Determined the function of HMG-R gene by RNAi.

**Bactrocera tryoni**

Participant Olivia Reynolds, Toni Chapman, Peter Crisp (Australia)

5 year plan
- Field cage trials assessing longevity and mating success of probiotic supplemented flies (subject to funding)
- Field releases of probiotic supplemented sterile *B. tryoni* (subject to funding)

Plan for the second 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.

Progress up to 2nd RCM
- Secured Plant Biosecurity Cooperative Research Centre PhD Scholarship funding
- Candidate, Deane Woruba, competitively selected and enrolled with University of Western Sydney

**Genus Ceratitis**

**Ceratitis capitata**

Participants: Dina Melgar, Pablo Rendón, Felipe Jerónimo (Guatemala)
5 year 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 in release centre and measure impact on insect fitness

Plan for the second 18 months
- No activities planned

Progress up to 2nd RCM
- No activities

Participants: Peter Crisp (Australia)

5 year 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 isolated from *D. pornia* for SIT *C. capitata*

Plan for the second 18 months
- Establish trials to assess effect of exposure to bacterial volatiles isolated from *D. pornia* for SIT *C. capitata*
- Establish laboratory cage trials to assess effect of bacterial feed at emergence for SIT *C. capitata*

Progress up to 2nd RCM
- No activities

Participants: Jamie Garcia de Oteiza Teresa Navarro (Spain)

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

Plan for the second 18 months
- Develop procedures for probiotic delivery to adults for mass rearing programmes

Progress up to 2nd RCM
- No activities
Background situation analysis

Certain symbiotic bacteria are known to manipulate the mating behaviour and reproduction of their hosts. After detecting and characterizing the occurrence of Wolbachia and other heritable symbionts in several Bactrocera and Anastrepha species there is a need to determine their phenotypes and their fitness effects on hosts, and their interactions with other associated organisms. This determination 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, desiccation 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, and Dacus. However, Wolbachia is absent from C. capitata, B. oleae, and A. ludens. 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 phenotype 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.

In addition, some of the fruit flies under study are cryptic species complexes. Accurate taxonomic identification of the fly specimens used for *Wolbachia* detection should be guaranteed in order to establish possible associations between *Wolbachia* strains and particular fruit fly species/populations.

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* but not in *A. ludens*. With exception of *A. striata* (carries both A and B supergroups) all other species carry *Wolbachia* strains that belong to supergroup A.

*Bactrocera*: *Wolbachia* detected in populations of *B. dorsalis*, *B. philippinensis*, *B. carambolae*, *B. correcta*, *B. tau*, *B. cucurbitae*, *B. zonata* and several Australian *Bactrocera* (e.g. *B. neohumeralis*). The prevalence of *Wolbachia* infection on *Bactrocera* species varies among and within countries.

*Ceratitis*: Although *Wolbachia* has been reported in a laboratory strain in Brazil and one French population of *C. capitata*, recent thorough surveys of natural populations demonstrate that *Wolbachia* is absent in Brazil.

*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*, *R. cingulata* and *R. completa*. 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
- Application of sensitive methods to detect low titer and additional strains of *Wolbachia*

(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
Knowledge about the effects of reproductive symbionts on behavioural traits is restricted to medfly.

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.

(iv) Trans-infection technology

- Lack of efficient methodology to transfer *Wolbachia* across fruit fly species.

A2. Genus level background:

**Anastrepha:**

- CI in *Anastrepha* species is not fully understood.
- Knowledge of infection frequencies and types in field populations is restricted to few species and/or regions.
- There are no studies on fitness effects of *Wolbachia* infection.

**Bactrocera:**

- CI in *Bactrocera* species is not fully understood.
- Knowledge of infection frequencies and types in field populations is restricted to few species and/or regions.
- There are no studies on fitness effects other than CI.

**Ceratitis:**

- Additional studies required to understand fitness effects in artificially infected medfly lines.
- Lack of compatibility studies between trans-infected Vienna 8 lines and different wild medfly populations preferably obtained from different geographic areas.
- Lack of feasibility studies regarding the application of IIT.
- Lack of data regarding *Wolbachia* presence in other *Ceratitis* species than medfly.

**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 besides some first efforts for A. ludens.
- Status of naturally infected populations by reproductive symbionts has been characterized for some species. Additional data are needed for other species and populations.

Bactrocera:
- No artificially infected lines have been established (except B. oleae).
- Status of naturally infected populations by reproductive symbionts has been characterized for some species. Additional data are needed for other species and populations.

Ceratitis:
- The status of naturally infected populations by reproductive symbionts other than Wolbachia 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 other than *Wolbachia* 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-directional 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 is limited knowledge regarding tools for the application of IIT and 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. There are recent data on effects of *Wolbachia* on medfly demographic and behavioural traits including the Vienna 8 strain. Also, the performance of trans-infected Vienna 8 strains under mass rearing conditions has been assessed.

*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*. The *Wolbachia* infection status (including multiple infections) has been determined for many European populations of *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 behavioural 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 except for preliminary attempts in *A. ludens*.
- 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 open field assessment of the performance of artificially infected (with *Wolbachia*) *C. capitata* 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 other than *Wolbachia* 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.
- Evaluation of *Wolbachia wAstriB* as potential incompatibility agent in the Mexican fruit fly (*A. ludens*)

Plans for the 2nd 18 months period
- Perform crosses to test CI in *A. ludens* using the recently established transinfected lines.

Progress made since 1st RCM
- *Anastrepha ludens*, *A. striata*, *A. obliqua* and *A. serpentina* specimens were collected in Chiapas, Mexico during the period 2012-2014.
- PCR screening for heritable endosymbionts (including *Wolbachia* and *Spiroplasma*) was performed in all collected individuals. *Wolbachia* was found in *A. striata*, *A. obliqua* and *A. serpentina*. Screening results suggest that *A. ludens* is not naturally infected with *Wolbachia*; however, sampling was restricted to several specific areas in south Chiapas.
- A *Wolbachia* strains *wAstriB* was characterized in *A. striata* with the MLST approach.
- Transinfections from *A. striata* to *A. ludens* were performed to evaluate the potential use of *Wolbachia* as a cytoplasmic incompatibility agent. Trans-infected females are currently maintained in the rearing facilities at ECOSUR, Chiapas and infection success is being monitored.

Participants: Diego Segura (Argentina)
Collaborators: Claudia Conte, Silvia Lanzavecchia (Argentina), Kostas Bourtzis (IAEA)

5 years plan
- Screen wild populations and laboratory strains from different morphotypes of *A. fraterculus* to determine presence/absence of *Wolbachia* strains.
- Genetic and phenotypic characterization of the *Wolbachia* strains found.
Plans for the 2\textsuperscript{nd} 18 months

- Continue the Wolbachia screening by increasing the number of individuals and populations to be analysed.
- Continue the genetic and phenotypic characterization of \textit{wAfraCast1} and \textit{wAfraCast2}.

Progress made since 1\textsuperscript{st} RCM

- Specimens from 3 wild populations from Argentina and one laboratory strain were analysed with \textit{wsp} gene and 16S RNA gene for \textit{Wolbachia} detection. All individuals screened were found to be infected.
- \textit{Wolbachia} strains were characterized with MLST, \textit{wsp}-HVR and \textit{gltA}, \textit{groe1} and \textit{dnaA} genes. Two \textit{Wolbachia} strains (\textit{wAfraCast1} and \textit{wAfraCast2}) were detected with \textit{wsp}-HVR. Preliminary data suggest that there is no co-infection.

Genus: \textit{Bactrocera}

Participants: G. Tsiamis (Greece), Mahfuza Khan (Bangladesh)
Collaborator: Kostas Bourtzis (IAEA)

5 years plan

- Genotype the \textit{Wolbachia} strains (time permitting genotyping of \textit{Arsenophonus} strains)

Plans for the 2\textsuperscript{nd} 18 months period

- Detect the prevalence of \textit{Wolbachia}, \textit{Cardinium}, \textit{Rickettsia}, \textit{Spiroplasma} and \textit{Arsenophonus} strains in populations of the \textit{Bactrocera dorsalis} complex (\textit{B. dorsalis} sensu stricto, \textit{B. carambola}, \textit{B. papayae}, \textit{B. philippinensis} and \textit{B. invadens}) and in \textit{B. zonata}, \textit{B. cucurbitae}, \textit{B. tau}, \textit{B. scutellaris} \textit{B. nigrofemoralis} and \textit{B. minax}. Genotype the \textit{Wolbachia} strains (time permitting genotyping of \textit{Arsenophonus} strains)

Progress made since 1st RCM (18 months plan)

- Detection of \textit{Wolbachia} and \textit{Spiroplasma/Mycoplasma} strains in natural populations.
- Partial MLST characterization of the \textit{Wolbachia} strains.

Participants: Ramesh Hire, Ashok Hadapad (India)
Collaborators: K. Bourtzis (IAEA), G. Tsiamis (Greece)

5 years plan

- Collect \textit{B. cucurbitae} adults from different agro-climatic conditions and regions for screening \textit{Wolbachia} presence.
- Screening of \textit{B. cucurbitae} cultures collected from different agro-climatic conditions for \textit{Wolbachia} infection using MLST approach (\textit{wsp}, \textit{fisz}) and 16S rDNA specific primers.
- Genotype the \textit{Wolbachia} strains found.
Plans for the 2nd 18 months period

- Collection of *B. cucurbitae* adults from different geographical locations will be continued for screening of *Wolbachia* presence.

