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Symbiotic chimeras: novel immunological properties of *Asaia* symbionts engineered to be covered by the *Wolbachia* surface protein

Ph.D. Thesis

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ABSTRACT

Vector-borne diseases represent one-sixth of all infectious diseases and cause more than 1,000,000 deaths every year. The World Health Organization (WHO) proposes the adoption of sustainable integrated vector management, which includes strategies such as environmental remediation, information and education of the population, but also the integration of classical chemicals (e.g. insecticides) with non-chemical control methods for the control of arthropod vectors. Among these strategies, the symbiotic control (SC), which exploits microorganism symbionts of the insects, is getting more and more attention as it is particularly promising for the control of vector-borne diseases.

Particularly, in this thesis, I studied the potentiality of bacteria belonging to two genera: *Asaia* and *Wolbachia*. The former includes acetic acid bacteria, symbionts of many insects such as mosquitoes and recently proposed as good candidates for paratransgenesis-based control strategies. The latter includes symbionts of arthropods and nematodes, with the capability to stimulate innate immune responses in mosquitoes with a reduction of their vectorial capability. We thus engineered the bacterium *Asaia* to express the *Wolbachia* surface protein (WSP), a potential inductor of innate immunity, derived from *Wolbachia* infecting the nematode *Dirofilaria immitis* (*Asaia*^{WSP}).

My project has two main aims:

i) to test the capability of the bacterium *Asaia*^{WSP} to stimulate the immune system of mosquitoes and thus to interfere with *D. immitis* development after infection;

ii) to verify if *Asaia*^{WSP} is able to induce a macrophage polarization during the immune response towards the M1/Th1 phenotype and if this polarization could determine an anti-*Leishmania* effect.

The obtained results to achieve these aims have been summarized in two articles:

Article 1

The first study, here presented, consolidates the previous evidence on the immune-stimulating property of WSP. In fact, WSP from *Wolbachia* of *D. immitis* was shown to stimulate the immune response in mosquitoes and mammals (humans, rodents, dogs) *in vitro*, but here for the first time an engineered bacterium expressing WSP was tested. *Asaia*^{WSP} activated the expression of immune genes coding for effector molecules in *Ae. aegypti* and *An. stephensi* mosquitoes. Once verified the stimulation of the immune system of the mosquitoes, the capability to inhibit the development of the nematode *D. immitis*, ethiological agent of dirofilariasis, in the vector *Ae. aegypti* was investigated. We obtained a first evidence of an inhibition of *D. immitis* larval development after the infection of mosquitoes with the engineered bacterium *Asaia*^{WSP}.

Article 2

In the second paper, I focused my attention on leishmaniases, vector-borne diseases widespread in more than one hundred countries in tropical, sub-tropical and temperate zones and caused by the protozoan parasite *Leishmania.* I investigated the capability of the chimeric bacterium *Asaia*^{WSP} to polarize the immune response towards the M1/Th1 phenotype, which is protective for the host; in fact, macrophage polarization towards the M1/Th1 or M2/Th2 side is crucial for the outcome of the visceral leishmaniasis. The

chimeric bacterium *Asaia*^{WSP} acted as a polarizing agent, stimulating the phagocytosis and inducing the release of M1/Th1 cytokines, ROS and the expression of iNOS. Then, *Asaia*^{WSP} determined an anti-leishmanial effect with a reduction of the number of intracellular parasites.

In conclusion the modified bacterium *Asaia*, here proposed, appears as a promising candidate for paratransgenesis-based control strategies for the control of vector-borne diseases in general, and as a successful immunomodulator, which could be used in combination with the classical chemotherapeutic agents for the treatment of leishmaniases and other M1-impaired diseases.

RIASSUNTO

Le malattie a trasmissione vettoriale rappresentano un sesto di tutte le malattie infettive e causano più di 1.000.000 di morti all'anno. L'organizzazione mondiale della sanità (OMS) propone l'adozione di un sistema di controllo integrato che include strategie come la bonifica ambientale, l'informazione e l'educazione della popolazione, ma anche l'integrazione dei classici metodi a base di insetticidi per il controllo dei vettori con metodologie non basate sull'utilizzo di sostanze chimiche. Tra queste strategie sta attirando sempre più attenzione il controllo simbiotico che sfrutta i microrganismi simbionti degli insetti in quanto particolarmente promettente per il controllo delle malattie a trasmissione vettoriale.

In particolare, per il presente lavoro di tesi, ho studiato le potenzialità di due batteri: *Asaia* e *Wolbachia.* Il primo è un batterio acetico, simbionte di molti insetti tra i quali le zanzare, ed è stato recentemente proposto come buon candidato per le strategie di controllo sfruttando la paratransgenesi. Il secondo è un simbionte degli artropodi e delle filarie in grado di stimolare le risposte immunitarie delle zanzare con una riduzione delle loro capacità vettoriali. Abbiamo così ingegnerizzato il batterio *Asaia* per l'espressione della *Wolbachia* surface protein (WSP), un potente induttore dell'immunità innata, isolata dal batterio *Wolbachia* del nematode *Dirofilaria immitis* (*Asaia*^{WSP}).

Il mio progetto ha un duplice ruolo:

i) verificare se il batterio *Asaia*^{WSP} sia in grado di stimolare il sistema immunitario delle zanzare e così interferire con lo sviluppo di *D. immitis* in seguito a infezione;

ii) verificare se la risposta immunitaria polarizzata dal batterio *Asaia*^{WSP} sia di tipo M1/Th1 e se tale polarizzazione possa determinare un effetto anti-*Leishmania* in saggi *in vitro*.

I risultati ottenuti per portare a termine tali obiettivi sono riassunti in due articoli:

Articolo 1

Il primo lavoro, qui presentato, consolida le precedenti dimostrazioni in merito alle proprietà immunostimolanti di WSP. Infatti, è noto che la WSP da *D. immitis* sia in grado di stimolare la risposta immunitaria in zanzare e mammiferi (umani, roditori e cani) *in vitro*, ma per la prima volta è stato testato un batterio ingegnerizzato per la produzione della proteina WSP. Dai nostri risultati è emersa una maggiore espressione dei geni del sistema immunitario delle zanzare *Ae. aegypti* e *An. stephensi* da parte del batterio *Asaia*^{WSP}*.* Una volta verificata la stimolazione del sistema immunitario delle zanzare, è stata investigata nel vettore *Ae. aegypti* la capacità di inibire lo sviluppo del nematode *D. immitis,* agente eziologico della dirofilariosi. I risultati ottenuti indicano una prima evidenza di inibizione dello sviluppo delle larve di *D. immitis* dopo infezione delle zanzare con il batterio ingegnerizzato *Asaia*^{WSP}.

Articolo 2

Nel secondo articolo, mi sono focalizzata sulle leishmaniosi, malattie a trasmissione vettoriale causate dal protozoo *Leishmania* e ampiamente diffuse in più di cento paesi delle regioni tropicali, subtropicali e temperate. Ho studiato la capacità del batterio chimerico *Asaia*^{WSP} di polarizzare la risposta immunitaria in senso M1/Th1, che risulta essere protettiva per l'ospite; infatti, la polarizzazione M1/Th1 o M2/Th2 è cruciale per

l’esito di questa infezione. Il batterio chimerico *Asaia*^{WSP} agisce come agente polarizzante, stimolando la fagocitosi e il rilascio di citochine M1/Th1, ROS e l’espressione di iNOS. Infine, *Asaia*^{WSP} ha determinato un effetto anti-*Leishmania* con una riduzione del numero di parassiti intracellulari.

In conclusione, il batterio modificato del genere *Asaia*, qui presentato, appare come promettente candidato per le strategie di controllo delle malattie a trasmissione vettoriale basate sulla paratransgenesi e come efficace immunomodulante che potrebbe essere usato in combinazione con i classici farmaci nel trattamento delle leishmaniosi e di altre patologie mediate dalla risposta Th1.

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1. INTRODUCTION

1.1 Microbiota of insects and symbionts

Insects harbor inside and outside a variety of microorganisms that have a strong impact on the physiology of the host. First, the microbiota plays a role on the immune response against pathogens; in fact microorganisms can produce secondary compounds with a toxic effect or stimulate the immune system of the host. For example, a toxic compound "pederin" is produced by the bacterium *Pseudomonas* sp. harbored by the beetle *Pederus* which protects the host from predators, while the bacterium *Wolbachia*, harbored by many arthropods, can alter the transmission of different pathogens upregulating the immune genes and affecting the vector capacities (Douglas et al., 2014).

Microbiota has also an impact on the insect nutrition with a dual role: microorganisms can supply nutrients or be a nutrient for the host. The insects can colonize different environments, also poor of nutrients or with unbalanced diets, due to the metabolites as vitamins or secondary compounds produced by the microorganisms and necessary for the survival of the insect. For example, vitamin B is provided by the bacterium *Rhodococcus rhodnii*, which lives in the blood-feeding *Rhodnius prolixus*, the Chagas disease vector. In the second case, bacteria localized in the gut can be lysed and used by the insect as nutrients. In addition, the gut bacteria can also degrade polymers such as cellulose, making available acetate for the host; this is the classical example of what happens in termites (Engel et al., 2013). In the study published by Wong and colleagues (2017) the authors also described the influence of the microbiota in altering the food preferences of the insect. The study was performed on *Drosophila melanogaster* model; they reported that if the microbiota changes, alternative dietary preferences can be observed, suggesting the huge role of the microbiota in the diet and forage preference (Wong et al., 2017). The last example about the nutritional role of the microbiota concerns the food detoxification, which makes compounds available that otherwise the insect would not be able to assimilate (Engel et al., 2013).

