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Chapter

Probiotics as a Beneficial Modulator of Gut Microbiota and Environmental Stress for Sustainable Mass-Reared *Ceratitis capitata*

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Abstract

The Mediterranean fruit fly *Ceratitis capitata* (medfly) is a major pest throughout the world and one of the most destructive. Several strategies for controlling this pest have been proposed, including the sterile insect technique (SIT). The SIT's effectiveness against the medfly is well documented. Sterile medflies, on the other hand, can perform poorly. Reduced mating compatibility and mating competitiveness in the field may be caused by genetic and symbiotic differences between natural and laboratory medfly populations. Probiotic gut symbionts have been shown to facilitate control strategies and improve male medfly fitness. They are equally effective in the live and inactivated forms when administered to medfly adults or larvae. They have been shown to modulate a large set of inducible effector molecules including antimicrobial peptides (AMP) and stress-responsive proteins. The selection procedures of probiotics for their use in the medfly rearing process are reviewed, and other pathways for selection are proposed based on recent in silico studies. This chapter summarizes the most relevant evidence from scientific literature regarding potential applications of probiotics in medfly as an innovative tool for biocontrol, while also shedding light on the spectrum of symbiotic relationships in medfly that may serve as a powerful symbiotic integrative control approach.

Keywords: *Ceratitis capitata*, probiotic, selection, *in vivo*, *in silico*, probiogenomics

1. Introduction

The development of insect farming is critical for achieving agricultural sustainability goals and dealing with rising food demand, ongoing natural resource depletion, and global climate change. Insects are now being mass-produced as entomophagous arthropods for pest management or for food and feed. During the 1950s and 1960s, the

field of insect mass-rearing began with the mass production and release of sterile males for autocidal control of flies such as the screwworm and later with natural enemies during the 1970s, 1980s, and 1990s. By far the sterile insect technique (SIT) is the technique that makes the most use of mass-rearing. Pests are reared in large numbers before being sterilized with ionizing radiation and released into the wild as a viable alternative to chemical pesticides. Male sterile insects compete with male wild insects of the target pest. Females inseminated with sterile sperm are not fertilized and will not give birth. The worldwide directory of SIT facilities (DIR-SIT) indicates that there are more than 142 facilities breeding mainly Diptera, Lepidoptera, and Coleoptera.

The innovation of mass-rearing necessitates the development of artificial diets, as well as a controlled environment with clear and reproducible procedures to achieve the best yields at the lowest costs. For the Mediterranean fruit fly *Ceratitis capitata* (medfly), which is a major key pest that attacks more than 400 hosts, standard rearing procedures were developed by the USDA, IAEA, and the FAO in the 2000s [1]. This document represents the recommendations, reached by consensus of an international group of quality control experts, on the standard procedures for product quality control (QC) that are used now for sterile mass-reared and released tephritid flies. Indeed, despite years of improving the various breeding and release procedures, laboratory sterile males tend to have reduced performance compared to their wild counterparts. Recently microbiome disturbance or dysbiosis has been increasingly recognized as a significant contributor to the poor performance of sterile medfly males, which play a key role in shaping health and fitness. The presence of minor communities such as *Pseudomonas aeruginosa* in the medfly gut at the expense of major communities such as Enterobacteriaceae would result in a decrease in host nutrients and energy metabolic activity in sterile medfly males [2, 3]. Both culture-dependent and culture-independent techniques were used to identify potential dysbiosis after domestication, irradiation, mass-rearing, and handling, highlighting the potential risks to host immunity, development, nutrition, and health. The dominant presence of the enterobacterial community in the medfly's gut contributes to the fly's nitrogen and carbon metabolism, development, and copulatory success [2, 4], as well as its host fitness by acting as a barrier against deleterious bacteria [2]. The dominant species in wild and laboratory medfly populations were identified as *Klebsiella oxytoca* and *Enterobacter agglomerans*, respectively [5].

Even though prevention is preferable to cure, the development of health-enhancing additives such as probiotics began in the 1950s–1980s [6]. Because of their prophylactic efficacy against bacterial infections of the gut and immunomodulating activity, there is agreement on the efficacy of supplementing probiotics to human health conditions [7], poultry [8], and, more recently, aquaculture [9].

With the development of mass-rearing, concern for insects' health increased. Probiotics are already sold to beekeepers to restore the gut microbiota of honey bees following antibiotic treatment. First, anaerobic gut bacteria obtained from bees were studied, along with strains from several additional sources [10]. The most popular probiotic strains for bees are *Lactobacillus* and *Bacillus*, two strains that are associated with honey bees and/or have been chosen from the bee environment [11]. Over the past decade, experimental supplementation of probiotics to the medfly diet has provided key insights. Probiotics stimulate production and modulate the immune system. To what extent are these probiotics thought to be a preventative measure for medfly mass-rearing? This chapter describes ongoing research in this field and attempts to analyze how probiotics might aid sterile medflies in fighting diseases, dealing with pesticides, and dealing with the effects of climate change.

2. What causes dysbiosis in the medfly gut microbiome?

Gut symbionts are claimed to positively influence the development and ecological fitness of tephritidae. It could be through the provision of essential nutrients such as amino acids, vitamins, nitrogen, and carbon compounds [12–15], the suppression of pathogen establishment [2, 16, 17], the enhancement of host resistance to pesticides [18], or the mediation of mate selection [19]. As a result, dysbiosis of the gut microbiota has recently emerged as the cause of the sterile medfly males' low fitness. Indeed, these males face a variety of constraints during mass-rearing, treatment with ionizing radiation, and release conditions that favor minor bacterial genera such as *Providencia* and *Pseudomonas*, which are considered potential pathogens for the fly [16, 20]. The reduced fitness of released sterile males usually means that they are less competitive [21–23].