Progress made since 1st RCM

- Adults of natural populations of *B. cucurbitae* and other *Bactrocera* species were collected from different agro-climatic regions of India (10 states, 14 districts) during 2012 – 2014.
- Eight species of *Bactrocera* were collected from the Indian subcontinent.
- Samples of all collected populations of *B. cucurbitae* and that of other *Bactrocera* species were screened for *Wolbachia* infection.
- Phylogenetic analysis based on 16S rRNA gene suggested that *Wolbachia* present in Indian *B. cucurbitae* belongs to the *Wolbachia* supergroup A.

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 (IAEA), 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 (inter population) and individual level (intra population)
• **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

**Plans for the 2\textsuperscript{nd} 18 months period**

• Assess the molecular diversity of each *C. capitata* population sampled by identification of the barcode region of the COI.

• Import the Vienna 8 *Wolbachia* trans-infected line and establish a colony in Brazil.

• Establish laboratory populations from several localities from Brazil.

• Test the compatibility of crosses between the Vienna 8 *Wolbachia* trans-infected lines with natural populations of *C. capitata* in Brazil.

**Progress made since first RCM:**

- Natural populations of *C. capitata* were obtained from eight localities representing five different states in Brazil: i) Canavieiras (7 specimens), Jequié (20 specimens), Vitória da Conquista (20 specimens) and Wenceslau Guimarães (7 specimens), all from state of Bahia; ii) Campina Grande (20 specimens), state of Paraíba; iii) Petrolina (18 specimens), state of Pernambuco; iv) Pelotas (20 specimens), state of Rio Grande do Sul; v) Campinas (20 specimens), state of São Paulo;

- Samples have been subjected to DNA extraction and diagnostic PCR using the several primers sets for *Wolbachia* detection (wsp81F/wsp691r, wsp106F/406R, wspecF/wspecR, MLST alleles);

- No *Wolbachia* infection has been detected to be associated with all natural populations examined.

**Participant:** Nikos Papadopoulos (Greece)

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

**5 years 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.

• Study the interactions between *Wolbachia* infection and probiotic diets.

• Study the interaction between *Wolbachia* infection and exposure of males to orange oil.

• Explore possible impact of *Wolbachia* infection in the chemical ecology of medfly.

**Plans for the 2\textsuperscript{nd} 18 months period**

• Determine the interactions between *Wolbachia* infection and probiotic diets in both larvae and adults.

• Study the interaction between *Wolbachia* infection and exposure of males to orange oil.

• Explore possible impact of *Wolbachia* infection in the chemical ecology of medfly.
Progress made since 1st RCM

- The progress of sexual maturation of both males and females of the *Wolbachia*-infected and non-infected cohorts has been determined.
- The effect of *Wolbachia* infection on the mating behaviour of both males and females has been determined.
- The remating rates of females mated with *Wolbachia* infected males have been characterized for both *Wolbachia* infected and non-infected females. Data analysis is in progress.
- The mating competitiveness of *Wolbachia*-infected males against wild males to mate to wild females has been determined in small laboratory cages. Data analysis is in progress.

**Participant: Aydin Tuncbilek (Turkey)**
**Collaborators: Kostas Bourtzis (IAEA) 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

**Plans for the 2nd 18 months period**
- Collect wild *C. capitata* populations from different region of Turkey and over different seasons.
- Explore whether reproductive symbionts other than *Wolbachia* are present in *C. capitata* populations.

Progress made since 1st RCM

- *Ceratitis capitata* specimens were collected from different parts of Turkey
- PCR screening for reproductive endosymbionts was performed in all collected samples.
- Analyses of specimens with *wsp* and 16S rDNA genes for *Wolbachia* infection suggest that medfly is not naturally infected with *Wolbachia*.
- Preliminary results based on analyses of *C. capitata* specimens with 16S rDNA genes suggest the absence of *Cardinium*, *Arsenophus* and *Spiroplasma*. Additional analyses should be conducted to confirm the above findings.

**Genus Dacus**
- No plans

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

**5 years plan**
- Determine the demographic and behavioural 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*.

Plans for the 2nd 18 months period
• Determine *Wolbachia* infection status in larvae, pupae and different ages of adult *R. cerasi*.

Progress made since 1st RCM
• The demographic profile of three German populations that are differentially infected by multiple *Wolbachia* strains has been determined
• The genetic structure of Greek populations of *R. cerasi* has been defined
• The *Wolbachia* strains in Greek populations of *R. cerasi* has been genotyped.

3.2. Interactions of reproductive symbionts and other microorganisms

**Genus: Anastrepha**

**Participants:** Diego Segura (Argentina)
**Collaborators:** Claudia Conte, Silvia Lanzavecchia (Argentina), Kostas Bourtzis (IAEA)

5 years plan
• Establishment of *Wolbachia* free *A. fraterculus* strains
• Evaluate the role of *Wolbachia* on the interactions between *A. fraterculus* larvae and *Diachasmimorpha longicaudata*.

Plans for the 2nd 18 months period
• Evaluate the role of *Wolbachia* on the interaction between *A. fraterculus* larvae and *Diachasmimorpha longicaudata*.

Progress made since 1st RCM
• The process of removal of *Wolbachia* to obtain a *Wolbachia* free strain was initiated and is on-going.

**Genus: Bactrocera**

**Participant:** George Tsiamis (Greece), Mahfuza Khan (Bangladesh)
**Collaborator:** Kostas Bourtzis (IAEA).

5 years plan
• Characterization of the gut symbiotic bacteria using 16S rRNA pyrosequencing libraries in populations of the *Bactrocera dorsalis* complex (*B. dorsalis* sensu stricto, *B. carambolae*, *B. papayae*, *B. philippinensis* 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)
Plans for the 2nd 18 months
- Characterization of the gut symbiotic bacteria using 16S rRNA pyrosequencing libraries in populations of the *Bactrocera dorsalis* complex (*B. dorsalis* sensu stricto, *B. carambolae, B. papayae, B. philipinnensis* and *B. invadens*) and *B. zonata, B. cucurbitae* that are infected and non-infected with *Wolbachia*.

Progress made since 1st RCM
- No activities were planned for the 1st 18 months

Participants: Olivia Reynolds, Peter Crisp (Australia)
Collaborators: Markus Riegler (Australia), Toni Chapman (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 (IAEA), George Tsiamis (Greece)

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

Plans for the 2nd 18 months period
- Characterize the effects of irradiation on the microorganisms of *C. capitata*
  Identify the gut microbial communities of medfly by classical microbiological approach and molecular techniques

Progress made since 1st RCM
- No work activities had been planned for the 1st 18 months.

Genus: *Dacus*
- No plans
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)
Collaborators: Claudia Conte, Silvia Lanzavecchia (Argentina), Kostas Bourtzis (IAEA)

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*.

Plans for the 2nd 18 months
- Perform crosses between the two strains to assess fertility and sex ratio.

Progress made since 1st RCM
- Singly infected *A. fraterculus* lines have been established carrying either the *wAfra*Cast1 or the *wAfra*Cast2 *Wolbachia* strain.

**Genus Bactrocera**
Participants: Ramesh Hire, Ashok Hadapad (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: Olivia Reynolds
Collaborators: Markus Riegler (Australia), 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 (IAEA), Jair Virgilio (Brazil)

5 years plan

• Establish in the laboratory a colony of the *C. capitata* Vienna 8 GSS (artificially infected with *Wolbachia*).

• Perform under laboratory conditions mating compatibility assays between the *C. capitata* Vienna 8 GSS 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* Vienna 8 GSS to suppress selected populations from Brazil.

Plans for the 2nd 18 months

• Establish in the laboratory a colony of the *C. capitata* Vienna 8 GSS (artificially infected with *Wolbachia*).

Progress made since 1st RCM

• No activities were planned for this period.

**Participants:** Nikos Papadopoulos (Greece)

**Collaborators:** Kostas Bourtzis (IAEA), Carlos Caceres (IAEA), and 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 behavioural profile of artificially *Wolbachia* infected medfly lines over time to analyse the evolution of the symbiotic relationships
• Evaluate under semi field conditions an IIT approach using the two available *Wolbachia* infected lines

Plans for the 2\textsuperscript{nd} 18 months period
• Determine the population increase parameters of *Wolbachia* infected medfly lines.

Progress made since 1\textsuperscript{st} RCM
• The performance of the *Wolbachia* infected lines under small scale mass rearing has been qualified.
• The quality properties and performance of mass reared (under small scale mass rearing) *Wolbachia* infected lines has been determined
• The mating performance of the two available *Wolbachia* infected lines in the context of an IIT application has been evaluated under semi field conditions.