It is well known that insects have developed relationships with other microorganisms (prokaryotes and eukaryotes), acquiring capabilities to colonize almost all ecosystems (Douglas et al., 2014). Gil and colleagues (2019) described different types of relationships: "according to the fitness effects on the two (or more) symbiotic partners, such relationships can be referred as mutualism when both species increase their fitness, parasitism when one species increases its fitness while the fitness of the other is adversely affected, and commensalism when one partner is increasing its fitness without affecting the other one. […] Depending on the location of the symbiont in relation to the host cells, it is referred as endosymbiosis when the prokaryote symbiont lives inside a specialized eukaryote cell, called bacteriocyte, and ectosymbiosis when the symbiont lives on the host's body surface. Finally, according to the degree of dependence, the association can be obligate (or primary) and facultative (or secondary)" (Gil et al., 2019).

The nutrition can be affected by symbionts: this is the case of the bacterium *Buchnera aphidicola*, symbiont of aphids, which supplements the diet of the host with essential amino acids and vitamins (Baumann, 2005). In addition, symbionts can protect host from natural enemies, as happens with *Hamiltonella defensa*, a symbiont of aphids, which defends the host from parasitoid wasp larvae (Oliver et al., 2010). Moreover, symbionts have impact on reproduction, speciation (Bandi et al., 2001) and immunity (Macdonald and Monteleone, 2005) of their hosts.

1.2 The use of symbionts for the control of vector-borne diseases: the symbiotic control

Until last century, the vector control strategies were based almost exclusively on insecticide-based interventions. Chemical agents such as dichloro-diphenyl-trichloroethane (DDT) were successfully used for the control of arthropods such as *Anopheles* and *Aedes*, two of the most important vectors of world-wide infectious diseases. The indiscriminate use of these products has caused the phenomena of mosquito resistance and the impact not only on non-target species, but also on the environmental, raising the need to find an alternative (Wilke et al., 2015).

It is a shared opinion that the control of vector borne diseases and the arthropod vectors can only be achieved through the adoption of sustainable Integrated Vector Management (IVM) (fig.1), which is described as “a rational decision-making process to optimize the use of resources for vector control” (WHO, 2012). IVM is based on five strategies among which environmental remediation, information and education of the population, and the integrated approaches for the vector control, described as “ensure rational use of available resources by addressing several diseases, integrating non-chemical and chemical vector control methods and integrating with other disease control methods” (WHO, 2012). Among these approaches, the symbiotic control (SC) is getting more and more attention as it is particularly promising for the control of vector-borne diseases. As reported in Ricci et al., 2012, symbiotic control means “a multifaceted approach that uses symbiotic microorganisms to control insect pests or reduce vector competence”. In fact, the microbial symbionts have a strong impact on the insect in terms, for example, of fitness and resistance to parasites.

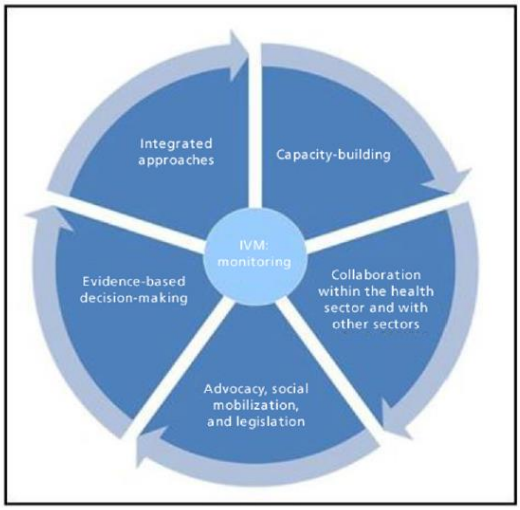

Figure 1. IVM framework (Beier et al., 2009)

Three main approaches are at the basis of the use of the SC for the control of insect pests and diseases:

i) killing of symbionts necessary for the survival of the host (Bandi et al., 1999);

ii) trasformation of the symbionts for the expression of anti-pathogen molecules (paratransgenesis) (Wang & Lorena, 2017);

iii) introduction of microorganisms into the insect influencing the life-span (Kambris et al., 2009).

1.3 The paratransgenesis

Here, I focused the attention on this strategy, one of the topics of my thesis. The paratransgenesis is a strategy which implies the manipulation of the insect symbionts to produce anti-parasitic molecules (Beard et al., 1998) (fig. 2), by exploiting the fact that symbionts and parasites harbor the same compartment (Wang & Lorena, 2017).

The choice of the most suitable symbiotic microorganism is important; Wang & Lorena reported the fundamental characteristics required to be a good candidate for the control of mosquito-borne diseases: i) "the symbiotic bacterium should preferably originate from the disease-transmitting vector and have a stable symbiotic relationship with the vector; ii) the symbiotic bacterium can be cultured; iii) the symbiotic bacterium can be genetically manipulated; iv) the effector gene product should not impair symbiont and vector fitness; v) the effector gene product should be secreted to assure interaction with the target pathogen; vi) an efficient means of introducing the engineered symbiont into field vector populations must be devised" (Wang & Lorena, 2017).

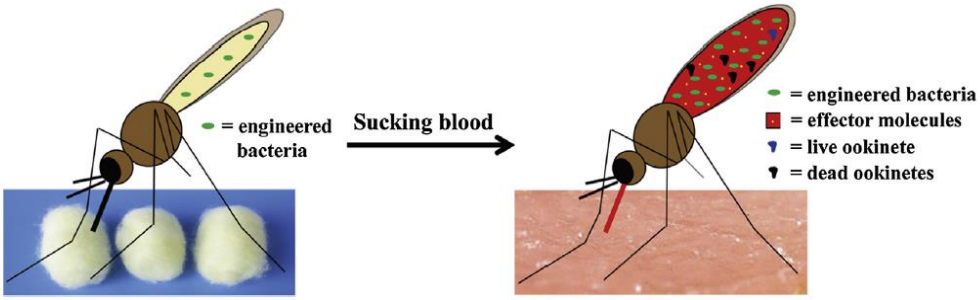

Figure 2. Graphical representation of the paratransgenesis approach (Wang & Lorena, 2017)

The first application of paratransgenesis was proposed for the control of Chagas disease (Durvasula et al., 1997; Hurwitz et al., 2011). Chagas disease is a parasitic disease caused by the protozoa *Trypanosoma cruzi* and transmitted by an insect belonging to the family of Reduviidae. The paratransgenesis approach exploits the presence of the symbiont *Rhodococcus rhodnii*, a cultivable bacterium, which has been engineered for the release of anti-trypanosomal effector molecules and then re-established into the host. This technique showed important results in the reduction of parasite transmission in semi-field trial.

Another example of the application of paratransgenesis for the control of mosquito-borne diseases is reported in Riehle et al. (2007) with the engineering of the bacterium *E. coli* for the expression of the two anti-plasmodial molecules SM1 and phospholipase-A (2). In this study, the re-introduction of the bacterium *E. coli* in the mosquito *Anopheles stephensi* 24h prior the blood meal determined a reduction of *Plasmodium berghei* development. If on one hand the authors showed an increase of bacteria after the blood meal with a consequent increase of the effector molecules released, on the other they recorded a poorly survival of the bacteria. In fact, *E. coli* could not survive at least two weeks, the average life span of an insect.

Recently, Wang and colleagues proposed the use of the transgenic bacteria *Pantoea agglomerans* modified for the expression of different anti-plasmodial molecules. *P. agglomerans* is a symbiont of different *Anopheles* mosquitoes and compared to the *E. coli* can resist inside the insect enough to exploit the inhibition of the parasite. They administered the engineered bacteria to the mosquitoes prior the infection and then, counting the oocysts, obtained an inhibition of the development of *Plasmodium falciparum* up to 98%, compared to the control.

Another candidate bacterium proposed for the control of mosquito-borne diseases through paratransgenesis belongs to the genus *Serratia*. This bacterium has been engineered for the release of different anti-plasmodial molecules and reintroduced in *An. gambiae* mosquitoes. The authors showed an inhibition of up to 92% of *P. falciparum* infection when engineered bacteria were present. In addition, this strain of *Serratia* can be efficiently transmitted and has not an impact on the lifespan, fecundity and fertility of mosquitoes (Wang et al., 2017).
Not only bacteria, but also fungi can be candidates for a paratransgenic approach. Unlike bacteria, fungi can survive for long time in the environment and can re-colonize the insect through the cuticle not only by feeding as for bacteria. Fang and colleagues engineered a fungus of the species *Metarhizium anisopliae* for the expression of three different molecules anti-*P. falciparum*. Thanks to the fact that the fungus can be in form of spore, it can be sprayed on the mosquitoes and colonizes through the cuticle. The results showed a high reduction of parasite development (Fang et al., 2011).
The choice of a good effector molecule is one of the prerequisites for a good application of paratransgenesis. In particular, effector molecules should not affect vector fitness or have a toxic effect against other organisms. Some of the effector molecules, grouped according to their modes of action and used for the control of mosquito-borne diseases through paratransgenesis, are reported below.