3. Probiotics used in mass-reared *Ceratitis capitata*: biological and functional properties

3.1 Current status and application of the probiotics to medfly sterile males production system

The term "probiotic" is derived from the Greek words pro and bios, which mean "life" [24]. It was coined in 1965 by Lilly and Stillwell [25] to contrast the term "antibiotic". The definition of probiotic' has evolved. The Food and Agricultural Organization and World Health Organization (FAO/WHO) define probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [26]. Many species have been designated as "Generally Recognized As Safe" (GRAS) with the origin of the strain, antibiotic resistance, and lack of pathogenicity determining the safety of probiotic strains [27]. Different Gram-positive bacteria belonging to the genus *Lactobacillus*, *Enterococcus*, *Bacillus*, and *Bifidobacterium* have been studied extensively for their role as probiotics.

Pioneering studies on the experimental use of probiotics were initiated following the interesting findings of Ben Ami et al. [16], working on medfly, that regenerating the original microbiota community could result in enhanced competitiveness of the sterile flies. We should also mention that this study, which partially replicated the work of Niyazi et al., [28], shed light on the composition of the intestinal microbiota in sterile males.

As demonstrated by Ben Ami et al., [16], the addition of Streptomycin-resistant *K. oxytoca* strain to the post-irradiation adult diet allowed this probiotic to colonize the guts of *C. capitata* sterile males. Currently, the most common method of medfly administration is oral administration via diet [17, 29, 30]. Indeed, probiotics could be given to medfly at two stages: larval and adult. If the addition occurs during the larval stage, there is only one option: add the probiotics as a suspension, usually 10^7, 10^8, 10^9 CFU/g mixed with the diet (carrot or wheat bran). If the addition occurs during the adult stage, there are two options: the first is to incorporate it into the adult diet as a bacteria-containing diet (granular sugar and yeast mixture or agar) [28], and the second is to introduce it through a cotton pad soaked with the bacterial suspension [2, 13, 16, 29, 31–35]. If multiple strain preparation is of interest in aquaculture, single administration for insects in general and medfly, in particular, is the option. As shown in **Table 1**, most of the studies exploited the probiotic strains as live; however, other

Strain	Orig	Stage	Diet	Stat	Single/ multi	C_i	Inoc T	Contact duration	Pf col	Ref
Enterobacter agglomerans Klebtiella pneumoniae		A	Granular sugar-yeast 3:1 ratio	Live	Single	50%		Ad libitum	yes	[28]
			Granular sugar-yeast diet 6:1 ratio							
			Prerelease sucrose-agar diet							
			Sucrose-agar diet containing a small amount of yeast							
Pectobacterium cypripedi Citrobacter freundii Enterobacter spp. *Klebtiella oxytoca Pantoea* spp.	Wild caught flies	A	Bacterial suspension in 20% sucrose solution		Multi	10^8 CFU/ml	Daily until death			[2]
Klebtiella oxytoca SmKo	Wild caught flies	A		Live/ inactive		10^6 CFU/ml			Yes	[16]
Klebsiella oxytoca N8-S	Wild caught flies	A	Cotton wool soaked with bacterial culture	Live		10^9 CFU/ml	Daily	5 days	Yes	[34]
Enterobacetr spp. *Klebtiella pneumoniae Citrobacter freundii*	Other	L	Wheat bran diet	Live	Multi	5.6 µg/g	Daily	10 days	No	[13]
Enterobacter spp.	Vienna 8 GSS	L	Carrot diet	Live/ Inactive		$10^6, 10^7, 10^8$ CFU/g			No	[29]
Klebtiella oxytoca	Vienna 8 [D53+]	L	Carrot diet	Live/ Inactive		$10^6, 10^7, 10^8$ CFU/g				[30]

Strain	Orig	Stage	Diet	Stat	Single/ multi	C_i	Inoc T	Contact duration	Pf col	Ref
Enterobacter AA26 *Klebtiella oxytoca*	Wild caught flies Vienna 8 [D53+,]	A	Cotton pad soaked with 5 ml of bacterial suspension	Live	Single	10^8 bacteria/ ml	Daily	5–6 days		[30]
Enterobacter AA26		L	Carrot diet with full yeast replacement with *EAA26* biomass	Dry biomass		7%, 3.5% and 0%				[32]
			Carrot diet with partial yeast replacement with *EAA26* biomass							
Morganella morganii *Enterobacter* spp. *Klebtiella oxytoca* *Rahnella aquatilis* *Lactococcus lactis* *Pluralibacter gergoviae* *Enterobacter asburiae*	Wild caught flies	L	Wheat bran	Live	Single	10^9 CFU/g			No	[35]
Enterobacter spp.	Wild caught flies	L	Wheat bran	Live/ inactive		10^5, 10^7, 10^9 CFU/g			No	[31]

Abbreviations: Origin (Orig), Stat (Status), Ci (species concentration), Inoc t (inoculation times), Pf col. (Proof of colonization), Ref (references), A (Adult), L (larvae).

Table 1.
Summary of probiotics use in medfly SIT application.

Parameters	Adult						Larvae										
	E. agglomernas, K. pneumoniae	P. cypripedi, C. freundii, Enterobacter spp., K .oxytoca, Pantoea spp.	K. oxytoca N8-S	K. oxytoca SmKo	Enterobacter AA26	Enterobacter spp., C. freundii, K. pneumoniae	Enterobacter spp.			Enterobacter spp.	Enterobacter AA26	K. oxytoca	M. morganii	R. aquatilis	L. lactis	P. gergoviae	E. asburiae
References	[28]	[2]	[34]	[16]	[30]	[17]	[29]	[35]	[31] #	[30]	[32] *	[30] #			[35]		
Egg to pupae recovery							+				+	—					
Egg to adult recovery							—	+			+	—	+	+	+	+	+
Sex ratio							—	—			—	—					
Egg to pupae development time							+	+			+						
Egg to adult development time							+	—				+					
Larvae development time										+		+					
Pupa stage duration (♀)							+					—					
Pupa stage duration (♂)												+					
Fecundity											—						
Pupal weight						+		—	+	+	+		+	+	+	+	+
Emergence						+				+			+	+	+	+	+
Flight ability						+		—	+	+	—	+	+	+	+	+	+
Morphometric traits						+											
Pheromone calling	—																
Latency time				+													
Remating			+														

	Adult							Larvae						
	E. agglomernas / *K. pneumoniae*	*P. cypripedi* / *C. freundii* / *Enterobacter* spp. / *K. oxytoca* / *Pantoea* spp.	*K. oxytoca* N8-S	*K. oxytoca* SmKo	*Enterobacter* AA26	*Enterobacter* spp. / *C. freundii* / *K. pneumoniae*	*Enterobacter* spp.	*Enterobacter* AA26	*K. oxytoca*	*M. morganii*	*R. aquatilis*	*L. lactis*	*P. gergoviae*	*E. asburiae*
Longevity	+/−	+	—			—	+	—	—	+	+	+	+	+
Sexual competitiveness	+/−		+	—		—	+	—	—	+	+	+	+	+
Sperm transfer						+								

Abbreviations: (*) inactivated, (#) live and inactivated (+) positive effect, (−) no effect, (+/−) inconsistent findings between diet substrates, field and laboratory.