**Genus: Dacus**
• No plans

**Genus: Rhagoletis**
• No plans
Table 1. Status of different Fruit flies of economic importance with respect their infection with the endosinbiont *Wolbachia spp*

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Location</th>
<th>Wolbachia status</th>
<th>MLST Strain(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anastrepha</td>
<td>ludens</td>
<td>Mexico</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>striata</td>
<td>Mexico</td>
<td>Detected</td>
<td>wAstriB</td>
</tr>
<tr>
<td></td>
<td>obliqua</td>
<td>Mexico</td>
<td>Detected</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>serpentina</td>
<td>Mexico</td>
<td>Detected</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>fraterculus (Br.1 morphotype)</td>
<td>Argentina</td>
<td>Detected</td>
<td>wAfra Cast1, wAfra Cast2</td>
</tr>
<tr>
<td>Bactrocera</td>
<td>cucurbitae</td>
<td>India/Bangladesh</td>
<td>Detected</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>zonata</td>
<td>India/Bangladesh</td>
<td>Detected</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>tau</td>
<td>India/Bangladesh</td>
<td>Detected</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>dorsalis</td>
<td>India/Bangladesh</td>
<td>Detected</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>correcta</td>
<td>India/Bangladesh</td>
<td>Detected</td>
<td>*</td>
</tr>
<tr>
<td>Ceratitis</td>
<td>capitata</td>
<td>Brazil/Turkey</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>Rhagoletis</td>
<td>cerasi</td>
<td>Greece</td>
<td>Detected</td>
<td>wCer1, wCer2, wCer4, wCer5</td>
</tr>
</tbody>
</table>


LOGICAL FRAMEWORK

<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 components, mandates improvement of the cost-effectiveness of this environment-friendly sustainable approach.</td>
</tr>
<tr>
<td>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. For example as part of area-wide integrated, pest outbreak situations and prophylactic pest management programmes applied against such pests.</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</td>
</tr>
<tr>
<td><strong>Specific Objectives:</strong></td>
<td>1. To develop methods of using beneficial bacteria to replace costly ingredients in larval and</td>
<td>N/A</td>
<td>1. Microorganisms contribute to the nutrition and health of their insect hosts and can be used to</td>
</tr>
</tbody>
</table>
adult diets in insect mass rearing facilities and improve the quality of the mass-reared insects.

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:

| 1. Costs of mass-rearing of target fruit pests reduced by incorporating microorganisms in larval and adult diets. | 1. A protocol developed and validated under mass-rearing conditions. | 1. Mass rearing data and cost-benefit analysis. | 1. Facilities are eager to reduce mass-rearing costs. The protocol developed is effective and can help reduce these costs. |
| 2. A better understanding of the impact of radiation on symbiotic associations of mass-reared insects. | 2. Development of a monitoring protocol for the characterization of post-radiation symbiotic communities. | 2. Data collected upon the implementation of the monitoring protocol. | 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 | | | 3. Performance of sterile males is |
3. Improved sexual performance of sterile males based upon probiotic supplements to pre-release adult diet.

4. An additional control tool that is based on symbiotic associations and complementary to the SIT is available.

3. A protocol developed and validated under operational conditions.

4. An additional control tool designed and validated.

3. Data collected and cost-benefit analysis completed.

4. Data collected and feasibility analysis completed.

not optimal and can be improved. Symbiont-based probiotic supplements to pre-release adult diet can improve sterile male sexual performance.

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

**Outputs:**

1.a. Egg and larval microflora of pests (natural and mass-reared) characterized.

1.b. The ability of beneficial bacteria to reduce production costs of mass-reared larval and adult diets is explored.

1.a. Characterized in at least 4 target species.

1.b. Explored in the mass-reared larval diets of at least two target species.

1.a. Reports and peer reviewed publications.

1.b. Reports and peer reviewed publications.

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

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
<table>
<thead>
<tr>
<th>2. The effect of radiation on the symbiotic associations is determined.</th>
<th>2. Determined in at least four target mass-reared species.</th>
<th>2. Reports and peer reviewed publications.</th>
<th>deleterious microorganisms.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.a. Adult associated microflora of pests (natural and mass-reared) characterized.</td>
<td>3.a. Characterized in at least four target species.</td>
<td>3.a. Reports and peer reviewed publications.</td>
<td>2. Irradiation damages symbiotic associations in a dose-dependent manner.</td>
</tr>
<tr>
<td>3.b The impact of symbiont-based probiotic supplements to adult pre-release diet on sterile male sexual performance is determined.</td>
<td>3.b Determined for at least three target species.</td>
<td>3.b. Reports and peer reviewed publications.</td>
<td>3.a. The microbial assemblages of adult insects are species-specific and amenable to characterization by classical and molecular microbiological approaches.</td>
</tr>
<tr>
<td>4.a. Microorganisms that affect host reproduction are characterized.</td>
<td>4.a. Characterized in at least two target species.</td>
<td>4.a. Reports and peer reviewed publications.</td>
<td>3.b. Symbionts can be used as probiotic supplements in diets provided to sterile males before their release to improve performance.</td>
</tr>
<tr>
<td>4.b. The tripartite interactions of host-parasitoid-symbionts characterized.</td>
<td>4.b. Characterized in at least one target species.</td>
<td>4.b. Reports and peer reviewed publications.</td>
<td>4.a. Microorganisms that are potential reproductive manipulators are amenable to characterization by molecular microbiological approaches.</td>
</tr>
<tr>
<td>4.c. Population suppression by</td>
<td>4.c. Explored in at least one target species.</td>
<td>4.c. Reports and peer reviewed publications.</td>
<td>4.b. The tripartite interactions of host-parasitoid-symbionts affect the host fitness and are amenable to manipulation.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.c. Symbiont-based population control strategies can be</td>
</tr>
</tbody>
</table>
microorganisms that manipulate host reproduction is explored.

<table>
<thead>
<tr>
<th>Activities:</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Announce project amongst established entomologists and symbiologists researchers and fruit and vegetable pest control programmes and establish CRP.</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>3. Organize first RCM to plan, coordinate and review research activities (2nd quarter 2012).</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>4. Carry out R&amp;D and draft technical protocols.</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>5. Second RCM to present data and coordinate future research as required (early 2014).</td>
<td>5. 2nd RCM held in May 2014, Bangkok, Thailand.</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>6. In conjunction with second RCM, hold workshop on &quot;Novel tools for the characterization and trainees capable of implementing</td>
<td>6. Workshop held in May, 2014. Harmonized procedures and</td>
<td>6. Workshop report.</td>
<td>6. There is need for training; techniques and instructors are available.</td>
</tr>
<tr>
<td></td>
<td>developed against target pest species.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

80
bioinformatics analysis of insect symbiotic communities".

7. Continue R&D.

8. Review the CRP after its third year.

9. Third RCM to present data and coordinate future research as required (late 2015).

10. In conjunction with third RCM, hold workshop on "Incorporating bacteria to larval and pre-release adult diets" (Idea: Imaging of bacteria and insects; marking and tracking bacteria).

11. Continue R&D.

12. Hold final RCM to review data and synthesize results (early 2017).

13. Evaluate the CRP and submit evaluation report.

7. Research carried out by contract and agreement holders.

8. Mid-CRP review carried out.

9. 3rd RCM held in 2015.


11. Research carried out by contract and agreement holders.


13. CRP evaluation carried out.

14. CRP members submit papers summarizing activities.

7. Reports and publications.


9. Working material printed and distributed for 3rd RCM; Research published in scientific literature and disseminated to member states and scientific community.

10. Workshop report.

11. Reports and publications.

12. Final CRP report completed.

13. CRP evaluation report completed.

7. Renewal requests and continued funding of RCM’s and CRP.

8. Mid-CRP review by Agency committee is positive.

9. Mid-CRP review approved by IAEA committee. Research activities continue; progress satisfactory.

10. There is need for training; techniques, equipment and instructors are available.

11. Renewal requests and continued funding of RCM’s and CRP.

12. Research and dissemination activities concluded.

13. CRP evaluation by Agency committee is positive.

14. Peer-reviewed manuscripts
LIST OF REFERENCES


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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ANNEX 2 (Agenda)

SECOND FAO/IAEA RESEARCH COORDINATION MEETING ON
“Use of Symbiotic Bacteria to Reduce Mass-Rearing Costs and Increase Mating Success in Selected Fruit Pests in Support of SIT Application”

6-10 May 2014 Bangkok, Thailand

The Montien Riverside hotel

TUESDAY, 6 MAY 2014

08:30 – 08:45 Opening remarks and welcome Mr Prasong Prapaitakul (Director of the Bureau of Agri. Product Quality Development, Department of Agricultural Extension)

08:45 – 09:00 Watchreeporn Orankanok / Carlos Caceres: Welcome statement, administrative issues and goals of the meeting.

SESSION I - Chairperson: Olivia Reynolds

09:00 – 09:30 Peter Crisp - Australia: Progress Report on “The physiological importance of bacteria isolated from the gut of Dirioxa pornia (Tephritidae)”

09:30 – 10:00 Marysol Aceituno - Mexico: Progress of the role of the associated bacteria on the biology, rearing and behaviour of Anastrepha obliqua, A. serpentina and A. striata.

COFFEE BREAK

10:30 – 11:00 Patrizia Sacchetti - Italy: Evaluation of the effects of diets based on two bacteria species on the olive fly physiology (Bactrocera oleae).

11:00 – 11:30 Michael Ben-Yosef - Israel: Nutritional complementation by bacterial symbionts promotes adult fitness and larval development in the olive fly (Bactrocera oleae).

11:30 – 12:00 Erin Schuenzel - US: Assessment of larval survivorship from exposure to suspect pathogenic bacteria.

LUNCH

SESSION II - Chairperson: Erin Schuenzel

13:00 – 13:30 Olivia L. Reynolds - Australia: Probiotic diets to increase sterile Queensland fruit fly male performance.