Mode of action	Effector	Function or mechanism
Parasite killing	Antibacterial peptides such as defensins, cecropin and gambicin	Lyses the parasite
Interaction with parasites	EPIP (*Plasmodium* Enolase–Plasminogen Interaction Peptide), single-chain Monoclonal antibodies (scFvs)	Blocks ookinete and sporozoite invasion of the midgut epithelium and salivary gland epithelium, respectively
Manipulation of mosquito immune system	Akt (a protein kinase) and Rel 2 (*Anopheles* Imd pathway transcription factor)	Stimulates the innate immune response

Table 1. Effector molecules used in the mosquito-borne disease control (Wang & Lorena, 2017)

1.4 The bacteria of the genus *Asaia*

Bacteria of the genus *Asaia* are other promising candidates for strategies based on paratransgenesis. *Asaia* spp. are extracellular acetic acid bacteria, gram-negative, strictly aerobic; the colonies are rod-shaped, yellowish/pink, measuring from 0.4 to 1 by 0.8 to 2μm (Yamanada et al., 2000). It grows at 30°C at pH 5 and it is easily cultivable in cell-free media. They colonize fruits and flowers of tropical regions like Indonesia and Thailand, but they are also isolated in temperate regions of Japan; more in general, *Asaia* has a cosmopolitan distribution (Yamanada et al., 2000).
Asaia spp. are also symbionts of different insects, among which mosquitoes such as *Anopheles stephensi*, *An. maculipennis*, *An. gambiae*, *Aedes aegypti* (Crotti et al., 2009), *Ae. albopictus* and *Culex quinquefasciatus* (De Freece et al., 2004). In *An. stephensi* *Asaia* sp. colonizes the gut, salivary glands and reproductive organs of both male and female mosquitoes allowing both vertical and horizontal transmission (Favia et al., 2007).
Asaia spp. also colonise the sand fly *Lu. longipalpis* vector of *L. mexicana* (Sant'Anna et al., 2014), *Phlebotomus papatasi, P. tobbi, P. argentipes, P. duboscqi* and *Sergentomyia* spp. (Akhoundi et al., 2012).

Asaia spp. are cultivable and easily transformable; in fact, an *Asaia* sp. has already been engineered for the expression of the green fluorescent protein (GFP) and the red fluorescent protein (DsRed) (Damiani et al., 2010). In this article the presence of these two modified strains of *Asaia* has been detected in the gut and in the reproductive organs of *An. stephensi* mosquitoes for at least 20 days; few days less in the salivary glands.
Moreover, this bacterium has also been transformed for the expression of antiplasmodial molecules. Bongio and colleagues (2015), modified *Asaia* sp. making it able to hider the development of *P. berghei* in mosquitoes with an inhibition up to 80%.
Moreover, the capability of *Asaia*-GFP to circulate in *Anopheles* mosquitoes was also evaluated in semi-field trial, highlighting the potentiality of this bacterium as candidate for paratransgenesis (Mancini et al., 2016).
Finally, regarding security concerns, *Asaia* bacteria can be considered as rare opportunistic pathogens, making them good candidates for paratransgenesis-based control strategies (Epis et al., 2012).

1.5 Introduction of microorganisms into the insect influencing the lifespan and vector capacity: the case of *Wolbachia*

The presence of endosymbionts inside the mosquitoes has an impact on pathogen transmission and this is well documented. Gonzalez-Ceron and colleagues, observed a 200-fold reduction of oocyst densities of *P. vivax* when aseptic mosquitoes were infected with *Enterobacter* spp. and *Serratia marcescens* bacteria (Gonzalez-Ceron et al., 2003); the inhibition of the parasite was to be attributed to the modulation of the immune system. In this respect, the symbiotic bacterium *Wolbachia* is the most relevant example, attracting great attention as a candidate for the control of mosquito-borne diseases. Bacteria of the genus *Wolbachia* are obligate intracellular gram-negative α-proteobacteria, order of Rickettsiales, found in the cytoplasmic vacuoles of insects, isopods, mites and nematodes (Wilke et al., 2015). Although these bacteria may be classified in different supergroups, the genus comprises the single species *W. pipientis* (Bouchery et al., 2013). Thus, researchers in the field now commonly refer to *W. pipientis* simply as *Wolbachia*. Dittmer and colleagues estimated that *Wolbachia* infects up to 65% of terrestrial arthropods, being the most abundant symbiont among insects (Dittmer et al., 2018).
The properties of *Wolbachia* are different; *in primis Wolbachia* has the ability to manipulate the reproduction of the host and to cause cytoplasmic incompatibility (CI), which in Engelstädter & Telschow (2009) is thus defined: “CI causes embryonic mortality in offspring when infected males mate with either uninfected females or with females that are infected with a different strain of bacteria”. In this way the number of *Wolbachia*-infected insects increases at each cycle and the females positive for *Wolbachia* show a selective advantage compared to uninfected females (Wilke et al., 2015).
In addition, *Wolbachia* can interfere with the transmission of human pathogens such as dengue and Zika viruses, malaria parasites and filarial worms through alteration of fatty acid intracellular trafficking, competition for cholesterol, manipulation of miRNAs expression and/or upregulation of innate immunity responses (Moreira et al., 2009; Bian et al., 2010). On details, while *Wolbachia* is present in the great majority of insects, this bacterium is almost completely absent in mosquitoes of the genus *Anopheles* and in *Ae. aegypti*, the vectors of human malaria and dengue virus, respectively. The strategy, based on the introduction of *Wolbachia* bacterium in un-infected mosquitoes, is considered a surprising strategy for the control of the vector borne diseases (Ricci et al., 2012). Indeed, field applications for the control of dengue virus transmission based on the release of

Wolbachia-infected *Ae. aegypti*-mosquitoes have been established since 2011, with very effective results (Walker et al., 2011; Frentiu et al., 2014).
In *Ae. aegypti, Wolbachia* determines: i) shortened lifespan (Kambris et al., 2009); ii) reduction of their potential to transmit pathogens (Bian et al., 2010; Dutra et al., 2016); iii) limited susceptibility to infection with the dengue or chikungunya virus or *Plasmodium* parasite (Moreira et al., 2009); iv) induction of cytoplasmic incompatibility (Zabalou et al., 2004). The life-shortening effect was first studied with a strain of *Wolbachia* called wMelPop isolated from *Drosophila melanogaster*. This strain of *Wolbachia* was used to infect *Ae. aegypti* -naturally uninfected- mosquitoes and the result was a reduced lifespan of the host. Moreover, these experiments proved that the wMelPop-*Ae. aegypti* mosquitoes were stable and that the bacterium can be maternally transmitted and can induce CI, spreading in the wild population (Kambris et al., 2009). The same strain of *Wolbachia* was used to infect *An. gambiae* mosquitoes, vectors of *Plasmodium.* The presence of this bacterium stimulated the expression of different immune genes and, among them, the activation of the gene coding for the complement C3-like molecule TEP1 was related to a reduction of *Plasmodium* infection. Therefore, the stimulation of the immune system by *Wolbachia* is one of the ways to control the pathogen, making this bacterium a suitable tool for the parasite control program. The up regulation of the immune system of *Ae. aegypti* mosquitoes was also shown when they were infected with another strain of *Wolbachia,* wAlbB, highlighting the role of the bacterium in the priming of the immune system of the mosquito especially through the Toll signaling pathway enhancing the expression of antimicrobial peptides and reactive oxygen species (Pan et al., 2012).
Many other studies were performed corroborating the previous results, highlighting the high rate of spreading of this bacterium in the population and the low fitness cost for the mosquito (Hoffmann et al., 2011). These important results made this system very attracting.
Another *Wolbachia*-based approach is called Insect Incompatible Technique (IIT) and consists in the application of the cytoplasmic incompatibility induced by *Wolbachia*. It is similar to the Sterile Insect Technique (i.e. a control strategy that uses radiation to produce genetic mutations or chromosomal breaks to generate sterile adult insect) with the only difference that the sterility of the insects is not induced by irradiation but with the CI mechanism.
Even though the efficient release of *Ae. aegypti*-infected mosquitoes in field for the control of dengue virus has been achieved (Walker et al., 2011), the use of *Wolbachia* in paratransgenesis is impaired because it is an obligate intracellular symbiont and it is not easy culturable in cell-free media and thus not transformable. Moreover, it is likely not very resistant outside cells, and thus not to be used in the environment.

1.5.1 *Wolbachia*, endosymbiont of filarial nematodes

Wolbachia, as said above, is not solely associated to the Arthropoda but it is also harbored by parasitic filarial nematodes of the family of Onchocercidae only; *Wolbachia* is absent in the other groups of nematodes (Fenn et al., 2006). Between *Wolbachia* and Onchocercidae there is a mutualistic relationship, since both benefit from this symbiosis. This is supported by the fact that the treatment based on e.g tetracycline, active against Rickettsiales group, causes sterilization and the death of worms (Bandi et la., 1999). In addition, the genome analyses of *Wolbachia* has shown genes such as for heme metabolism absent in the filarial genomes; this corroborates the idea that *Wolbachia* can contribute for the iron acquisition in favor of the host. In filarial nematodes, *Wolbachia* can be found in all larval stages at the level of the ovaries, oocytes and in the uterus, while it is absent in the male reproductive system.

Wolbachia bacteria have a critical role in the pathogenesis of filariases. It is known that in presence of filarial parasites a predominant Th2 cell response is induced with high levels of IL-4, IL-5, IgE, IgG1 and eosinophils (Babu et al., 2014). Previous studied showed that extracts of *Brugia malayi* filarial parasite were able to stimulate a pro-inflammatory response with high levels of cytokines like TNF and IL-1β, and nitric oxide. The authors discovered that the activation was mediated by lipopolysaccharide-like activity (LPS) with the consequent binding to the LPS binding protein (LBP), activation of TLR4 and the cascade of inflammatory cytokines activation (Taylor et al., 2000). The demonstration that a pro-inflammatory response was caused by *Wolbachia* has been shown exploiting the nematode *Acanthocheilonema viteae*, a *Wolbachia* free parasite; in this case no inflammatory response was detected, underlying the fact that *Wolbachia*, not only was the source of inflammatory response in filarial infection, but it was the only mediator present in the nematode. Moreover, the same experiments, but using live parasites, did not exploit the same results, pointing out the need of the release of the bacteria after the death of the parasite (Taylor et al., 2000). In fact, after treatment with anti-parasite drugs such as diethylcarbamazine or ivermectin, *Wolbachia* DNA or bacteria were detected in plasma within one week in infected people with the activation of a pro-inflammatory response; the death of the worms as a result of the filaricidal treatment, released the bacteria in the bloodstream (Hise et al., 2007). Afterwards, Hise and colleagues studied the role of TLRs and which of them was used by *Wolbachia* for the innate immune activation. They demonstrated that the activation was mediated by TLR2 and TLR6, but not via TLR4 (Hise et al., 2007). Same results were achieved by Daehnel and colleagues (2007) which examined the role of TLRs in dendritic cell activation. They confirmed that in filarial infection the immune response was TLR-2 dependent and TLR-4 independent with a consequent activation of Th1 instead of Th2 response (Daehnel et al., 2007).