Table 2.
Summary of the affected parameters after probiotic supplementation to the reared sterile medfly males.

forms such as inactivated (autoclaved suspension) [16, 29–31] or biomass as a replacement for yeast in the diet can be used [32]. Until now, the use has been limited to non-spore-forming bacteria, with the exception of Hamden et al. 2013's work, which used *Citrobacter* sp. of non-host origin. Spores are chemically resistant forms that could be a good candidate as a probiotic, particularly in the medfly larvae diet, which contains acidulants and antimicrobials [33].

Furthermore, Hamden et al. [17] tested the administration of a probiotic mixture, and as previously stated, the strains were of non-host origin, which is one of the agreed-upon selection criteria for a good probiotic candidate. The intervals of administration were also variable across experiments, with adult diet supplementation being frequent [2, 28, 34], whereas larval diet administration is limited to diet preparation, except for Hamden et al. [17].

3.2 Ameliorative effects on medfly colonies productivity and biological quality of sterile males

The initial interest in probiotics for medfly was focused on their use to improve colony productivity and the biological quality of released sterile males, such as longevity, flight ability, and mating competitiveness; however, new areas have been found, such as their effect on stress tolerance, although this requires more scientific development. The following section discusses some functional properties of gut bacteria supplemented as probiotics in medfly feeding. **Table 2** provides an overview of the main results obtained in several studies. There have been several studies in which potential bacterial strains such as *K. oxytoca* and *Enterobacter* sp. have been used to improve the egg to the adult recovery of medfly colonies [29, 32, 35] as well as the biological quality of released sterile males in the laboratory and/or field cages [16, 17, 28, 31, 32, 34, 35]. These studies revealed that the incorporation of gut bacteria in larval or adult artificial diets can positively affect pupal weight [17, 31, 35], adult size [17], survival ability [2, 17, 28, 32, 35], flight ability [17, 30, 35], mating competitiveness [17, 28, 34, 35], and sperm transfer [17].

However, **Table 2** also demonstrates that inconsistencies between results for the same bacterial strain can be found for some parameters, including pupal weight and sexual competitiveness [28, 29, 35]. This might be explained by the methodological setup used in each study. Since experiments are conducted with different medfly strains, isolated bacterial taxa, feeding stages, and lab or field-based applications, the different effects of the bacteria additives on medfly fitness may be explained. Probiotic bacteria have the potential to establish themselves, modify the existing gut microbial community, and play a more discrete role in nutrition and development. Follow-up experiments regarding the localization/quantification of these bacteria after incorporation in larval or adult artificial diets in the medfly's gut during development can provide more insight into how probiotic diets work. More research could enhance mass-rearing even further by upscaling the experimental design, using more replicates and generations, and potentially combining these beneficial isolates (consortium) or testing new bacteria isolated either from the medfly or other insect species. In general, increased pupal and adult productivity, decreased developmental time of the immature stages, and improved fly longevity would result in increased production of insects in shorter periods. This would facilitate mass-rearing of this insect pest species for SIT applications as well as small-scale laboratory rearing required for research.

3.3 Colonization of the probiotics and host origin importance

An effective probiotic should be able to adhere to and colonize the mucus layer of the insect gut [36]. According to **Table 1**, some studies chose to supplement the probiotic daily [17], whereas others only did so once. The initial step in establishing a symbiotic relationship between a microorganism and its host is colonization. Since the ingested food moves from the oral to the anal opening, the digestive tract is exposed to the environment. The term "colonization" can therefore be used for a wide range of associations, ranging from the simple transition of environmental bacteria to the replication, proliferation, and persistence of specific symbionts in the insect gut [37, 38]. The research on *Drosophila* revealed that each strain had a different capacity to reside in the gut following initial colonization [39]. The first day after consuming probiotics, the gut's probiotic levels grew quickly. After ceasing the probiotics, their number in the *Drosophila* intestine dropped and remained at a low level [39]. On the contrary, Lee et al., [40] did not find any differences in the extent of colonization and proliferation in the *Drosophila* gut among the tested bacteria. Successful colonization of the probiotics was demonstrated for medfly by [16, 28, 34]. However, to confirm the presence of *E. agglomerans* and *K. pneumoniae* in the guts of the probiotically treated insects, Niyazi et al., [28] only stated that the later strains were retrieved from the treated males, whereas control flies were found to be largely free of these bacteria (90% of the cases) (**Table 1**). There was no information provided about the isolates' identification procedure. Similarly, Gavriel et al., [34] confirmed that they recovered probiotics (*K. oxytoca* N8-S stereptomycine-resistant strain) from enriched sterile flies even after more than 7 days with no bacteria replacement by comparing bacterial counts on an antibiotic (Sm) treated LB agar and LB agar without antibiotics. However, Ben Ami et al., [16] went further in their explanation of the colonization by comparing the total bacterial count (SmKo strain) from adult guts on chromogenic medium and LB medium containing antibiotics for five consecutive days for the enriched diet and two additional days with a diet devoid of bacteria. Colonization is a fairly complex phenomenon that would also depend on stochastic factors and preexisting populations. The latter reduces the chances of subsequent colonization as was suggested for irradiated males of *B. dorsalis* fed with *K. oxytoca* BD177 [3], thus increasing the stability of the highly-diverse guts [41]. The direct and indirect colonization resistance from the commensal gut microbiota will limit the long-term effect of the probiotic. Indeed, Akami et al., [42], working on *Bactrocera dorsalis*, discovered that axenic flies preferred probiotic diets over symbiotic flies, confirming colonization resistance due to resident microbiota. They hypothesize that the native probiotic isolates were able to recolonize their natural habitat in the axenic flies' guts and revive appetitive behaviors that had been slowed due to bacterial suppression.