13:30 – 14:00 Carlos Cáceres / Antonios Augustinos - IAEA: Manipulating the gut microbiota of the Mediterranean fruit fly, Ceratitis capitata (Diptera: Tephritidae): implications for SIT improvement.
14:00 – 14:30 **Malini Alleck - Mauritius:** Feeding of *Klebsiella oxytoca* to improve the quality of sterile fruit flies in an SIT programme.

14:30 – 15:00 **Changying Niu - China:** Pyro-sequencing reveals a shift in symbiotic bacteria populations across life stages of *Bactrocera dorsalis*.

**COFFEE BREAK**

15:30 – 16:00 **Dyna Melgar - Guatemala:** Comparative identification and evaluation of microorganisms associated to both sterile insect production and release facilities.

16:00 – 16:30 **Jaime Garcia de Oteyza - Spain:** Mass reared and wild medfly microflora isolation and identification.

16:30 – 17:00 **Fernando Consoli - Brazil:** Identifying symbiotic associations with fruit flies targeted for SIT application in Brazil.

**WEDNESDAY, 07 MAY 2014**

**SESSION III - Chairperson: Humberto Martinez**

08:30 – 09:00 **Carolina Yañez Prieto - Chile:** Characterization of bacterial communities in the gut of mass-reared Mediterranean fruit fly *Ceratitis capitata* (Wied.).

09:00 – 09:30 **Lida E. Pimper - Argentina:** Identification of bacterial community associated to *Anastrepha fraterculus* (Diptera: Tephritidae).

09:30 – 10:00 **Teresa Vera – Argentina:** Antibiotic treatment effect on male mating success in *Anastrepha fraterculus*.

**COFFEE BREAK**

10:30 – 11:00 **Mahfuza Khan - Bangladesh:** Mating success of melon fly, *Bactrocera cucurbitae* (Coq.) and the oriental fruit fly, *Bactrocera dorsalis* (Hendel) in support of SIT application in Bangladesh.

11:00 – 11:30 **George Tsiamis / Kostas Bourtzis – Greece / IAEA:** Unravelling tephritid-associated microbiota with 16S rRNA gene pyrosequencing approaches.

11:30 – 12:00 **Ashok Hadapad - India:** Isolation and characterization of associated bacterial endosymbionts from melon fly, *Bactrocera cucurbitae*.

**LUNCH**

**SESSION IV - Chairperson: George Tsiamis**

13:00 – 13:30 **George Tsiamis - Greece:** Characterization of SymBioKosmos of *Bactrocera dorsalis* complex of fruit flies.

13:30 – 14:00 **Ayidin Suzu Tuncbilek - Turkey:** *Wolbachia*-based strategies to increase sterile *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) quality in support of SIT.
14:00 – 14:30  **Nikos Papadopoulos - Greece**: Effects of *Wolbachia* infection on the biology and behaviour of the Mediterranean fruit fly, and the European cherry fruit fly

14:30 – 15:00  **Humberto Martinez Montoya - US**: Evaluation of *Wolbachia* wAstriB as potential cytoplasmic incompatibility agent in the Mexican fruit fly (*Anastrepha ludens)*.

**COFFEE BREAK**

15:00 – 15:30  **Carlos Cáceres / Georgios Kyritsis - IAEA**: *Ceratitis capitata*-*Wolbachia* symbiosis: the impact of the symbiont on host development and male mating competitiveness.

**SESSION V Presentations Discussion - Chairperson: Nikos Papadopoulos / C. Cáceres**

15:30 – 16:30  All participants

16:30 – 16:45  Working groups definition

16:45 – 17:00  Group leaders meeting

18:30  **GROUP DINNER**

**THURSDAY, 08 MAY 2014**

**SESSION VI: Review of the individual proposals (Chairperson: Carlos Cáceres and Group Leaders)**

08:30 – 10:00  Working Groups: Background situation analysis, baseline knowledge from the last RCM, progress made and review of research gaps that need to be addressed.

**COFFEE BREAK**

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

**LUNCH**

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

**COFFEE BREAK**

15:30 – 17:30  General Discussion: Review baseline knowledge at start of CRP, progress made and review of research gaps that need to be addressed.

**FRIDAY, 09 MAY 2014**

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.

SATURDAY, 10 MAY 2014

SESSION VII: RCM report (Chairperson: Carlos Caceres)

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 Comments on the http://www.sit-symbiosis.net, agreement on information exchange mechanisms, on location of 3rd RCM/workshop, and closure of the RCM.
# ANNEX 3 (Working groups)

<table>
<thead>
<tr>
<th>1. Larval diets and radiation effects</th>
<th>2. Probiotics (adult diets)</th>
<th>3. Wolbachia and novel control tools</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erin Schuenzel*</td>
<td>George Tsiamis*</td>
<td>Nikos Papadopoulos*</td>
</tr>
<tr>
<td>Marysol Aceituno</td>
<td>Peter Crisp</td>
<td>Humberto Martinez Montoya</td>
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<tr>
<td>Dina Melgar</td>
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<td>Ashok Hadapad</td>
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<td>Jaime García de Oteyza</td>
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<td>Fernando Consoli</td>
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<tr>
<td>Boaz Yuval</td>
<td>Mahfuza Khan</td>
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*Group leader
ANNEX 4 (Abstracts of presentations)

Probiotic diets to increase sterile Queensland fruit fly male performance

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¹Graham Centre for Agricultural Innovation (an alliance between New South Wales Department of Primary Industries and Charles Sturt University), ²NSW DPI and ³University of Western Sydney, Australia.

Recently, studies on insects have revealed seminal contributions of microorganisms to the nutrition, health and reproductive success of their hosts. The sterile insect technique (SIT) is a key approach to manage Australia’s most significant horticultural pest, Queensland fruit fly (Qfly), *Bactrocera tryoni* (Froggatt), in some of Australia’s most valuable horticultural production areas. There is evidence that during the mass-rearing and radiation processes, the native microflora of fruit flies is disrupted, with symbiosis directly affecting host fitness in the field.

Male sterile flies must be sexually competitive with their wild counterparts. Fitter sterile males could lead to a reduced sterile to wild ratio required to control a population, resulting in increased efficiency and effectiveness of a control program and overall reduced costs.

The main objective of this project is to identify the key intestinal microbial flora associated with *B. tryoni* and related species and to exploit these microorganisms to improve the performance and quality of sterile male flies through the development of an adult probiotic diet.

Initially, we aim to sample adult fly populations from various regions and vegetation types (orchards through to rainforest) every 3 months for a period of 12 months, to determine if variations in intestinal flora are evident in the field. Using molecular techniques (16S rDNA and next generation sequencing), the microorganisms within the gastrointestinal tract associated with *B. tryoni* and related species from field populations and both irradiated and non-irradiated *B. tryoni* from the Fruit Fly Production Facility, NSW DPI will be identified. The microbiome comparisons of these different fly populations will reveal important candidates for probiotic adult diets that will then be tested to increase fitness of released sterile flies.

A Cooperative Research Centre Plant Biosecurity PhD Scholarship candidate will be employed to work on this project. The candidate will be enrolled at the University of Western Sydney (UWS) and will work closely with a team at the Centre of Excellence for Plant and Animal Health, Elizabeth Macarthur Agricultural Institute (EMAI), NSW DPI and the Hawkesbury Institute for the Environment, UWS.
The physiological importance of bacteria isolated from the gut of Dirioxa pornia (Tephritidae)

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

South Australian Research and Development Institute

This first study was carried out as a starting point for identifying bacteria from the gut of adult D. pornia. The gut bacteria were identified by analysing the 16S rRNA gene utilising two molecular diagnostic methods. Firstly, the gut bacteria DNA was amplified by running a PCR (Polymerase Chain Reaction) using individual bacteria cultures, which was then sequenced. Secondly, a whole gut was sequenced without culturing individual bacteria which provided data for cultural and Candidatus species of bacteria and was carried out to ensure that all gut bacteria were identified. The identified bacteria, from both methods, belonged to three main bacterial groups (phylum), six classes and 23 families. The predominant bacteria, in most of the examined samples, belonged to two orders, Lactobacillales (Firmicutes: Bacilli) and Pseudomonadales (Gammaproteobacteria: Proteobacteria). Some of these bacteria contribute critically in mating preference, longevity, development and nutrition of fruit flies.

Secondly diet supplementation trials have been conducted with Enterobacteria isolated from the gut of D. pornia which improved reproduction success or suppressed next generation emergences when provided as a dietary supplement. These bacteria induced similar changes in next generation emergences in B. tryoni, either acting as a pro-biotic and increasing population growth in subsequent generations or as an entomopathogen as was observed for D. pornia in earlier experiment. The level of suppression of reproduction induced the flies feeding on the bacteria appeared to vary, and may be associated with the level of feeding occurring or the method of providing the supplements. Minimising this effect is required before any commercial application can be considered. This may be able to be achieved by elucidation of active compounds in the bacteria that induce the effect and developing a synthetic delivery system.