1.6 The *Wolbachia* surface protein (WSP) from nematodes

An alternative approach to exploit *Wolbachia* could be the identification of molecules from this bacterium. Among its molecules, the major surface protein of *Wolbachia* (WSP) is one of the most abundant. “This protein contains transmembrane domains and a standard signal peptide for secretion and shows homologies to the major outer membrane proteins of *Ehrlichia* spp. and related. This indicates that WSP is a membrane protein of the bacterial outer envelope”. The features of the protein are thus described in Bazzocchi et al. (2000). WSP from *Wolbachia* of the nematode *D. immitis*: i) can inhibit the apoptosis in human polymorphonuclear cells (Bazzocchi et al., 2007); ii) stimulates the expression of iNOS, the production of NO and antibodies related to the Th1 immune response (Morchon et al., 2007).

Similar results were obtained in Brattig et al. (2004) where the activation of the innate immune system by WSP was studied in mammalian cells; high levels of pro-inflammatory cytokines (e.g TNF-α, IL-1β, IL-6 e IL-8) were obtained after co-incubation of mammalian cells with WSP and the induction of innate immune response was via TLR2 and TLR4, making the WSP a Th1-promoting factor. The difference with the study of Hise and colleagues, cited above, which reported an activation of TLR2 only, may be due to the use of a recombinant protein with a probably less amount of endotoxin than using the whole bacterium (Hise et al., 2007).
The protein WSP is also able to stimulate an innate immune response in mosquito cells. As reported in Pinto et al. (2012) in *An. gambiae* cells, naturally uninfected with *Wolbachia*, an upregulation of immune genes such as TEP1 and *Anopheles Plasmodium*-responsive Leucine-rich repeat 1 (APL1) was detected after challenging with WSP from the nematode *D. immitis*. The stimulation of the innate immune system was less intense when *Ae. albopictus* cell lines were incubate with WSP, pointing out a tolerance to WSP in this mosquito naturally infected with *Wolbachia* (Pinto et al., 2012).
In summary, WSP appears as a promising candidate molecule to induce innate immune activation, not only in insects but also in mammals.

1.7 Rational and aim of the project

Based on the evidences and assumptions discussed up to now, the control of vector-borne diseases could be fought with new systems in which the use of chemicals gives the way to methods that exploit microorganism symbionts of the insects.
In this scenario, two bacteria appear very promising: *Asaia bogorensis* and *Wolbachia pipientis*. As already said before in chapter 1.4, bacteria of the genus *Asaia* are extracellular symbionts of *Aedes* and *Anopheles* mosquitoes capable of spreading in the wild population. In addition, *Asaia* spp. have already been transformed with anti-plasmodial molecules for a paratransgenesis approach. *Wolbachia*, on the contrary, is an intracellular bacterium widely diffused in the arthropods and filarial nematodes. This bacterium can affect the transmission of pathogens like dengue virus or filarial worms due to its ability to upregulate the innate immune response of the host or reduce the vector capabilities. For these reasons, the infections with *Wolbachia* of mosquitoes which naturally not harbor the bacterium, have already been tested under field conditions with excellent results. However, the bacterium cannot be used in paratransgenesis due to difficulties in cultivation in cell-free media and engineering.
In order to join both qualities of these symbionts, a chimeric bacterium was generated: the bacterium *Asaia bogorensis* was engineered with the *Wolbachia* surface protein (WSP) of *Wolbachia* from the nematode *D. immitis*. Consequently, the obtained engineered bacterium *Asaia*^{WSP} has both the immune-activating capacities of *Wolbachia* and the culturable characteristics of the symbiont *Asaia*.
The bacteria *Asaia*^{WSP} and the control bacterium *Asaia*^{pHM4} (engineered with the empty plasmid) were tested against:

- the heartworm *D. immitis*, *in vivo* (see paper 1);
- the protozoan parasite *L. infantum*, *in vitro* (see paper 2)

In the first article, *Aedes* mosquitoes were colonized by the bacterium and the ability to stimulate the immune system was evaluated *in vivo*. Then, the capability to interfere with the dirofilariasis was assessed in response to mosquito infection with *D. immitis* larvae.
In the second article, the chimeric bacterium was studied in co-infection experiments against the parasite *L. infantum*. Since it is known that: i) WSP induces a Th1 immune

response in mammalian cells; ii) Th1-cells activation is protective for the host with the inhibition of the parasite, the immunomodulant properties and the leishmanicidal effect of the engineered bacterium *Asaia*^{WSP} were studied in *in vitro* assays.

Why Wolbachia from D. immitis as a M1/Th1 inductor?
As I stated above, *Wolbachia* has been shown to be a major player in in immunity to filarial infections, being involved in innate immunity activation, inducing a Th1 modulation, as well as in acquired responses. In addition, the *Wolbachia* surface protein (WSP) has been shown to elicit the release of Th1 cytokines. In *D. immitis*-infected dogs, the immune system is exposed to huge amounts of dead-end *Wolbachia* bacteria, released daily in the bloodstream by fertile females, and after the turnover of the microfilariae. Intracellular bacteria classically drive the immune response toward the Th1 side, with an effector role of classically activated macrophages. In evolutionary terms, selection is expected to act on pathogenic bacteria to reduce such modulation, as a M1/Th1 response clearly harms them. *Wolbachia* is an intracellular bacterium, but it is hosted by the filarial worm, and its evolutionary success is that of its host. The overall immune modulation it elicits, including the partial Th1 bias, is thought to have a positive effect for the nematode, thus for *Wolbachia* itself, as it protects the worm from an excess of Th2 response. For this reason, I suggest that selective pressure acts on *Wolbachia* to preserve its "Th1-inductor" phenotype: its survival is closely tied to the survival of the nematode host. At any case, while other intracellular bacteria benefit from a reduced Th1 response, *Wolbachia* is for sure not directly harmed by this type of response.

Why Leishmania has been selected as parasite model?
Here we reported several considerations about *Dirofilaria immitis* and the filaria-*Leishmania* interaction. Till a couple of decades ago, the heartworm *D. immitis* was widespread in dog populations in several temperate and tropical countries, with very high prevalence values (Morchón et al., 2012). In Italy, the area of highest prevalence values for dogs and cats was the Po River Valley (northern Italy), where the prevalence rate for dogs ranged from 50 to 80% in animals untreated with preventive drugs (Genchi et al., 2001), and approximated fixation in endemic rural areas of the Po river valley (Claudio Genchi, personal communication). *D. immitis* was thus a major component of the microbiota of the dogs living in this area, as well as in other highly endemic areas (Morchón et al., 2012; microbiota is used *sensu lato*, being referred to a nematode worm). During the last three decades, after the introduction of anti-filaria prophylaxis, the heartworm *D. immitis* virtually disappeared from the dog population of the Po valley. Prior to the introduction of heartworm prophylaxis, no cases of autochthonous leishmaniasis had been detected in dogs in the Po river valley (Otranto et al., 2009). Then, during the last 25 years, autochthonous cases of the disease have been detected in dogs in this area, with growing incidence and novel records of micro-endemic foci (Maroli et al., 2008). This invasion of the Po valley by *Leishmania* is generally attributed to the 'global warming' resulting in *de novo* colonization of this territory by phlebotomine vectors. Here, we propose that another anthropogenic environmental change contributed to the ingression of this parasite: almost the whole population of owner's dogs in the valley is now subjected to prophylactic treatment to prevent infection by the heartworm, *D. immitis*; in our view, the removal of this parasite from owner's dogs in the Po valley has determined a population-wide bias in the immune milieu of the animals, opening an immunological gate to *Leishmania* invasion.

1.8 Leishmaniases

1.8.1 Epidemiology

Leishmaniases are world-wide vector-borne diseases (see box 1) caused by the protozoan parasite of the genus *Leishmania* transmitted by the bite of a female sand fly. Leishmaniases are widespread in 102 countries in tropical, sub-tropical and temperate zones and over 20 different species of *Leishmania* are infective causing 700.000-1.2 million cases per year and 20.000-30.000 deaths annually (Tomiotto-Pellissier et al., 2018; www.who.int/leishmaniasis). Moreover, 220.000 and 58.000 new cases are reported for cutaneous and visceral leishmaniasis, respectively (Alvar et al., 2012).

The notification of the cases of leishmaniases is critical mostly in developing countries; in fact, out 200 countries, about half of the countries are reported to be endemic in 2017. In particular, 65 were found to be endemic for both visceral leishmaniases (VL) and cutaneous leishmaniases (CL), 10 for VL only and 22 for CL only (www.who.int/gho/neglected_diseases/leishmaniasis).

Not only humans are vertebrate hosts of the parasite *Leishmania*, but about 70 species of mammals can be infected in nature. Rodents and canids are the more susceptible to the infection, but there are also xenarthrans, hyraxes, marsupials, chiropterans, lagomorphs, procyonids, felids and primates (Rio Riberio et al., 2018).