The provenance of the strain studied, however, is something we want to highlight here since it is crucial. All of the aforementioned studies used the *Drosophila* model to examine the probiotic human strains. Isolating putative probiotics from the host or environment where the bacteria are intended to exert their beneficial effect, on the other hand, makes more sense. The origin of the host should be considered even if for human purposes this requirement was negated since some strains showed to be effective even if they were of not human origin [43]. Recently, a study used a mixture of non-native and native bacteria for honey bees [44], however, without any proof of persistence in bee guts.

3.4 Isolation and characterization strategies of probiotics for mass-reared *Ceratitis capitata*

The majority of probiotics have thus far been isolated from medfly using the classical methods. Culture-dependent approaches have been used and adjusted to isolate and identify most of the probiotics. In the culture-dependent approach, the culture is using solid media allowing growth of bacteria such as Luria Bertani (LB), tryptic soy agar (TSA) [28], or a chromogenic medium such as CHROMagar orientation [16]. However, the morphological characterization by itself is unresponsive because bacteria's morphological characteristics, such as their color and shape, are not always constant. Further accurate identification approaches have been used such as the 16SrRNA gene amplification and sequencing. To reassemble bacterial colonies in haplotypes while minimizing sequencing, Hamden et al., [35] used the universal primers S-D-Bact-1494-a-20 and L-D-Bact-0035-a-15 to perform DNA amplification of the 16S–23S rRNA internal transcribed spacers region (ITS-PCR) (**Table 3**). While Augustinos et al., [29] combined morphological examination of colonies and RFLP assays, Ben Ami et al., [16] chose amplified rDNA restriction analysis (ARDRA), both techniques are based on restriction enzymes that provide the same digestion pattern.

Probiotics	Isolation	Identification	Reference
Enterobacter agglomerans *Klebsiella oxytoca*	Tryptic soy agar	—	[28]
Pectobacterium cypripedi *Citrobacter freundii* *Enterobacter* spp. *Klebsiella oxytoca* *Pantoea* spp.	—	16S rRNA eubacterial GC-clamp 968F-1401	[2]
Klebsiella oxytoca SmKo	Antibiotic LB medium CHROMagar medium	16S rRNA eubacterial 63F-907R 784F-1401R	[16]
	LB medium	16S rRNA	[17]
Klebsiella oxytoca	LB medium	16S rRNA ubacterial 63F-907R 784F-1401R	[34]
		16S rRNA	[30]
Lactococcus lactis *Rahnella aquatilis* *Pluralibacter gergoviae Klebsiella oxytoca* *Enterobacter* spp. *Enterobacter asburiae*	LB medium	16S–23S rRNA S-D-Bact-1494-a-20 L-D-Bact-0035-a-1	[35]
Enterobacter spp.	LB medium	16S rRNA 27F/1492R	[29]

Table 3.
Isolation and selection approaches of probiotics for medfly mass-rearing.

4. Mechanism of action and selection process of probiotics

Probiotics' mechanisms of action are not fully understood [45]. These mechanisms have been reviewed for humans through *in vitro* and *in vivo* animal models such as *Drosophila* [46, 47]. The effects of probiotics on medfly were studied, but the mechanisms underlying this were not explored. In general, probiotics affect microorganisms through antimicrobial secretion, competitive adhesion to epithelium and mucosa, intestinal epithelial barrier reinforcement, and immune system regulatory impact [48].

The probiotics used in the initial studies were selected from the prevailing population. The effectiveness of the aforementioned probiotic was then confirmed using the quality control criteria, which can be referred to as *in vivo* analyses, that were used to rate the quality of sterile males [1]. None of the studies adopted the basic selection approaches developed for human or aquaculture. The recent study by Hamden et al., [35] was the first to select strains based on specific criteria established in accordance with probiotics selection criteria and SIT requirements. Stress tolerance (tolerance to irradiation), adhesion ability (hydrophobicity, autoaggregation and coaggregation assays (biofilm formation), and antipathogenic activity (Exopolysaccharides production (EPS)) at specific diet incubation temperatures were the minimum criteria for a probiotic strain prior to integration into medfly food for SIT application. It consists of a series of *in vitro* tests that allowed all of the isolated strains to be screened as a first step before being proven *in vivo*. **Table 1** also shows that *Enterobacter* AA26, isolated from the gut of the Vienna 8D53+ genetic sexing strain (GSS), is a promising probiotic for medfly. When this strain was added to the larval diet, it increased the strain's productivity. Azis et al., [49] thoroughly investigated this strain *in vitro* for its biokinetic properties and nutritional values. Indeed, as demonstrated by this strain, a probiotic can be chosen for its functional molecules' secretory abilities, which could provide amino acids, vitamins, and increased α- and β-glucosidase activities.

From a scientific standpoint, the selection criteria for medfly probiotics could be expanded to include immunostimulatory activity, anti-inflammatory activity, and safety assessment [50]. Combined "omics" approaches including genomics, proteomics, transcriptomics, and metabolomics analyses in a novel scientific discipline called "Probiogenomics" [51] could provide a better comprehension and new insights about the selection of the "best" probiotic strain (see Section 5).