The increased reproduction associated with bacterium A may be associated with improved nitrogen assimilation (Behar et al. 2005) and improved nutritional status and competitiveness (Ben-Yousef et al. 2008; Niyazi et al. 2004). This improvement in reproduction will be further assessed for application in commercial facilities where of B tryoni are mass reared for sterile insect technique applications.
Pyrosequencing reveals a shift in symbiotic bacteria populations across life stages of *Bactrocera dorsalis*

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The oriental fruit fly, *Bactrocera dorsalis*, causes a huge menace to horticultural industry in China and around the world. The intricate relationship between insects and symbiotic bacteria provides a promising option that could be exploited for the control of these pests. In order to develop a comprehensive understanding of the bacteria structure associated with this fly, we carried out 454 pyrosequencing of 16S rRNA gene amplicons in different life stages. At ≤ 97% nucleotide similarity, the total reads (8212 -10895) could be assigned to 998 Operational Taxonomic Units (OTUs) thus a large diversity of bacteria is associated with this fly. Proteobacteria had the most abundant number of reads in immature stages while *Firmicutes* dominated in adult stage. Of the total dominant OTUs (*Vagococcus, Comamonas, Orbus,* and *Enterobacter*), only *Comamonas* were not present in all life stages, indicating a possibility of vertical transmission for *Vagococcus, Enterobacter and Orbus*. *Vagococcus* was the most abundant in all life stages except the pupa and third instar larva (BD3L). In contrast, *Comamonas* was abundant in the pupa but completely absent in the adults, indicating *Comamonas* are possibly transferred by relocalization or horizontal transmission. Interestingly, the adult and the immobile stages each had unique bacterial groups. Selection pressures exerted by the host insect could be the reason for the observed shifts in the population dynamics of symbiotic bacteria. The possibility of these shifts assisting the host to adapt to their lifestyle and environment cannot be ruled out. These findings increase our understanding of the intricate symbiotic relationship between the bacteria and the oriental fruit fly and provide clues to develop novel biological control techniques against the target fruit flies.
Evaluation of the effects of different diets based on two bacteria species on the Olive Fly physiology

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A research aimed at evaluating the effects of bacteria on the olive fly physiology was carried out using a *Bactrocera oleae* lab strain. Flies were fed on probiotic diets based on the bacteria *Pseudomonas putida* and *Acetobacter tropicalis*. Alive and dead bacteria have been supplied at two different concentrations in order to highlight possible probiotic effect of the microorganisms. Bioassays showed how living *P. putida* bacterium positively affected longevity, fecundity and fertility, moreover female survival was increased by the twofold concentration of the bacterium. Differently from alive bacteria, diet containing dead *P. putida* did not positively affect adult life span, indirectly proving that alive bacteria are involved in the host metabolism and may play an important role within the host gut. Diet containing dead or living *P. putida*, did not produce any remarkable effects on fecundity.

Flies fed on *A. tropicalis* showed a negative effect on olive fly survival compared to those fed on sugar. No differences have been found in flies fed with diets containing dead or alive *A. tropicalis*.

Samples of flies fed on different diets were dissected in order to ascertain the presence of supplied bacteria.

Further, preliminary data concerning bacterial transfer of *Candidatus Erwinia dacicola* from wild flies to lab flies are reported.
Nutritional complementation by bacterial symbionts promotes adult fitness and larval development in the olive fly (*Bactrocera oleae*).

Michael Ben-Yosef, Edouard Jurkevitch & Boaz Yuval

Department of Entomology, Hebrew University of Jerusalem

Many fruit flies (Tephritidae) associate with environmental, free living bacteria (*Enterobacteriaceae*) which densely inhabit the adult esophageal bulb and midgut lumen. Olive flies (*Bactrocera oleae*) are similarly associated with bacteria. However, adults and larvae host a non-cultivable bacterium (*Candidatus Erwinia dacicola*), which is considered as a co-evolved obligate symbiont of this fly. We postulated that these bacteria contribute to larval and adult nutritional ecology, by enabling the fly to overcome the limited nitrogen availability of its natural food substrates.

By suppressing bacteria in the gut and monitoring female fecundity we demonstrate that bacteria contribute essential amino acids and metabolize urea into an available nitrogen source for the fly, thus significantly elevating egg production. In an ecological context, bacteria were found to be beneficial to females subsisting on bird droppings, but not on honeydew – two natural food substrates.

Additionally, we show that larvae require their natural complement of bacteria to develop within unripe (green) olives. Nevertheless, in ripe (black) olives development occurs independently of the symbiotic microbiota. Our experiments suggest that bacteria counteract the inhibitory effects of oleuropein - an active component in green but not in black olives, which leads to a decrease the nutritive value of the fruit's protein.

We suggest that by means of their microbiota adult flies gain the ability to utilize diets which are low or imbalanced in assimilable nitrogen. At the larval stage bacteria seem to facilitate the utilization of protein in the green olive. This enables the olive fly to expand its host range from ripe, hospitable olives, to highly toxic ones. We assume that other Tephritids (Dacinae and Trypetinae) may similarly benefit from their gut bacteria.
Progress of the role of the associated bacteria on the biology, rearing and behaviour of *Anastrepha obliqua*, *A. serpentina* and *A. striata*

M. Aceituno Medina¹, A. Vázquez Gómez², I.S. Gómez², P. Montoya¹, P. Liedo³ J. Toledo³ & E. Hernández¹

¹Programa Moscafrut (SAGARPA-IICA), ²Centro de Biociencias, (UNACH), ³El Colegio de la Frontera Sur. Midgut morphology in the irradiated males of *Anastrepha ludens*.

The irradiation damage on the cells of the midgut in adult males of *Anastrepha ludens* (Loew) was described. The pupae were irradiated at 60 and 80 Gray and the control that was not irradiated. The irradiated and control flies were dissected when the adult reach the age of 1 and 7 day old. Fertile males of 1 and 7-days old showed and intestinal epithelium with degenerated possibly due to early aging and result of the intense activity by the cells. Irradiation caused cellular changes in the midgut of sterile males, the severity of these alterations in the cells depended on the dose, the age of the insect section and evaluated. Regarding the presence of bacteria in the midgut of these were found in lesser amounts on the first day old, fertile males had the gut bacterial load of irradiated males, meanwhile the intestines of male 7-days old contained bacteria mature established along midgut biofilms, bacteria are presented in smaller quantities in male fertile irradiated. Bacteria that formed bacterial biofilms were *bacilli, cocci, coccobacilli and diplococci*. The changes caused by irradiation were described and the images indicated that the midgut of male degenerates completely days after treatment with irradiation as in some species of insects, although bacteria found we cannot ensure the physiological state in which these are found, it could be that they are not fulfilling the functions have been attributed.

Survival and competitiveness of males of *Anastrepha obliqua* and *Anastrepha serpentina* fed with enriched food with bacteria.

Consistently, the sterile males of *A. obliqua* and *A. serpentina* during the competitiveness test showed lower values than the wild males. Previous experiments indicate that flies increase their sexual competitiveness when consume enriched foods with bacteria during the sexual maturation period. In this experiment, bacteria of flies of the same species were isolated and subsequently the effect of foods containing such bacteria on biological attributes *A. obliqua* and *A. serpentina* were tested. The bacteria isolated from the midgut of *A. obliqua* were *Providencia rettgeri*, *Serratia marcescens*, *Citrobacter freundii*, *Erwinia spp.*, *Staphylococcus aureus*, and *Enterobacter cloacae*. While, in *A. serpentina* the isolated species were *Serratia marcescens*, agglomerans *Pantoea*, *Providencia rettgeri*, *Staphylococcus aureus*, *Staphylococcus xylosus* and *Chryseobacterium indologenes*. These bacteria were added in Mb® food and sucrose concentration of 5x10⁴ and 5x10¹⁰ UFC/g. The results indicated that the survival under stress conditions of the males of *A. obliqua* increased from 3 to 7 days when fed with the mixture enriched with sucrose containing all the bacteria at a concentration of 5x10⁴ UFC/g. *A. serpentina* did not showed any significant effect. The male sexual competitiveness of *A. obliqua* and *A. serpentina* increased in a 21.1 and 19% when *P. rettgeri*
and *P. aglomerans* were added in Mb® food 21.1 and 19%, respectively, whereas in induction of the sterility not was observed any significant benefit in both species of fruit flies.
Evaluation of Wolbachia wAstriB as potential cytoplasmic incompatibility agent in the Mexican fruit fly (Anastrepha ludens)

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Current efforts to eradicate A. ludens include population surveillance, chemical and biological control using both parasitoid release, and Sterile Insect Technique (SIT), although the SIT has been successful in population control of fruit flies as A. ludens and other insects, it is expensive. More recently, a related control strategy, the Incompatible Insect Technique (IIT), is being explored as a viable alternative or addition to the SIT for control of Tephritid flies. IIT relies on the ability of certain Wolbachia strains (and other endosymbionts) to cause Cytoplasmic Incompatibility (CI). Accordingly, Wolbachia-infected males of the target pest species are mass produced and released into a target pest population that does not harbour Wolbachia, or that harbours a strain of Wolbachia that is incompatible with the one being released. Wild females that mate with such males will produce none or few viable offspring, depending on the strength of CI.

Wolbachia has been reported in several members of Anastrepha genus from Mexico Anastrepha striata, A. serpentina and A. obliqua. Nevertheless, Wolbachia has not been detected in the most economically harmful species A. ludens, opening the possibility of employing novel control strategies relying in Wolbachia-induced cytoplasmic incompatibility, as an addition or alternative to the current management strategies.