There are three main forms of human leishmaniases: visceral (also known as kala-azar and the most serious form of the disease), cutaneous (the most common), and mucocutaneous (www.who.int/leishmaniasis). They are in turn divided in anthroponotic and zoonotic depending on the reservoir of the parasite, humans or animals, respectively.

VECTOR-BORNE DISEASES

The WHO defines the vector-borne diseases as “human illnesses caused by parasites, viruses and bacteria that are transmitted by mosquitoes, sandflies, triatomine bugs, blackflies, ticks, tsetse flies, mites, snails and lice”. Every year the vector borne diseases cause more than 700.000 deaths and represent 17% of all the infectious diseases. The pathogen transmission is modifying because of the global travel and trade, unplanned urbanization and climate change making transmission season longer or more intense (who.int/vector-borne-diseases).

Box 1. Definition of vector borne diseases

1.8.2 Clinical forms

VISCERAL LEISHMANIASIS

The zoonotic visceral form (ZVL) is endemic in Latin America, Mediterranean basin and Asia and the causative agent is the species *Leishmania infantum*. In the New World it is caused by the subspecies *L. infantum chagasi* (fig. 3).

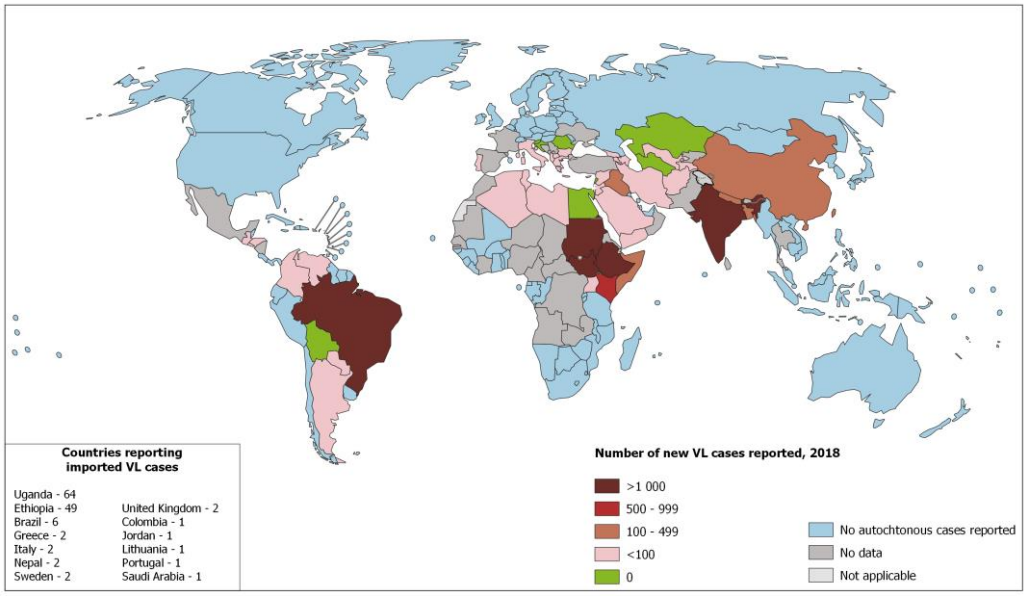

Figure 3. Geographical distribution of VL in 2018 (www.who.int/leishmaniasis/burden/en)

WHO reported that in countries like Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan the percentage of new cases exceeded the 90%, while in Bangladesh there was a significant reduction (www.who.int/leishmaniasis/burden/en).
The phlebotomine vectors in the Mediterranean basin belong to the genus *Phlebotomus* (*Phlebotomus perniciosus* e *P. ariasi*), while in the new world the exclusive vector is *Lu. longipalpis*. Jackals and foxes are the main reservoirs in the wild environment, while in the Mediterranean area the major domestic reservoir is the dog. Emerging new reservoirs of the parasite are rats (Galán-Puchades et al., 2019), cats (Iatta et al., 2019) and donkeys (Nardoni et al., 2019).
In the developing countries the pathology affects almost exclusively children under the age of five, while in southern Europe 50% of the cases are adults, generally immunocompromised. The pathology is characterized by the dissemination of the parasites and parasite-infected macrophages from the initial site of cutaneous infection to the spleen, liver, bone marrow, lymph nodes, intestinal mucosa.
The anthroponotic form of visceral leishmaniasis (AVL) is caused by the parasite *Leishmania donovani* and occurs in the African and Indian regions (Bangladesh, India and Nepal) where is called kala-azar (Desjeux, 2001). It is the most severe form with the following major symptoms: fever, loss of weight, splenomegaly, hepatomegaly and anemia (www.who.int). It is the most lethal form if left untreated (the mortality can reach the 95%) and the people at risk are children and adults above 50 years and more in general, people affected by malnutrition or immune suppression (Dayakar et al., 2019). Three-10% of patients develop, after successful treatments, post kala azar dermal leishmaniasis (PKDL) with nodules over the face and extensor surface of the limbs (Awasthi et al., 2004).

It is reported that the risk to develop VL increases when people are infected with HIV. The co-infection *Leishmania*-HIV is present in 35 countries where there is a high rate of people living with HIV. From 3% to 25% of HIV patients have developed visceral leishmaniasis, so people living with HIV are more susceptible to leishmaniasis. Parasitological diagnosis, in this case, is more suitable instead of serological methods and a critical point is the treatment of the co-infection because the mortality is high and the successful of the therapy is low (Lindoso et al., 2018).

CUTANEOUS LEISHMANIASIS (CL)

CL is the most common form of leishmaniasis and almost all the reported cases are localized in the following countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia, and Syria (De Vries et al., 2015) (fig. 4).

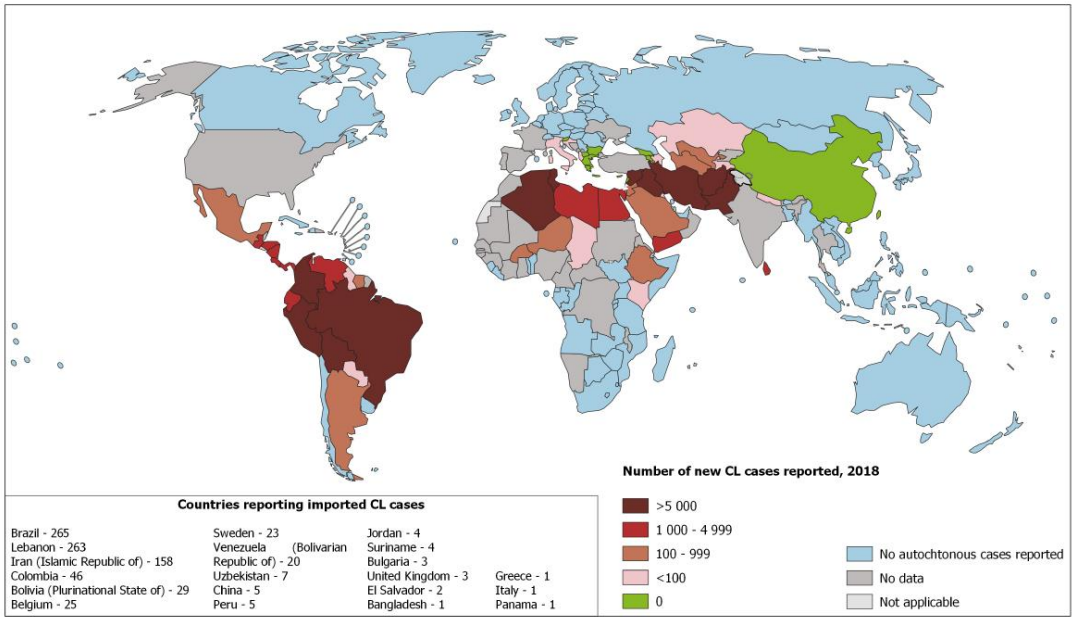

Figure 4. Geographical distribution of CL in 2018 (www.who.int/leishmaniasis/burden/en)

The zoonotic form of cutaneous leishmaniasis (ZCL) is diffuse in South America, Asia and North Africa and is caused by the species *Leishmania brasiliensis* and *Leishmania mexicana* in the new world, while *Leishmania major* in the old world and here is transmitted by the sandfly *Phlebotomus papatasi*. The animal reservoirs are typically small rodents (gerbils) (Desjeux, 2001). The anthroponotic form (ACL) is only diffuse in the old world transmitted by *P. sergenti* and the species involved is *L. tropica*. In general, the cutaneous form is characterized by lesions on the skin that can ulcerate on exposed parts of the body with an incubation period between 2-6 weeks (Awasthi et al., 2004). Lesions may begin as small red papules that can get worse in erythematous nodules or plaques.

MUCOCUTANEOUS LEISHMANIASIS (ML)

Contrary to cutaneous leishmaniasis, mucocutaneous leishmaniasis is potentially life threatening and requires treatment. The species involved are of the *Viannia* subgenus, typically found in the Americas (L. (V) *braziliensis*, *Leishmania* (*Viannia*) *amazonensis*, *L.* (V) *panamensis*, and *Leishmania* (V) *guyanensis*). This form of leishmaniasis is characterized by a partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues. Up to 10% of patients are prone to this

type of mucosal worsening, whose clinical progression is dependent on a combination of host cell-mediated immunity and parasite virulence (David et al., 2009).

CANINE LEISHMANIASIS (CanL)

CanL is diffuse in the Old and New World, even in the northern area of the Europe due to the spreading of sand flies in previously free areas and un-controlled dog movement (Dantas-Torres et al., 2019).