5. In silico approaches for probiotics selection

The conventional approaches of validating and selecting new probiotics using *in vitro* and *in vivo* assays are still not yielding robust results. Indeed, the molecular mechanisms through which probiotic microorganisms benefit insect health are, in fact, largely unknown. Thus, in order to fully benefit from probiotics, methodological evolution is required to discover a new potential probiotic. The advancement of sequencing technologies and related bioinformatic techniques enables the development of predictive models tailored to insect rearing conditions for the rational selection of new probiotics. In this context, the complete genome sequencing data of potential probiotic candidates have enabled the development of new effective approaches that serve as the basis for "in silico" screening of metabolic capability prediction and microbial interactions that operate in a microbial

community following probiotic treatment [52, 53]. Furthermore, the reproducibility of metagenomics results can enter interpretative variations at many steps of the SIT protocol, including long-term mass-rearing conditions, pupae irradiation, insect diet variability, etc., all of which may map variations in *C. capitata* intestinal microbiota. Such data could be combined with bioinformatics tools to modulate microbial composition within insects on a personalized beneficial population basis. Currently, the taxonomic microbiome characterization as well as the relative abundance of each taxonomic level is increasingly being combined with metagenomics sequencing of 16S rRNA V3-V4 hypervariable regions data through various existing NGS platforms sequencing technologies (pyrosequencing (www.454. com); sequencing-by-synthesis (www.illumina.com); sequencing-by-ligation (www.solid.appliedbiosystems.com); semiconductor sequencing (www.lifetechnolog ies.com); and nanoball sequencing (www.genomics.cn)). As a result, the taxonomic classification of metagenomic sequencing data of intestinal microbiota as well as diversity studies after probiotic treatment can reveal the probiotic potential parameters of bacteria candidates such as viability after mass-rearing, persistence or transience post-irradiation, capacity for intestinal colonization in the host, and effect on gut community structure [54]. Moreover, the integration of metagenomic data in various software programs (e.g., Prodigal, PICRUST, etc.) and Web-based bioinformatic pipelines (e.g., MicFunPred, available at: http://micfunpred.microdm. net.in/ [55]; Microbiome Analyst, available at: https://www.microbiomeanalyst.ca [56]; Galaxy/Hutlab, available at: https://huttenhower.sph.harvard.edu/galaxy [57]) can be used as a metagenome genes prediction approach to identify the likely functions of the intestinal microbiota before and after probiotic treatment for interpretive variations. Various functional databases, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) level 1 to 3, Gene Ontology Resource (GO), Clusters of Orthologous Genes (COG), and Carbohydrate Active Enzymes (CAZY), can be used for the identification and functional analysis of genes related to metabolic pathways. For instance, using NGS and bioinformatics platforms to examine changes in the composition and metabolic processes of medfly intestinal microorganisms after probiotic supplementation in the diet of the larval and adult stages serves as a reference for further studies and application of probiotics for SIT improvement.

This approach can be associated to the novel scientific discipline known as "Probiogenomics", which is a combination of "omics" methods using genomics, transcriptomics, metabolomics, and proteomics assays, that has been successfully applied in human health and aquaculture [51–53]. The "omics" assays provide in-depth details of the molecular features related to physiology, functionality, and mechanisms of action of the microorganism [58]. Based on the available whole genome sequence (WGS), "Probiogenomics" approach can be used to gene prediction of probiotic metabolic function [59]. However, there are a number of stressors that the probiotics must deal with during insect mass-rearing, including the composition of the larval and adult diets, irradiation, etc., which can affect their viability and abundance in the insect's digestive system. Consequently, the functional prediction would not be sufficient. Such models can be used not only for discovery and prediction, but also for elucidating the mechanisms of action of potential probiotic microbes on insect health, as well as for accurately identifying probiotics in multistrain mixes and the presence of potential contaminants [60]. Nonetheless, none will replace the need for *in vivo* assessments, which remain the gold standard for probiotic efficacy in the SIT mass-rearing process (**Figure 1**).
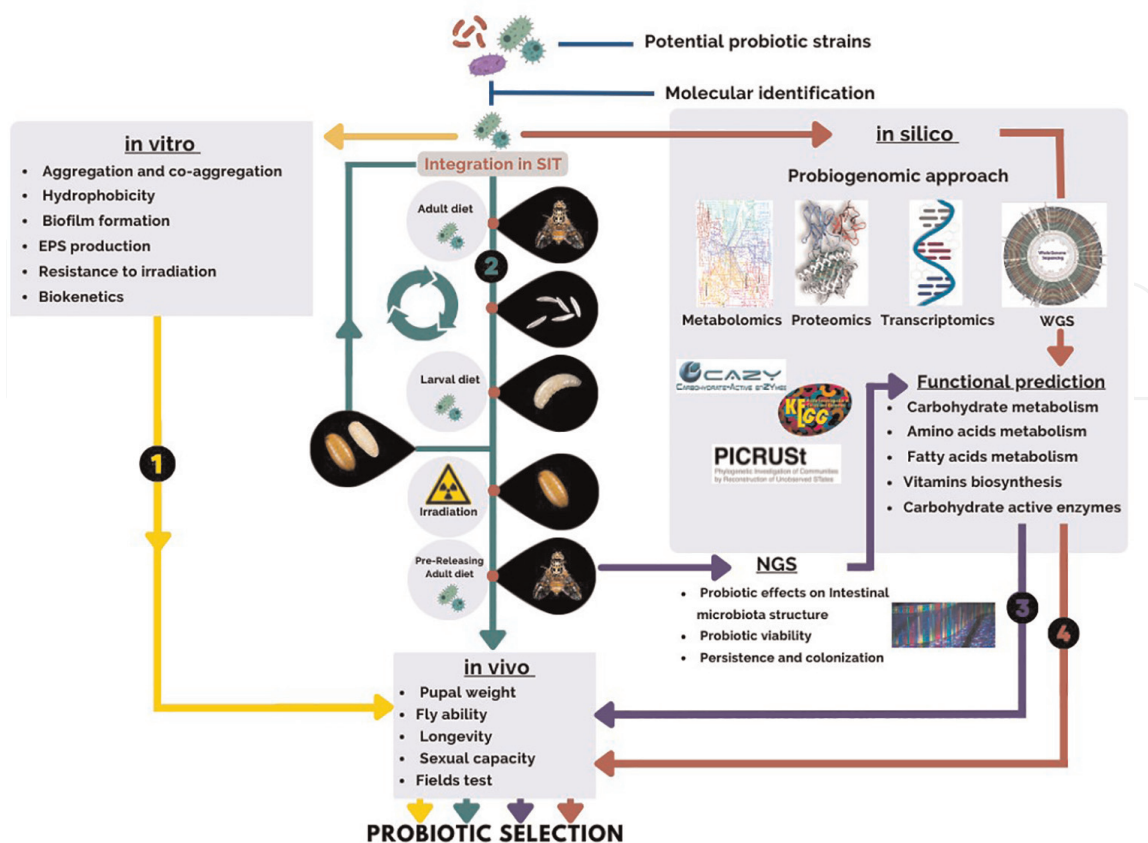
Figure 1.
Probiotics selection strategy for mass-reared Ceratitis capitata *for SIT application. Pathway1: Classical approach using "in vitro" and "in vivo" assays; Pathway2: Integration of potential probiotic strain into SIT procedures; Pathway3: Probiogenomic approach using different "omics" methods and functional prediction; Pathway4: Probiotic selection using metagenomics analysis and functional prediction of genes related to metabolic pathways.*