To date, 2531 wAstriB embryonic transfections have been performed in A. ludens. Of these, 65 larvae emerged from the eggs (i.e., 2.5% egg-larva survivorship), 17 individuals (i.e., 26%) reached third-instar and pupal stages (sex ratio 1:1). Eight transfected females were allowed to mate separately. PCR screening to confirm presence/absence of Wolbachia will be performed until each isoline is stable.
Comparative Identification and Evaluation of Microorganisms associated to both Sterile Insect Production and Release Facilities.

D. Melgar D., F. Jerónimo, P. Rendón.

Comisión, MOSCAMED, Guatemala

In order to determine which species of microorganisms are present inside fruit during the egg/larval development infested fruit samples were collected from the field. Wild insect larvae from fruit host plants were allowed to pupate. Wild adults emerging from these field samples were placed inside four sets (4 cages = replicates) of screened aluminum cages (24 x 27 x 27 cm). Two sets for *C. capitata* and two for *A. ludens*. Adults were caged in the presence of the following fruits, *C. capitata*: mango (*Mangifera indica* L.) and pear (*Pyrus spp.*), *A. ludens*: mango and Guava (*Psidium guajava*). In parallel, identification of larval microbiota was also conducted and later compared to wild insect infested fruit. The microorganisms have been identified using molecular biology techniques RT-PCR (16S rDNA), 1500 bp.

After their identification, colonization assays were carried out. Air, bubbling, stones were used to test both pathogenic and symbiotic bacteria identified in the mass rearing system. Using air stones could establish a practical and cheap methodology to introduce symbiotic bacteria in the mass rearing process. Air (bubbling) stones were used to keep in suspension the insect eggs prior to be transferred to the larval diet, the normal air piping system was replaced by autoclaveable tubes. The symbiotic bacteria used for the colonization of the egg suspension in the bubbling process was *Klebsiella oxytoca*, the following criteria were used for this decision: (a) sugar utilization profile (able to grow in the presence of different sugars from the larval diet), (b) Urease positive (able to grow in larvae excretion products, i.e. uric acid). Colonization and magnification tests for *Klebsiella oxytoca* were carried out with the methodology previously described, initial results showed that the addition of *Klebsiella oxytoca* (LOG10 UFC/mL 5.22 ± 0.80), other genera were not detected, for the control test the microbial composition showed, *Enterobacteriaceae* genera (LOG10 UFC/mL 4.94 ±0.87), other genera (LOG10 UFC/mL 5.34±0.90) and *Chromobacterium violaceum* (LOG10 UFC/mL 3.00, ±1.72).
Ceratitis capitata-Wolbachia Symbiosis: the Impact of the Symbiont on Host Development and Male Mating Competitiveness

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Background: Wolbachia pipientis is an obligatory intracellular maternally inherited alphaproteobacterium, perhaps the most widespread endosymbiont on Earth. This symbiont commonly induces Cytoplasmic Incompatibility (CI) in insect species. In Diptera, CI is expressed as embryonic lethality in crosses between infected males and uninfected or infected females with different bacterial strain. Wolbachia-infected lines of Ceratitis capitata have been established and express 100% CI and could potentially be useful to enhance SIT programs. However, these lines have not been assessed under standard quality control tests in order to evaluate the impact of the symbiont on host development and male mating competitiveness.

Methods: We compared five (5) Ceratitis capitata laboratory strains: (a) Vienna 8 genetic sexing line (GSS), (b) Benakeio, (c) Vienna 8 GSS carrying Wolbachia strain wCer2, (d) Benakeio 88.6 carrying Wolbachia strain wCer2 and (e) Benakeio S.10.3 carrying Wolbachia strain wCer4. These five strains were investigated for the effect of Wolbachia on host development and male mating competitiveness.

Results: Significant differences in egg-to-adult survival and male mating competitiveness were observed between the five Ceratitis capitata laboratory strains. Statistical analysis is in progress to evaluate whether these differences are due to host genetic background, symbiont strain or both.

Conclusion: This study provides important information about the effect a widespread symbiont, Wolbachia, may have on Mediterranean fruit fly biology and mating behaviour and could serve as a model for similar studies in other agricultural pests and disease vectors. This knowledge is a prerequisite to evaluating the potential of Wolbachia symbiosis and CI for application and the enhancement of SIT.
Manipulating the gut microbiota of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae): implications for SIT improvement

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The Mediterranean fruit fly is a pest of major economic importance worldwide. Moreover, it represents a model species in the Tephritidae family of agricultural pests, regarding the development of Genetic Sexing Strains (GSSs) and their application in programs incorporating the Sterile Insect Technique (SIT). In the last few years, a number of studies have focused on the characterization of the gut symbionts of the Mediterranean fruit fly, presenting evidence for their significance on host’s biology and fitness. In the present study we isolated gut bacterial species from the Vienna 8 GSS and assessed their impact on host life history traits.

Methods: Culture dependent methods and different growth media were used to isolate gut bacterial species from larvae and adults of the Vienna 8 GSS. Taxa identification was performed with 16S *rRNA* gene sequencing. Selected bacterial strains were used to evaluate their impact on the Vienna-8 strain fitness following standard Quality Control (QC) tests.

Results: A limited number of gut-associated bacterial species were isolated from the Vienna-8 GSS. Preliminary results of the QC tests clearly confirm the importance of this community to the host’s fitness.

Conclusions: The manipulation of the gut-associated microbiota can be very important, leading to laboratory mass reared strains with better performance, thus increasing SIT efficiency. Additional experimental work is required in order to evaluate the role of the different bacterial species and their impact during Mediterranean fruit fly development. This study could be a model for similar studies with other SIT-targeted species and will allow having a better understanding of insect-symbiont interactions.
Assessment of larval survivorship from exposure to suspect pathogenic bacteria

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In order to assess the effects of microorganisms on the survivorship of \textit{Anastrepha ludens}, over 100 bacteria and fungi were isolated from egg washes and abnormal larvae shipped from Guatemala to the United States. All organisms were identified by molecular classification, 16s rRNA for bacteria and ITS for fungi. In addition to identification, survivorship testing was started on microorganisms with priority given to those microorganisms found on dead and dying larvae. The typical survivorship test was as follows; varying concentrations of the microorganism of interest and controls were applied to male \textit{A. ludens} eggs. All concentrations were tested in triplicate. A pathogenic microorganism was identified as one with significantly lower survivorship to pupation, lower survivorship to adult, and lowered or no flight ability in surviving adults than the controls. A beneficial microorganism would have the opposite effect. Several bacteria that had a severe detrimental result were \textit{Morganella} sp., \textit{Providencia rettgeri} and \textit{Providencia alcalifaciens}. \textit{Morganella} sp. caused a near 99% mortality rate for larvae. Of those that make it to adulthood, less than 20% had any flight ability. Both \textit{Providencia} species reduced adult survivorship by more than 50%. All three bacteria pose major threats to mass rearing facilities and method development to control them is ongoing. As more pathogenic bacteria are identified, next generation whole genome sequencing will be done to create specific diagnostics or identification methods. Any beneficial organisms will also be sequenced to more accurately identify which strain has the most beneficial effect.
Mass reared and wild medfly microflora isolation and identification

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TRAGSA, Valencia, Spain

Quality and cost of mass rearing programmes are main factors for its sustainability. Larval diet cost reduction by yeast substitution and better performing sterile males are to objectives which could be achieved by using probiotics / endosymbionts.

In order to identify the microorganism to which could be of application on an SIT programme, field infested fruits, wild adults and insects obtained from the mass rearing process at Bioplanta (Caudete de las Fuentes) will be analized. Culture dependent, as well as culture independent approaches were used for microflora characterization.

Samples were processed and colonies isolated. DGGE analysis after PCR amplification of the colonies allowed a reduction on the samples sent to sequencencing.

In order to identify non culturable enterobacteriaes associated to wild and mass reared medfly, adult midgut and larvae gut were analysed by pyrosequencing using Roche 454 FLX.
Characterization of bacterial communities in the gut of mass-reared Mediterranean fruit fly
*Ceratitis capitata* (Wied.)

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The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most serious agricultural pests in the world, with infestation levels reaching 100% in some of its hosts. Because of its wide distribution over the world, it is ranked among economically important fruit fly species. Chile was declared a fruit-fly free country in 1995. Since then, the National Fruit Fly Programme has been able to prevent the introduction and establishment of fruit flies of economic importance. The Sterile Insect Technique (SIT) was the key to the successful eradication of Mediterranean fruit fly populations in Chile.

The aims of this study were 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 in Arica, Chile. We studied the diversity of microbial communities in eggs, larvae, pupae and adult Mediterranean fruit flies. Analyses were based on 16S rRNA sequences obtained from isolates and denaturing gradient gel electrophoresis (DGGE).