As in humans, also in the dog the outcome of the disease is multifactor: parasite strain, immune system of the host and other factors can contribute to the intensity of the pathology. Therefore, there are dogs which are asymptomatic for all of their life, while other can show severe clinical manifestations, which untreated could cause death. It is reported that only in 40% of infected dogs the parasite progresses and causes severe manifestations, while in the others become resistant (Gradoni et al., 2015).

The diagnosis is not so simple, because the dog could be asymptomatic or shows a multisystemic manifestations. In the early stage the dog is usually asymptomatic for few months, becoming an excellent reservoir; the symptoms can be shown from three months to several years after the infection (Santos-Gomes et al., 2002). Coetaneous lesions can occur in 90% of the dogs and include: dermatitis, alopecia, nasal hyperkeratosis which can be localized or disseminate and usually involve other symptoms like renal disease, anorexia, enteritis, weight loss, hepatomegaly, arthritis and neurological disorders. Different studies report the vertical transmission of the parasite through the placenta; other suspected via of transmission can be by blood transfusion, infected donors or dog bites (Rio Riberio et al., 2018).

The rate of *L. infantum* infections in dogs is unknown globally. It is estimated that in Europe the percentage of infected dogs is about 10% of 500,000 analyzed dogs and the number tends to increase when molecular methods are used. Lately, the methods of diagnosis have undergone an improvement, but in absence of an effective canine vaccine, the cutaneous treatment based on pyrethroids represents the unique solution to reduce the incidence of infection (Gradoni et al., 2015).

1.8.3 Leishmaniases in Italy

As reported by WHO, Italy is endemic for visceral and cutaneous leishmaniases and the main endemic zones are the Tyrrhenian littoral, the southern peninsular regions and the islands. If 40 cases of human VL per year were not exceeded in 1980s, more than 200 cases were reported throughout the country in 2000, with new foci in the northern regions previously non-endemic (Gradoni et al., 2003). As for the canine leishmaniasis, the average seroprevalence is about 12-15% up to a maximum of 40% in the rural areas of central and southern Italy making it a re-emerging disease (Gramiccia et al., 2013; Burani et al., 2012). A spreading of canine leishmaniasis from the typical southern regions to the north was documented with an outbreak in 2011 in Emilia Romagna region which involved 35 residents of the zone (Cesinaro et al., 2017). Phenomena such as the increasing migrations and globalization have led to the importation of cases of human leishmaniasis (generally of the cutaneous form) in Italy, carried by exotic species of *Leishmania* typical of southern and eastern Mediterranean countries but also from the New World. Not only Italy, but also non-endemic countries/regions of North Europe, like Austria or Germany, have registered and documented the increase of imported leishmaniases (Di Muccio et al., 2015).

1.8.4 The pathogen *Leishmania* and the life cycle in the arthropod

SCIENTIFIC CLASSIFICATION	
Domain	**Eukariota**
Kingdom	**Protista**
Phylum	**Sarcomastigophora**
Class	**Zoomastigophora**
Order	**Kinetoplastida**
Family	**Trypanosomatide**
Genus	***Leishmania***

Table 2. Scientific classification of *Leishmania* spp**.**

Leishmania is an obligate intracellular protozoan parasite whose life cycle alternates between an intracellular developmental stage in mammals and an extracellular developmental stage in the insect, a female phlebotomine sand fly, its biological vector (Roberts et al., 2006). *Leishmania* presents two different morphological forms: the amastigote, round, non-motile localized inside the macrophages (3-7 μm) and the promastigote, motile, flagellated, elongated (10-20 μm), which lives in the alimentary tract of the sandfly (Awasthi et al., 2004). Infection starts when sand flies ingest blood containing macrophages infected with amastigotes. *Leishmania* parasites, migrating anteriorly from the posterior midgut to the stomodeal valve, undergoe to different developmental stages, all of which are characterized by morphological and functional changes aimed at ensuring their survival in the fly (fig. 5). First, amastigotes differentiate into small, sluggish procyclic promastigotes with short flagella and make the first multiplication cycle in the fly. They are localized in the early bloodmeal separated from the midgut by a peritrophic matrix (PM), whose function is to form a barrier that protects the midgut epithelium from abrasive food particles and microbes and slows down the diffusion of digestive proteases. Procyclics develop into nectomonads which migrate forward towards anterior ‘thoracic’ midgut, escaping the PM and anchoring themselves to epithelial cells (Kamhawi et al., 2006). From nectomonads derive both haptomonad promastigotes (Bates et al., 2018), highly specialized leaf-like parasites with short flagellum, and leptomonads, producers of the promastigote secretory gel plug (PSG) and developmental precursors of the mammal-infective metacyclic promastigote form (Gossage et al., 2003).

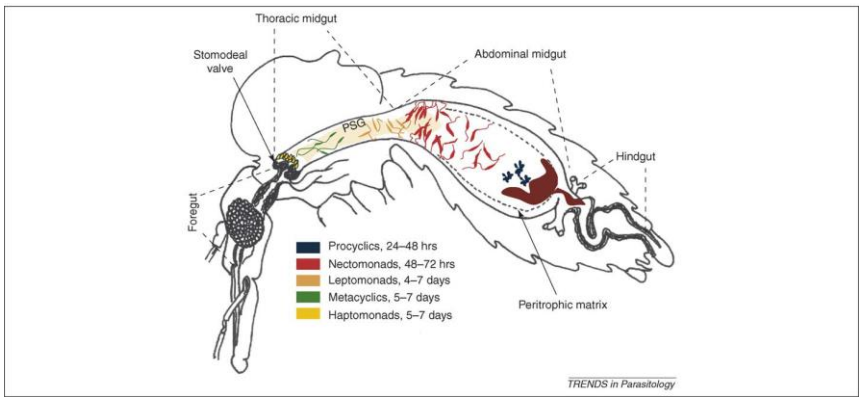

Figure 3. The *Leishmania* life cycle inside the sand fly (Kamhawi et al., 2006)

Up to now it thought that the only function of metacyclic promastigotes was to be inoculated into the vertebrate host, but in Bates (2018) the life cycle of *Leishmania* and the role of metacyclic promastigotes were modified (fig. 6). Briefly, not all metacyclics are transmitted, but can remain in the gut and de-differentiate into retroleptomonads increasing the parasite population with a higher number of metacyclics.

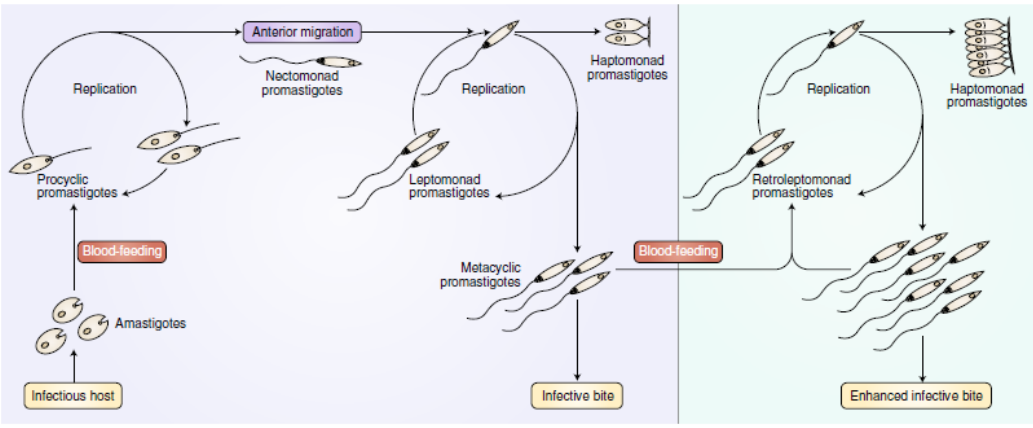

Figure 4. Revised life cycle of *Leishmania* (Bates, 2018)

During a new blood feeding, the PSG which surrounds the metacyclic promastigotes is able to block the lumen of the insect's anterior midgut and stomodeal valve, forcing the sandfly to regurgitate parasites and improving the efficiency of *Leishmania* transmission (Rogers, 2012).
The duration of life cycle of *Leishmania* inside the sand fly is extremely variable and fluctuates between six to nine days, depending on the species (Kamhawi et al., 2006).

1.8.5 Life cycle in the mammalian host

During the bite of a mammalian host, the metacyclic promastigotes, present in the insect's saliva, are regurgitated in the skin of the mammalian host. Being obligate intracellular parasites, they need macrophages for survival and replication (Liu et al., 2012). Proteophosphoglycans secreted by the parasite are inoculated into the host and are powerful stimulators of macrophage recruitment; the components of phlebotomine saliva have antihemostatic, chemotactic and immunomodulatory properties influencing the

parasite infection. Initially, the parasites are mainly found in neutrophils where they do not differentiate into amastigotes. The neutrophils are short-lived phagocytes which do not participate in the *Leishmania* elimination but produce cytokines and chemokines for the recruitment of different cell types. Then, due to the apoptosis, the parasites may subsequently infect the macrophages and transform into the amastigote. For this reason, the neutrophils are reported as “Trojan horses” used by parasites to rapidly enter the macrophages avoiding cell activation (Tomiotto-Pellissier et al., 2018). In the phagocytic cell the parasite is confined in a phagosome, which then fuses with lysosomes forming a parasitophorus vacuole filled with lytic enzymes and low pH which trigger the promastigote-to-amastigote differentiation (Awasthi et al., 2004; Podinovskaia et al., 2015). The amastigotes multiply by binary fission eventually leading to the lysis of the macrophage and thus infecting other macrophages in turn. At this point, depending on the *Leishmania* species and its tropism, the infection can remain localized in the skin or spread, from here, to the visceral districts (Bongiorno, 2007).