6. Probiotics' role in stress mitigation

6.1 Stress related to long-term mass-rearing and irradiation procedures

The biological quality of sterile males can be affected by a variety of significant stressors, including handling, artificial conditions for rearing, and radiation exposure. The ability of male medflies' to fly, attract females, compete for mates, and maintain longevity are all negatively impacted by sterilizing irradiation techniques used for SIT, which are also a significant source of microbiome perturbation [16, 61]. As a result, more focus has been placed on evaluating the impact of irradiation on the survival and mating abilities of the medfly sterile males in order to identify and pinpoint the primary drawbacks of these treatments. The changes in the diversity of the gut microbiota and the decline in the physical quality of sterile males are related. According to Ben-Ami et al. [16], industrial strains exhibit an increase in potentially pathogenic species like *Pseudomonas* and *Providencia*, which are known to harm insects, while levels of dominant gut bacteria (such as *Klebsiella* spp.) decrease after sterilization. It is interesting to note that adding *K. oxytoca* to the post-irradiation diet promotes colonization of these bacteria in the gut while lowering *Pseudomonas* spp. levels. The same authors, Ben Ami et al. [16], indicate that copulatory success tests show that the addition of these bacteria to male diets significantly improved sterile male performance. Similarly, a probiotic adult diet enriched with *E. agglomerans* and

K. pneumonia significantly improved the gut environment of medflies whose alimentary canal had been damaged by the radiation used in the sterilization process of medfly [61]. A more recent study on the effect of irradiation on medfly immunity discovered that molecular changes occur at different time points via regulation of stress and immunity genes such as *Hsp* 70, *Hsp* 83, *cecropin*, *attacin*, and *PGPR*. The expression of *attacin* and *PGPR-LC* was increased, whereas *cecropin* was decreased. *Hsp* genes, on the other hand, showed decreased levels between 0 and 18 h, peaking at 72 h. Only the *attacin* was induced after supplementation with the probiotic *Enterobacter* sp. [35].

6.2 Environmental stress

Along with the increase in agrochemicals, climate change and modifications in land use can all lead to unfavorable stress conditions for sterile males in agroecosystems. Sterile males are regularly exposed to unfavorable environments, including cold, heat, ultraviolet stress, lack of food resources, insecticide exposure, parasites, and infectious diseases or pathogens. Stress conditions can impair sterile males, physiology, biochemistry, and gene regulation, as well as the interaction between medfly and microorganisms, which lowers male performances. Given the range of beneficial functions provided by microbiota, it may also shape the ability of hosts to tolerate environmental stress [62]. Beneficial bacteria can help sterile males maintain their inherent resistance to these challenges; thus, adding these bacteria to the medfly diet can help reduce the negative impact of environmental stress conditions on sterile males. However, novel approaches are needed to explore medfly–bacteria and bacteria–bacteria interactions under abiotic and biotic stress conditions to identify potential stress-tolerant or -resistant bacteria to improve medfly performance.

6.2.1 Temperature tolerance

Among multiple stress factors, the temperature has profound effects on the physiology, behavior, and performance of insects [63]. There is evidence supporting that the ongoing climate change is expected to impose strong selection pressures on the heat tolerance of insects [64], and that gut microbiota can contribute to host thermal tolerance [65–67]. Alteration of energy reserves, metabolism, or gene expression by microbiota may indirectly affect thermal tolerance, which strongly depends on these traits [68]. Since the global surface annual temperature has increased at an average rate of 0.1°C, almost double compared to 20 years ago, and increases of 1.5°C and 2–4°C are expected by 2050 and 2100, respectively [69], rising temperatures can severely affect an AW-IPM program because temperature changes can influence the longevity, flight ability, and mating performance of sterile males. An elevated temperature could lead to the death of sterile males released during SIT [70]. Numerous studies have recently suggested that the gut microbiota is sensitive to environmental temperature, which induces changes in its composition and diversity, and may have significant consequences on host phenotype and fitness [71–73]. For instance, it has been shown that *K. michiganensis* was implicated in promoting insect resistance to long-term low-temperature stress in the tephritid fly *B. dorsalis*. The mechanisms by which gut symbionts modulate host physiologies and the molecules involved in these changes have been reported as follows: Gut symbionts, particularly *K. michiganensis*, help the host *B. dorsalis* upregulate the levels of "cryoprotectant" transcripts and metabolites,

which increases its resistance to long-term low-temperature stress by stimulating the host arginine and proline metabolism pathway [74]. It has also been noted in *Drosophila melanogaster*, the disruption of its gut microbiota leads to decreased cold tolerance [75] that can be rescued by supplementing a single member of its natural microbiota, the yeast *Lachancea kluyveri*. Similarly, increases in temperature have been associated with increased relative abundances of *Proteobacteria*. Developmental temperature has been shown to impact the composition of the gut microbiota of fruit flies, with higher temperatures (31°C) leading to increased abundances of *Acetobacter*, a genus of *Proteobacteria*, relative to lower temperatures (13°C) [76]. Additionally, in aphid, obligatory endosymbionts contribute to host performance at high temperatures [77, 78], whereas facultative endosymbionts also confer tolerance to high temperature in aphids [79, 80] and *Drosophila* [81]. Although *C. capitata's* acute tolerance of extreme temperatures, under ecologically relevant conditions, and the relative costs and benefits of acclimation have attracted significant attention [82–87], little is known about how microbial symbionts affect medfly sensitivity to toxins, desiccation resistance, and thermal tolerance.