Our results showed that the structure of the bacterial populations changes during development. Members of the Enterobacteriaceae constitute the dominant populations in the gut of *C. capitata*. *Stenotrophomonas maltophilia* was found only in eggs and larvae, while *Morganella morganii* was present in all stages. *Enterobacter* sp., *Klebsiella* and *Raoultella* sp. were found in adult flies. Moreover, *Pseudomonas* sp. were identified in pupae. In addition, we carried on analysis of bacterial populations in irradiated pupae and sterile male flies. Results will be presented.
Isolation and characterization of associated bacterial endosymbionts from melon fly, 
*Bactrocera cucurbitae*

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The melon fly, *Bactrocera cucurbitae* (Coquillet) (Diptera: Tephritidae) is an economically important insect pest of cucurbits and other vegetable crops in India. Several microorganisms exist inside the insect body either intracellular or extracellular and such associations play an important role in fitness and reproduction of insects. We have collected melon fly samples from different agro-climatic regions of India. These samples were used for detection of *Wolbachia* infection, isolation and characterization of gut microbiota. *Wolbachia* association was studied in natural populations of *B. cucurbitae* using *Wolbachia* specific 16S rRNA gene sequencing. *Wolbachia* were detected in majority of melon fly samples analysed and prevalence ranged from 25 to 100%. Phylogenetic analysis based on 16S rRNA gene suggested that *Wolbachia* present in Indian *B. cucurbitae* belongs to the *Wolbachia* supergroup A. Multi locus sequence typing (MLST) was performed using *gatB*, *coxA*, *hcpA*, *fbpA* and *fisZ* gene specific primers. The midgut bacteria were isolated from the natural population of adult melon fly. Based on 16S rRNA sequence analysis, 26 isolates were distributed in 9 genera and 15 species of bacteria. The dominant species in the midgut of melon fly were *Enterobacter* sp (38%), *Klebsiella* sp (19%), *Citrobacter* sp (8%), *Bacillus* sp (11%), *Providencia* sp (8%) and 4% of *Micrococcus*, *Staphylococcus*, *Leclercia* and *Exiguobacterium* sp. These isolates were further characterised for growth pattern, effect of salt concentrations and antibiotic sensitivity. The melon fly cultures were established and mass produced pupa were used for studying the effect of gamma radiation (10-80 Gy) on gut microflora. *Enterobacter* and *Bacillus* sp. were predominantly isolated from lab reared *B. cucurbitae*. Further, the purified bacterial cultures recovered from before and after irradiation are being identified and characterized.
The peach fruit fly, *Bactrocera zonata*, and the melon fly, *Bactrocera cucurbitae*, are key pests of fruits and cucurbits, respectively in Mauritius. The integration of the sterile insect technique (SIT) as a sustainable and environment friendly technique against these pests is being attempted. However, for SIT to be successful, the mass-reared flies should be as competitive as the wild flies. Since mass-rearing and irradiation disrupt the native microflora in the fruit fly gut, a study was conducted to assess the effect of supplementing the adult diet of *B. cucurbitae* with live symbiotic bacteria on the quality of the sterile flies. Symbiotic bacterial communities were extracted from wild flies, identified and fed to lab-reared irradiated and non-irradiated fruit flies. The crop and part of the mid-gut were removed and streaked on to sterile plates of bacteriological media. Inoculated plates were incubated for 24 hours at 37 °C. Predominant colonies were sub-cultured to obtain pure cultures of gut bacteria. Selected isolates were identified using standard bacteriological techniques namely gram staining, oxidase test and analytical profile index. *Klebsiella oxytoca* obtained was cultured on nutrient agar and fed to non-irradiated and irradiated *B. cucurbitae* to assess its effect on the longevity of the flies and on fertility and fecundity. Results show that mortality of flies fed on *K. oxytoca* and adult diet was less as compared to those not fed on bacteria. Fertility and fecundity of flies were higher in flies fed on bacteria and adult diet. Hence, supplementing fruit fly artificial diets with endogenous symbiotic bacteria can improve the quality of mass-reared sterile flies thereby improving the efficiency of SIT.
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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Tephritidae is a large family that includes many fruit pests which are usually adopted for housing large quantities of bacteria in their digestive tract. Explorations on the bacterial community of the mid-gut of third instar larvae of the Melon fly, Bactrocera cucurbitae (Coq.) and the Oriental fruit fly, Bactrocera dorsalis (Hendel) using API kits revealed that Enterobacteriaceae constituted the dominant population of the bacterial community. In total, 11 genera and 12 species of bacteria were identified from both fly species. The common bacterial spp. were Enterobacter cloacae, Delftia acidovorans, Pseudomonas putida and Stenotrophomonas maltophilia. In addition, the gut bacterial community of the larvae of both B. cucurbitae and B. dorsalis was 50% similar irrespective of different rearing media. No pathogenic bacteria were identified from whole body extracts of third instar larvae from either species. Present findings showed inconsistency of bacterial community identified from the third instar larvae of B. cucurbitae and B. dorsalis from our previous experiments using conventional biochemical techniques. The effect of bacteria on different fitness parameters of B. cucurbitae and B. dorsalis was found to be diet-dependent. Adult fly’s gut bacteria spp., Erwinia herbicola and Serratia liquefaciens help to enhanced mating success in terms of early mating of B. cucurbitae and B. dorsalis when incorporated with sugar diet compared to those fed on only sugar diet. E. herbicola and S. liquefaciens enriched protein and sugar diets have no significant effect on the ovariole number of B. cucurbitae and B. dorsalis after 14 days of adult emergence. However, delayed ovariole development and egg production were observed between 24-33 days when fruit flies fed on E. herbicola and S. liquefaciens enriched sugar diets. On the contrary, no egg production was recorded for both the fly species fed on only sugar diet. The response of B. cucurbitae in terms of egg production to bacteria enriched sugar diets was greater than B. dorsalis. Mortality rate of B. cucurbitae and B. dorsalis fed on Proteus rettgerti, Klebsiella oxytoca, E. herbicola and S. liquefaciens enriched sugar diet was higher than bacteria enriched protein diets. No significant difference was observed between mean mortality of control and sterile B. dorsalis fed on different diet treatments under semi-field cage experiment. The prevalence of Walachia infections differed significantly among various populations of the Bactrocera complex of Bangladesh. Higher level of Wolbachia infections were detected for population of B. dorsalis, followed by B. zonata, B.cucurbitae, and B. tau. The presence of three new allelic profiles or Sequence Types (ST) in the Wolbachia strains was determined from the
Bactrocera populations examined so far. A total of 8 WSP HVR profiles were identified with all of them being new in the Wolbachia WSP database.
Identification of bacterial community associated to *Anastrepha fraterculus* (Diptera: Tephritidae).

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Bacterial symbionts were largely characterized in fruit flies from Tephritidae family. They have been associated to nutritional status (gut bacteria) and reproductive behaviour (*Wolbachia*). In other dipteran species, bacteria influence the ability to cope with the attack of parasitoids through an enhancement of their immune response.

The South American fruit fly, *Anastrepha fraterculus*, is a key fruit pest in America. Little is known about the composition and role of gut and gonads microflora. The main objective of the present project is to describe the gut bacteria in *A. fraterculus* and explore its importance to the reproductive success of sterile males. We also aim to understand the role of *Wolbachia* on *A. fraterculus* fitness, in particular in terms of resistance to natural enemies.

Two methods were used in the characterization of the bacterial diversity after DNA extraction from the gut of *A. fraterculus* adult individuals. The first one involved PCR amplification of 16S rDNA gene with subsequent cloning and sequencing. The second approach consisted in a direct-band-sequencing after DGGE of bacterial 16S rRNA gene fragments of two different regions (included in V3 and V6-V8). Using the first approach, sequencing of 20 clones led to the identification of at least 5 distinct bacterial types: *Klebsiella oxytoca*, *Citrobacter freundii*, *Providencia rettgeri*, *Burholderia* sp., Uncultured *α* proteobacterium. With the second approach, after sequencing 18 samples from DGGE gels we identified 7 species of gut bacteria: *Erwinia chrysanthemi*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Kluyvera* sp., *Serratia* sp., Uncultured Bacilli, Uncultured *gamma* proteobacterium. Both methods allowed the molecular identification of almost the same bacterial species, the vast majority belonging to the Gamaproteobacteria class, order Enterobacteriales, as was previously described for other fruit flies species.

With respect to bacteria associated to reproductive behaviour, we continued the genetic characterization of *Wolbachia* strains in a laboratory strain and wild populations of *A. fraterculus* using *wsp* and MLST databases. We identified two strains of *Wolbachia* (Afra_Cast_1 and Afra_Cast_2) in the *A. fraterculus* laboratory strain by *wsp* nucleotide variation. We analyzed three wild populations of this fruit fly and found mainly Afra_Cast_2 *Wolbachia* strain, with very low frequency Afra_Cast_1 in the field. Both *Wolbachia* strains presented in all populations analysed were identical in the allelic profile of 5 genes (gatB, coxA, hcpA, fbpA and ftsZ) used for MLST analysis. For a further characterization, we analyzed a portion of the nucleotide sequences of gltA, groEl and dnaA genes. Preliminary results showed no differences at nucleotide level between the two *Wolbachia* strains for these loci. In order to evaluate potential phenotypes associated with the infection and the incompatibility between *Wolbachia* strains, we initiated the curing process of Afra_Cast_2 strain.

The molecular identification of gut microflora and *Wolbachia* strains, and the elucidation of their biological effects on the host will be useful to understand the role of *A. fraterculus* immune response and the effect of bacterial infections in the physiology and behaviour of this pest applied to the improvement of *A. fraterculus* mass rearing
Insects have established sophisticated symbiotic associations with diverse microorganisms, mainly with bacteria, which have allowed them to thrive in various ecosystems and to become perhaps the most evolutionary successful animal group on the planet. These symbiotic associations affect many biological processes including nutrition, reproduction, mating behaviour, immunity, vectorial capacity, pest status and insecticide resistance. In the frame of this CRP, we aim to characterize the microbiota associated with major agricultural pests of the Tephritidae family and to harness it in support of sterile insect technique (SIT) applications.