1.8.6 The sand fly vector

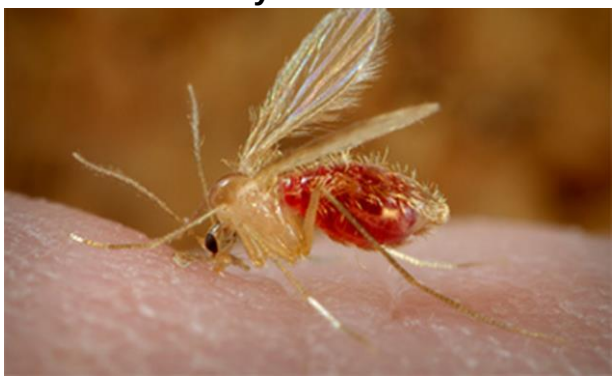

Figure 5. Sand fly female during the blood meal (www.ecdc.europa.eu)

The vector responsible of the transmission of *Leishmania* parasite is the sand fly (phylum Artropoda, class Insecta, order Diptera, family Psychodidae, sub-family Phlebotominae).
There are five genera of sand flies: *Phlebotomus*, *Sergentomyia*, *Warileya, Lutzomyia e Brumptomyia*, but only two are considered relevant in terms of clinical importance: sand fly species of the subgenus *Phlebotomus* (*Larroussius*) (e.g. *Phlebotomus perniciosus, P. ariasi, P. neglectus, P. tobbi*, and others) typical in most of the Old World, whereas members of the *Lutzomyia longipalpis* complex are the main vectors in Latin America (Gradoni, 2015). Phlebotomine sand flies are small (3 mm) and light-coloured. They have peculiar characteristics: 1) when at rest, they hold their wings at an angle above the abdomen; 2) their attack is silent; 3) they are hairy (fig. 7).
The preimaginal stages of sand fly are the following: the egg, four larval stages (instars), and the pupa; the larvae are known not to be aquatic, but terrestrial and their development requires a range of constant temperature (24-30°C) and high humidity (~90%) (Busani et al., 2012). They do not fly long distances and tend to bite only during crepuscular or nocturne hours, while during the day they prefer to stay in humid places like fissures in walls, rocks or soil. Only female sand flies make the blood feeding, necessary to complete the oogenesis, on the contrary males feed only on natural sources of sugar (Killick-Kendrick, 1999).
In Italy eight species of sand flies of the genera *Phlebotomus* e *Sergentomyia* are present and the most abundant are *P. perniciosus*, *P. perfiliewi* e *Sergentomyia minuta* (Maroli et al., 1994). *P. perniciosus*, the vector of *L. infantum,* etiological agent of the visceral and canine leishmaniasis, is the most important in Italy because is present in 18 out 20 regions both in domestic and wild environment (Busani et al., 2012).

1.8.7 Mechanisms of immune evasion by *Leishmania*

Aside from the species of the infecting parasite, the outcome of leishmaniasis is mainly determined by the host immune response and the mechanisms of immune evasion used by the parasite. The parasite, to develop in the macrophage, needs to evade the immune system of the host, deactivating the host complement system. Metacyclic promastigotes express protein kinases with the goal to phosphorylate the complement proteins C3, C5 and C9. In this way the complement system of the host is deactivated (Cupta et al., 2013).
Leishmania benefits from pro-inflammatory properties of the sand fly saliva and molecules like LPG and gp63 on the surface of the promastigotes that play an essential role in phagocyte chemo-attraction (Rossi et al., 2017). Furthermore, *Leishmania* must resist the adverse conditions inside the phago-lysosome preventing the fusion of the phagosome and lysosome and the following acidification, allowing the amastigote maturation. In addition, there is a modulation of the cytokine production and T cell activation towards a Th2 immune response; all the pro-inflammatory cytokines like IL12, TNF are suppressed in favor of an upregulation of IL-10 and TGF-ß, necessary for the parasite maintenance and survival (Cupta et al., 2013).

1.8.8 Immunity of the host

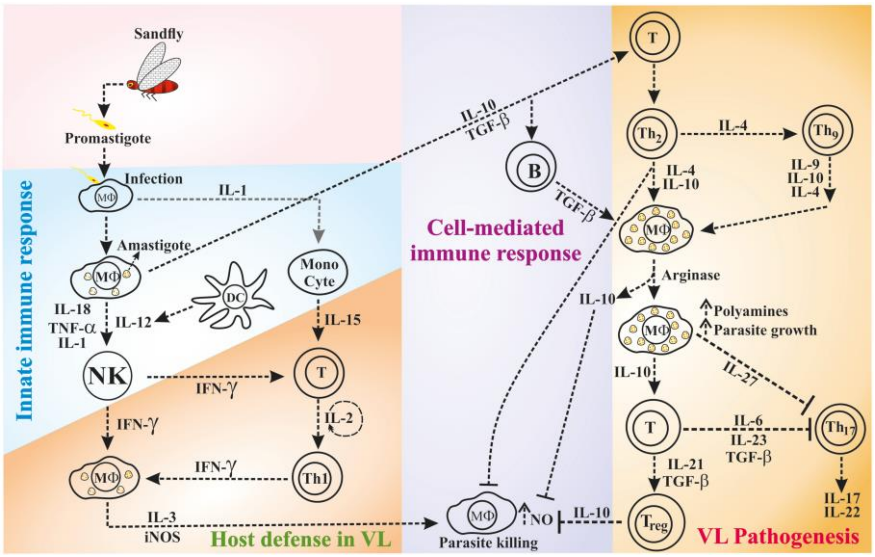

Figure 6. Innate and cell-mediated immune response during host defense or pathogenesis in VL (Dayakar et al., 2019).

When macrophages phagocyte the parasites active TLR-9 signaling with the production of IL12 which in turn stimulates NK cells to produce IFNγ, a key cytokine for the differentiation of Th1 cells, which produce INFγ stimulating the macrophages to produce ROS and to express iNOS with the final goal of killing the parasite. If there is the production of cytokines like IL10 by parasitized macrophages, the differentiation of Th2 cells is induced with the following production of IL-4 which in turn stimulates the macrophages to express arginase and inhibit iNOS and ROS with the consequent survival of parasite (fig. 8). Therefore, the Th1/Th2 dichotomy, based on the cytokines produced by these cells, is fundamental for the resolution of the disease: Th1 differentiation is linked to host protection, while Th2 with parasite survival.

From the nomenclature Th1/Th2 we moved to the M1-M2 concept, as testified in many recent papers, pointing out the macrophage phenotype (e.g production of cytokines, expression of co-stimulatory molecules) and how important are the microenvironment and signals for their activation (Martinez et al., 2013; Atri et al., 2018; Dayakar et al., 2019, Tomiotto-Pellissier et al., 2018).

The macrophages naïve (M0) in presence of IFNγ and TNF are activated to M1 macrophages also called "classically activated macrophages" (fig. 7) which produce inflammatory cytokines (TNF, IL1beta, IL-6, IL12, IL23, IL18, IFN), nitrite oxide, ROS and exhibit a high phagocytosis rate. All these markers are associated with tumor control and microbicidal properties (Mosser et al., 2008; Tomiotto-Pellissier et al., 2018).

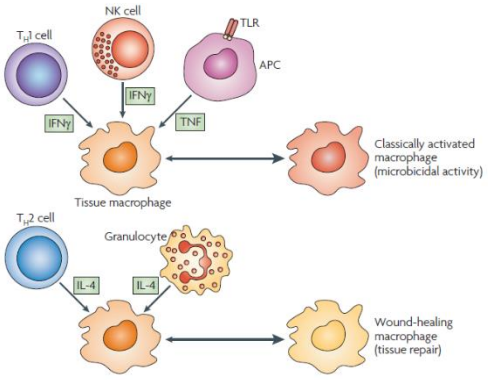

Figure 7. M1 and M2 dichotomy (Mosser et al., 2008).

Moreover, M1 macrophages activate transcription factors for the expression of proinflammatory genes, costimulatory molecules and chemokines and through the IL-12 production, stimulate the differentiation of Th1 cells (Mills et al., 2014). They recognize intracellular pathogens, being the first line of defense, and their activation is mainly due to the presence of lipopolysaccharide (LPS), IFN-γ or TNF-α (Tomiotto-Pellissier et al., 2018).

On the contrary, in presence of IL4 and IL13 produced by Th2 cell type, the macrophages are differentiated in M2 cells or "alternatively activated macrophages". M2 response is linked with anti-inflammatory cytokines, growth factor production and the activation of the Th2 response (fig. 9). The expression of iNOS is inhibited in favor of arginase, urea and L-ornithine production which are used by *Leishmania* for its growth (Tomiotto-Pellissier et al., 2018).

Recently, the M2 classification has been updated subdividing in M2a, M2b, M2c, M2d depending on the stimulus (Mantovani et al., 2004). The stimuli necessary for M2a macrophage polarization are colony-stimulating factor (M-CSF) and IL4 or IL-13. Among the molecules produced, IL-10 and expression of arginase can be highlighted. The M2b phenotype can be induced by apoptotic cells or LPS; they attract eosinophils and produce IL-10 and inflammatory mediators determining Th2 activation and immunoregulation. M2c macrophage is activated by IL10 and can down-regulate the pro-inflammatory cytokines production in favor of IL10 and TGF. In addition to these, a last phenotype was added: the M2d macrophage. It is associated with the inhibition of the immune response, promotion of angiogenesis and is induced by IL-6, toll-like receptor (TLR) ligands (Tomiotto-Pellissier et al., 2018). A summary of all markers associated with the two phenotypes M1-M2 are reported in figure10.

Development of leishmaniasis-related pathology depends not only on the infecting parasite species or strain, but it is mostly related to host genetics and the immunological state of the host. This could explain the presence of asymptomatic individuals in endemic regions, which can induce an effective anti-*Leishmania* response, but without achieving sterile immunity, since persistent infection with a low number of parasites guarantees resistance to reinfection (Iborra et al., 2018).