Medflies are exposed to a variety of environmental stresses in the wild. The wild flies seem to be remarkably temperature-variation resistant [83, 84]. Even if this is true, it does not follow that laboratory sterile medfly males will be the same once released. The performance of released sterile males could be improved by enhancing their phenotypic characteristics with probiotic bacteria that confer thermal tolerance. This might be a simple and affordable way to improve the effectiveness of an SIT program. The role of the gut microbiota in the adaptive response to climate change is a new area of study, and future research must balance mechanistic approaches to understand host-microbiota interactions with holistic approaches to understanding the role of the gut microbiota in insect ecology and evolution.

6.2.2 Pesticides tolerance

The management of *C. capitata* is currently based on the implementation of an integrated pest management (IPM) program that employs a variety of techniques, including insecticides [88, 89], mass trapping [90], the sterile insect technique [91, 92], and also biological control using parasitoids [93]. However, the area under IPM includes a large number of cultivated plant species that are attacked by other pests [94]. Pesticides are usually used when these pests exceed their economic thresholds. The compatibility of the existing programs will be determined by the interaction between SIT and other pest management strategies when SIT is used [95]. The impact of pesticides and their residues on sterile Vienna-8 males has been investigated in citrus-integrated pest management. San Andrés et al., [96] observed high mortality of sterile Vienna-8 males on proteinaceous malathion and spinosad baits under laboratory conditions. Additionally, Juan-Blasco et al., [97] showed that both chlorpyrifos and spinosad formulations at authorized concentrations against other citrus pests were toxic by contact with Vienna-8 males, resulting in significant mortality. Pesticides have deleterious effects on Vienna-8 males. Thus, a solution is needed to limit these off-target effects. Naturally, reducing pesticide use would expose Vienna-8 males to fewer pesticides, but this solution may reduce crop yield and burden the food supply. The use of alternative, non-chemical control methods, particularly against serious pests, is another suggestion. However, these approaches are subject to the legislative process and competing interests and do not give growers the ability to address the pesticide issue on their own.

According to recent findings, the insect-associated microbial community, that is exposed to pesticides, as a source of selection pressure, may help the host metabolize these substances by enhancing enzyme activity through a wide range of metabolic pathways able to break down and/or modify xenobiotics [98–100]. It might also act as a source of variation, which would make the host less vulnerable to pesticides [101]. In some model organisms, it has been demonstrated that administering bacteria as probiotics lowers toxicity and has protective effects on the host. Future studies can use this foundation to explore the possibility of enhancing SIT to control medfly [102–104]. It might be a novel idea to include probiotics in the diet of sterile medfly males to lessen the effects of pesticides. Recently, some authors have drawn attention to the capacity of bacteria, such as lactic acid bacteria, to be developed into probiotic products capable of reducing the oxidative damage brought on by pesticides *in vivo* [105, 106]. These authors also emphasized how bacterial strains differ in their resistance to organophosphorus pesticides and their capacity to degrade them [107].

Pesticide-degrading bacteria are common in nature and have been found in a variety of insect orders, including Lepidoptera [108, 109], Hemiptera [110], Diptera [18, 111], and Coleoptera [101]. The surface communities of the Tephritid fruit fly *Rhagoletis pomonella* contained the first bacteria with this characteristic to be identified [112] (**Table 4**). It has been demonstrated that this bacterial symbiont degrades up to six different insecticides from three major groups (chlorinated hydrocarbons, organophosphates, and carbamates). Since then, evidence has shown that various other bacterial microbiota, such as those in the guts of herbivores, are capable of degrading insecticides [113]. For instance, it was found that in *Bactrocera tau*, bacteria were involved in the degradation of the toxic substances the host insect ingested, leading to insecticide resistance [111]. *Bactrocera dorsalis*, an oriental fruit fly, detoxifies trichloroethylene as another fascinating example of symbiont-mediated detoxification in Tephritid fruit flies [18]. The findings of this study showed that a bacterium

Pesticides families	Pesticides name	Gut microbiota	Tephritidae pests	References
Carbamate	Carbaryl	*Pseudomonas melophthora*	*Rhagoletis pomonella*	[12]
Organochloride	Dieldrin	*Pseudomonas melophthora*	*Rhagoletis pomonella*	[12]
	Endosulfan	*Klebsiella oxytoca, Pantoea agglomerans, and Staphylococcus* sp.	*Bactrocera tau*	[111]
Organophosphate	Dichlorovos, Diazinon, Parathion, Diisopropyl phosphorofluoridate	*Pseudomonas melophthora*	*Rhagoletis pomonella*	[12]
	Malathion	*Klebsiella oxytoca, Pantoea agglomerans, and Staphylococcus* sp	*Bactrocera tau*	[111]
	Trichlorphon	*Citrobacter freundii*	*Bactrocera dorsalis*	[17]
Neonicotinoid	*Imidacloprid*	*Pantoea agglomerans, Staphylococcus* sp	*Bactrocera tau*	[111]

Table 4.
List of tephritidae gut microbiota involved in pesticide degradation.

called *Citrobacter freundii*, isolated from the gut of the *B. dorsalis*, can break down the toxin trichlorphon into less toxic compounds called chloral hydrate and dimethyl phosphite, possibly by activating genes called organophosphorus hydrolase (OPH-like) genes and conferring host resistance in the oriental fruit fly [18]. Higher tri-chlorphon resistance was seen when isolated *Citrobacter* species were inoculated with *B. dorsalis*, whereas flies treated with antibiotics exhibited lower resistance. Based on this evidence, it is possible to reduce pesticide uptake and increase pathogen resistance by supplementing the diet of larval and adult sterile medfly males with suitable bacteria that degrade insecticide (multiple strains or single strain). This would reduce the sublethal effects of pesticides. The ability to supplement sterile medfly males with probiotics could aid the insects in combating the unintended pernicious effects and improving the SIT application while chemical agents are still being used in agriculture.