As a first step, we employed 16S rRNA gene pyrosequencing approaches to unravel the bacterial communities associated with species and strains maintained at the Insect Pest Control Laboratory (IPCL) in Seibersdorf including: (a) the Mediterranean fruit fly Ceratitis capitata (Benakeio wild type strain and Vienna-8 genetic sexing strain); (b) the olive fly Bactrocera oleae (Greek strain); (c) the Mexfly Anastrepha ludens; (d) Anastrepha fraterculus (Argentinian strain from Tucuman); (e) Anastrepha fraterculus (Peruvian strain from Ica) and (f) Anastrepha grandis (Brazilian strain). The associated bacterial communities were characterized in respect to developmental stage, diet, sex and age.
The globally occurring Tephritidae family contains more than 4000 species, most of which are polyphagous. The *Bactrocera dorsalis* complex of the Tephritidae family (subfamily Dacinae) contains 75 described species, which are mainly endemic to Southeast Asia. Several species, however, have also been reported in Africa, Middle East, Australia, Papua New Guinea, Indian Ocean and South America. This complex, also known as the Oriental fruit fly complex, is considered one of the most damaging insect pests globally, being responsible for annual losses of billions of USD. 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. One of the aims of this study was to detect and genotype reproductive parasites in laboratory and natural populations of *B. dorsalis* sensu stricto, *B. papayae*, *B. philipinnensis*, *B. invadens*, and *B. carambolae*. Emphasis during the last year has been on *Wolbachia* and *Spiroplasma*. The study on the distribution of *Wolbachia* from laboratory and natural population of *B. dorsalis* sensu stricto, *B. papayae*, *B. philipinnensis*, *B. invadens*, and *B. carambolae* has been completed. In total 497 natural populations from India and Bangladesh have been screened for the presence of *Wolbachia* and *Spiroplasma*. The genotyping of the *Wolbachia* and *Spiroplasma* strains are still in progress. At the same time we will use amplicon pyrosequencing in order to characterize the symbiotic flora associated with the gastrointestinal tract of laboratory and natural populations of *B. dorsalis* sensu stricto, *B. papayae*, *B. philipinnensis*, *B. invadens*, and *B. carambolae*. In order to identify the optimum DNA extraction method and the best primer set we have isolated DNA using three different methods and used three different primer sets. Comparative results will be presented.
Mediterranean fruit fly (Medfly), Ceratitis capitata (Wiedemann) is a key pest of citrus and other crops in many countries throughout subtropical and tropical regions. It exists in all the coastal 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 these pests. Strategies for area wide integrated management of this invasive pest increasingly use the environmentally friendly approach of the sterile insect technique (SIT). Although SIT is widely used to control C. capitata, the sterilizing irradiation procedure affects the 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. Endosymbiotic bacteria (Wolbachia, Cardinium, Hamiltonella, Arshenophonus, Spiroplasma and Rickettsia) were searched for in Turkey to control medfly. Wolbachia, Hamiltonella and Rickettsia infections were not detected in the investigated locations. We obtained unexpected bands and sequences in İzmir population for Cardinium by using Cardinium specific Ch and Clo primers. We found that Klebsiella sp and Bacillus sp presence by using primers designed to amplify 16S rDNA from Arsenophonus (ArsF-ArsR2) and Spiroplasma (63F-TK55) and sequencing the bands. So these primers amplified the other bacteria.

Efforts were made to isolate and to identify the gut bacterial community of laboratory host reared C. capitata using cultural and morphological tests. The gut bacteria of C. capitata identified by test strips VITEK 2 were: Klebsiella oxytoca, Enterobacter cloacea, Citrobacter freundii and Providencia rettgeri.
Identifying symbiotic associations with fruit flies targeted for SIT application in Brazil.

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In this paper we will report on the association of *Wolbachia* with natural populations of *Ceratitis capitata* and on the culturable microbiota associated with the gut of the South American fruit fly *Anastrepha fraterculus*. In the first case, our interest is to investigate the potential of using the incompatible-insect technique to control *C. capitata* in Brazil, while the second case is target to assess the microbiota diversity to investigate their role in host nutrition and development. We will report the absence of Wolbachia infection in the natural populations of *C. capitata* that were investigates, and describe the culturable microbial community associated with the larval and adult gut of a lab-reared *A. fraterculus*. 
Effects of Wolbachia infection on the biology and behaviour of the Mediterranean fruit fly, and the European cherry fruit fly

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Wolbachia pipientis is a widespread endosymbiont of insects exerting a wide range of biological effects on its hosts, including Cytoplasmic Incompatibility (CI), which is commonly expressed as embryonic lethality in crosses between infected males and non-infected females or infected females with different Wolbachia strains. The recent infection of laboratory adapted Mediterranean fruit fly (medfly), Ceratitis capitata lines with Wolbachia strains, obtained from a naturally infected Rhagoletis cerasi population, paved the way for the development of an Incompatible Insect Technique (IIT) for the control of medfly. Wolbachia infection may affect various life history traits of both naturally infected insect population and trans-infected ones. Recently we have demonstrated that Wolbachia infection (a) shortens the egg-to-adult developmental duration of two C. capitata lines, (b) shortens the adult life span (to a different extent in males and females), and (c) reduces female fecundity. Interestingly, different Wolbachia strains differentially affect both immature and adult demographic traits. The current paper explores whether Wolbachia infection affects behavioural and reproductive traits of trans-infected medflies, and whether multiple, naturally occurring infections of three German R. cerasi populations are correlated with variation in adult demographic traits. The results of our experiments demonstrate that regarding medfly, Wolbachia infection (a) negatively affects both the progress of sexual maturity and the sexual signalling rates after attaining maturity, especially on males having access to protein rich diet (yeast hydrolyzate plus sugar), (b) does not affect female mating rates regardless of the infection status of the male sexual partner, (c) seems to increase the remating frequency of females depending on the infection status of the two male partners, (d) does not affect the female reproductive maturity regardless of the adult food, (e) increases spermatozoa storage in females having access to protein rich diet regardless of the infection status of the male partner. Interestingly, there is no effect of Wolbachia infection on spermatozoa storage of sugar-fed females. Regarding R. cerasi, the variability in adult demographic traits among the three geographically isolated, German populations that we found could be attributed to possible cost of multiple Wolbachia infections. The importance of our findings for the development of IIT projects against the Mediterranean fruit fly, and for understanding the complex interactions between Wolbachia and its hosts are discussed.
Antibiotic treatment effect on male mating success in *Anastrepha fraterculus*

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Fruit flies have symbiotic bacteria in their digestive tract and this can result in an increase of their reproductive success. Additionally, male mating success is conditioned by the nutritional quality of the diet. Yet, it is still not clear to what extent the bacteria can contribute towards counteracting nutritional deficiencies. Our objective was to evaluate the effect of adding antibiotics in different adult diets on the mating success in wild and laboratory-reared *A. fraterculus* males. Wild (W) and laboratory-reared (L) males were provided one of two different diets sugar (S) or sugar + protein (S+P), which in turn could have or not antibiotics (AB). This resulted in four treatments: 1) S; 2) S+AB; 3) S+P; 4) S+P+AB. To evaluate the mating competitiveness of the males from different treatments, one virgin wild female was released in a laboratory cage containing two males from the same origin as well as diet, but with different antibiotic treatments (i.e., W-S vs W-S+A or L-S+P vs L-S+P+AB). The number of copulations obtained by each type of male, copula duration and the time elapsed since the release of the females and the initiation of copula (latency) were recorded. Subsequently, the individuals were stored at -20°C to characterize the symbiotic flora by DGGE of 16S rRNA gene fragments. The diet in itself had an impact on male mating; males fed with S copulated less in relation to males fed with S+P, irrespective of having received antibiotics or not. Moreover, the addition of antibiotics resulted in a significantly lower number of matings while the diet and the origin of the males had no effect on this variable. The interaction between antibiotic treatment and male origin was close to significance (p = 0.065) and when the origins were included in the *post-hoc* comparisons, the effect of the antibiotic was significant only for laboratory males. Latency to mate depended on male origin; laboratory-reared males mated earlier than wild males with no effect of antibiotic treatment while for wild males the antibiotic treatment increased latency. Mating duration was affected by the diet for wild males but not for laboratory males; wild males fed with S have shorter copula than males fed with S+P. Samples collected from the flies treated with antibiotics have similar DGGE patterns, with dominant bacteria from the Alphaproteobacteria group and the genus *Stenotrophomonas* (Gamaproteobacteria), and no bacteria from the family Enterobacteriaceae. In addition, wild males fed with S+P showed the greatest diversity in the gut community. The three factors evaluated affected male sexual success in *A. fraterculus*. The addition of antibiotic to the adult diet decreased the number of copulations; the origin and the antibiotic treatment had an effect on latency and the diet and origin on copula duration. These results support the hypothesis of a symbiotic relationship between gut bacteria and their hosts, but yet more evaluations are needed to confirm such interaction.