7. Safety and efficacy of probiotics

7.1 Safety considerations

Probiotics formulated for use in mass-rearing facilities have been shown to be beneficial due to their ability to improve a multitude of parameters and contribute to the restoration of dysbiosis in the medfly digestive tract. The probiotics selected so far are exclusively from the family of Enterobacteriaceae, and they are the cause of enteric human diseases that can lead to illness and death [114]. The use of Enterobacteriaceae in medfly mass-rearing procedures is still under experimentation; researchers have not yet addressed the issue of handler safety and environmental risk in general. The use of the probiotic in the larval rearing medium at the rearing facility and the administration of the probiotic to the adult sterile males intended for release are the two processes to be considered for safety issues. In the first case, it has long been recognized that facility workers can become infected by the agents they manipulate, thus making the nature of their work an occupational hazard. In the second case, introducing pathogenic bacteria into the adult diet allows bacteria to be transmitted horizontally to the environment. Implementing biosecurity procedures in rearing units, such as daily decontamination of all surfaces and equipment with specific disinfectants and limiting ventilation inside production modules, is difficult and will incur additional costs. However, it is clear that an increasing number of experiments are based on the use of the inactivated form of the probiotic, which is prebiotic, which appears to be less complicated to handle and yields comparable results [29, 31, 35].

7.2 Microencapsulation of probiotics for medfly mass-rearing

Acidulants are present in the mass-rearing medfly larval diet and play an important role in preventing microorganism growth, buffering diets, decreasing diet rancidity, and modifying the viscosity and consistency of the diet [115]. The pH of the larval diet is adjusted to 3.5–4.5 in insectaries. Acid stress inhibits bacterial proliferation and changes the phenotypes and morphology of bacterial cells in the medfly diet as a result [116, 117]. This is not in the probiotic's favor because it will be subjected to pre-ingestion stress, reducing its stability and effectiveness. Encapsulation will stabilize the probiotics during processing, storage, and the site of action to safeguard them in the medfly diets. Given that edible polymers can be used as coating materials to

provide a protective environment for the long-term viability of microorganisms, encapsulation is a successful food industry technique [118]. The polymer systems used to encapsulate probiotics are alginate, carrageenan, gelatin, chitosan, cellulose acetate phthalate, locust bean gum, modified starch, chitosan, gellan, xanthan, gum arabic, and animal proteins [119].

Probiotic encapsulation in mass-rearing is a new and unexplored area. Remarkably, some research has suggested that entomopathogenic bacteria be microencapsulated for pest control. Due to its low residual activity in the field, the most notable example is the microencapsulation of *Bacillus thuringiensis* (B.t.) with arabic gum, gelatin, and chitosan against some Coleoptera, Lepidoptera, and Hemiptera at larval and adult stages. Laboratory tests on *Trichoplusia ni* larvae (Lepidoptera: Noctuidae) revealed that the microencapsulation process had no effect on B. t. bioactivity. After 12 days, the mean number of larvae in microencapsulated formulations in colloidosomal microparticles (50 mm) was significantly lower than in a commercial B. t. formulation, and the effect of microencapsulated formulations was comparable to that of a chemical pesticide (lambda-cyhalothrin) [120]. The spray dryer produced a particle size of 32 nm against *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae damaging cotton, and the results show that even low doses of this encapsulation significantly reduced the larval population [121]. These and other experiments show promise for the use of microencapsulation to ensure the stability of probiotics throughout the medfly rearing process while paying attention to functionality, which is impaired in some experiments [122].

8. Waste conversion in mass-rearing facilities

The most common insect for which the sterile insect technique has been used is *Ceratitis*. Following that, a large number of mass-rearing facilities were established around the world. Mexico and Guatemala have facilities that rear over 1.5 billion medflies per week. The most important factor in mass-rearing is diet. Each mass-rearing facility generates a large amount of waste on a daily basis, the majority of which comes from the remaining rearing diet that does not respond to increasing requirements for economic efficiency and environmental standards [123], combined with global warming. At the El Piño biofactory in Guatemala, 31 tons of larval diet per day are produced [124]. Waste recycling initiatives are not published even if they exist. It is obvious that this waste is autoclaved before being used in order to eliminate any stage of the pest. Mastrangelo et al., (2009) [124] stated after conducting analyses on medfly diet that it has the potential as an alternative ruminant feedstuff. Likewise, Sayed et al., [125] showed that this diet is a potential feed ingredient for the production of BSF pre-pupae and could be applied to valorize this rearing waste into high-value feed.

The conversion of waste, such as agricultural by-products and food preparation wastes, into novel animal feeds, has received a lot of attention. The addition of exogenous probiotics is a promising strategy that enhances the biotransformation of food wastes [126], water treatment [127], and compost production [128]. The probiotics were shown to exert a positive effect through the extracellular enzyme secretions to break down carbohydrates, proteins, and fats into micronutrients in the waste that is transformed into feed [126]. Consequently, the probiotics added to the medfly larvae diet in the rearing facilities could improve the degradation of the diet and its use as feed for livestock after the larvae have left the medium. Probiotics may

also reduce antinutritional compounds and lignocellulose from the finisher diet bran, which is used as a substrate [129], and inhibit endogenous pathogens [130]. Therefore, WHO specifies that converted products for the animal feed chain should not be degraded or contaminated while maintaining an acceptable nutritional value [131].

9. Conclusion

The introduction of probiotics into the insect industry and their mass-rearing could be game changers. Insect farming is useful for biocontrol, such as the sterile insect technique, but it is also useful for edible insects. Probiotics used in mass-rearing can provide enormous benefits by increasing production quality and quantity. However, when using them, certain security aspects must be considered. We believe that the proposed schemes for probiotic selection in medfly rearing are well suited to all insects mass-reared for SIT application and can be adapted for other types of rearing and modified according to the specificity of the insect in question. However, the global approach incorporating new OMICs techniques is applicable to all types of insect farming and can provide answers to all of the interactions that the selected probiotic will have with the host microbiota.

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Conflict of interest

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