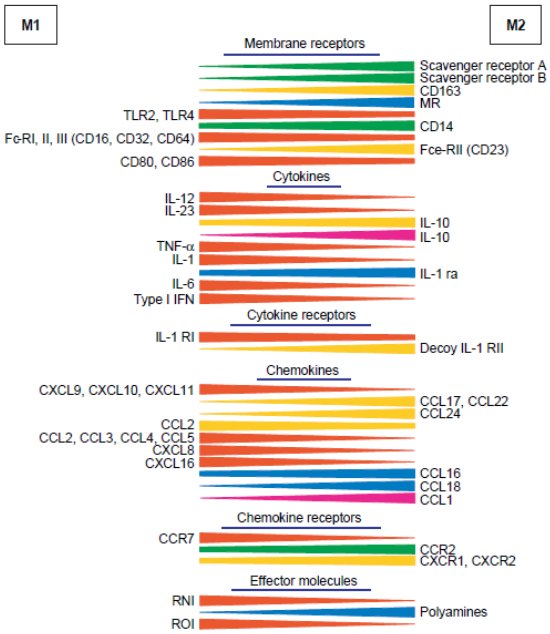

Figure 8. Stimuli and molecules of M1 and M2 macrophages (Mantovani et al., 2004)

1.8.9 Treatment of leishmaniases

If on a one hand the only cure for leishmaniasis is the pharmacological treatment, which can reduce the transmission of the infection, on the other, most of the actual drugs show toxicity problems or loss of efficacy; another major problem is the high cost of these drugs in the developing country. For this reason, new formulations and combinations of standard drugs have been approved against leishmaniases, recently (Reguera et al., 2019).

The pentavalent antimonial monotherapy in form of meglumine antimoniate and sodium stibogluconate has been used for decades for the treatment of visceral leishmaniasis. Two drugs based on these components, meglumine antimoniate (Glucantime®) (85 mg SbV/100 mL) and sodium stibogluconate (Pentostam®) (100 mg SbV/100 mL), have been used widely in India where phenomena of non-response led to increase the dose (Burza et al., 2018). There are many side effects life anorexia, vomiting, nausea, abdominal pain, malaise, myalgia, arthralgia, headache, metallic taste and lethargy (Control of the leishmaniases, WHO). Despite this, Pentostam is still used in Africa alone or in combination. Another drug used for therapy is Amphotericin B, an antibiotic with a strong antileishmanial effect. Given the low absorption at the gastrointestinal level, this drug must be intravenously administered and this a limiting point mostly in the low-income country. In addition, Amphotericin can cause hypokalemia and myocarditis, which require close monitoring and hospitalization.

Miltefosine was originally developed as an oral anticancer drug but now is also effective against *Leishmania*. The common side-effects are restricted to the gastrointestinal tract such as anorexia, nausea, vomiting and diarrhea and show also a teratogenic effect (Control of the leishmaniases, WHO).

Paromycin is a safe and effective antibiotic against *Leishmania* in combination with miltefosine. Another point in favor is the affordability, in fact the use of this drug costs only ~$10 per patient. The route of administration is intramuscular or intravenous since the low adsorption in the gastrointestinal tract (Reguera et al., 2019). For cutaneous leishmaniasis it could be used as topical formulation (Control of the leishmaniases, WHO).

Pentamidine is another drug for the treatment, but due to the side effects like insulin-dependent diabetes mellitus, it has been used inconsistently (Reguera et al., 2019).

1.8.10 Vaccines

Among all methods for the control of leishmaniasis, vaccination appears to be the best option, as individuals who recover from the disease develop immunity to subsequent infections (Iborra et al., 2018).

- Vaccines based on live parasites (first generation of vaccines)

It is so called “leishmanization” the century-old practice of inoculating live virulent parasites in minimally exposed areas. Even if this is the only vaccination strategy that has proven effective against leishmaniasis in humans to date, the development of resistances to treatment makes this practice unrecommendable (Palatnik-de-Sousa et al., 2012).

A further strategy is the use of attenuated parasites, which can elicit a subclinical infection of the host, avoiding pathology or symptoms. Attenuation can be obtained by techniques such as gamma irradiation, maintenance of the cultures for long periods of time, chemical induction of un-characterized mutations etc. Otherwise, the pathogen could be genetically modified, silencing the genes for virulence (Iborra et al., 2018).

- Vaccines with *Leishmania* extracts

Total protein extracts, obtained from autoclaved or chemical-treated parasites (phenol, merthiolate), can be administered either without adjuvants, or more often, formulated with BCG (Bacillus Calmette–Guerin) to increase the immunogenicity of the proteinaceous preparation. The success of this type of vaccine seems to be low (Iborra et al., 2018).

- Vaccines based on antigens (second generation of vaccines)

This type of vaccines is based on recombinant proteins with adjuvants or expressed in heterologous microbial vectors and they are more suitable for mass vaccination campaigns (Duthie et al., 2013). Since 80s of the last centuries, many leishmanial antigenic proteins with different cellular locations and functions have been identified such as glycoprotein GP63, KMP-11 (a kineto plastid membrane protein of 11 kDa), or the hydrophilic surface protein B1 (HASPB) (Iborra et al., 2018).

There is currently no licensed vaccine for human use, but many are in clinical trial or in development (Gillespie et al., 2016). For human leishmaniasis the most evolved vaccines in clinical trial are based on recombinant molecules, such as LEISHF1, LEISHF2, and LEISHF3. (Iborra et al., 2018).

Regarding the canine leishmaniasis, the first vaccine commercialized in Europe was CaniLeish®. It was launched in 2011 in different European countries like Portugal, Spain, France, Greece, and Italy and it is a second-generation defined peptidic antigen composed of excreted–secreted proteins of the supernatant of *L. infantum*. CaniLeish® showed an efficacy of 68.4%. Two CanL vaccines were registered in Brazil in the past years: Leishmune® and Leish-Tec®. Leishmune®, is a second-generation vaccine candidate introduced in Brazil in 2004 and finally licensed in 2011 for the canine visceral leishmaniasis. The immunogen of this vaccine is a fucose–mannose ligand (FML) glycoprotein from *L. donovani* promastigotes associated with a saponin-based adjuvant. It showed 92% protection and 76% vaccine efficacy, and it well tolerated (Palatnik-de-Sousa et al., 2012), but is not currently recommended by the Brazilian Ministry of Health.

In 2007, the use of Leish-Tec® vaccine was licensed in Brazil and it is composed of the A2 antigen, a recombinant amastigote-stage-specific protein of different *Leishmania* species containing saponin as adjuvant. It is reported that almost all (92.9%) immunized dogs with this vaccine remained healthy during the 11-month follow-up period (Grimaldi et al., 2013).

The last vaccine commercialized in Europe is LetiFend®. It is a novel vaccine based on the recombinant Protein Q, well tolerated and with an overall efficacy of about 72% (Fernandez-Cotrina et al., 2018).

1.8.11 Live Bacterial Vaccines

The idea of using live bacterial cells as vehicles to deliver recombinant antigens has emerged as an interesting alternative for the development of new vaccines.

Their capacity to stimulate an efficient long-term immune response against carried antigens is due to their intrinsic properties: the presence of the lipopolysaccharides in Gram-negative bacteria, or lipoteichoic acid in Gram-positive bacteria and other pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs), mediate different signaling pathways with the final production of inflammatory cytokines and the expression of other antimicrobial genes (Da Silva et al., 2014).

The bacteria typically used for a delivery system are recombinant bacteria that have been genetically modified to delete the virulence genes creating a non-virulent organism, ensuring the safety of the host (Yurina et al., 2018). In addition to the capacity to mimic the route of entry of many pathogens and stimulate the mucosal immune response, as said above, they can be administered orally or nasally avoiding the risk associated with contaminated needles. Among different suitable bacteria, lactic acid bacteria (LAB) such as *Lactococcus lactis* and some strains of *Lactobacillus* are attractive candidates for the delivery of heterologous antigens, *in primis* for their safe status. The only disadvantage is their non-invasive property to deliver not efficiently the plasmid DNA to the cytoplasm of antigen presenting cells. Other attenuated bacteria used as live bacteria vaccines belong to the genera *Shigella*, *Salmonella* and *Lysteria* (Detmer et al., 2006).

Considering the role of the immune system in the outcome of leishmaniasis, it has long been known the use of bacterial vehicles to stimulate the immune system and fight the disease; e.g. in Xu et al. (1998) the use of *Salmonella typhimurium* strains that express cytokines, known to be involved in the Th1 responses or in the activation of macrophages, is reported (Xu et al., 1998).

1.9 Dirofilariasis

Dirofilariasis is a vector-borne disease caused by nematodes *Dirofilaria* spp. and transmitted by mosquitoes of the genera *Aedes, Armigeres, Culex, Anopheles*, and *Mansonia*. Fourty species of *Dirofilaria* are known and six of which can be pathogens (*Dirofilaria immitis*, *Dirofilaria repens*, *Dirofilaria striata*, *Dirofilaria tenuis*, *Dirofilaria ursi* and *Dirofilaria spectans*). The typical reservoir is the dog, but also wild animals (coyotes, jackals, and wolves) and humans are not immune. Occasional hosts are domestic cats, bobcats, ferrets, and foxes. In general, the vectors and the species of *Dirofilaria* change according to the geographical distributions (Reddy et al., 2013; www.cdc.gov/parasites/dirofilariasis/biology).

The geographical distribution of human dirofilariasis is almost similar to that canine dirofilariasis, except for some differences related to the lack of data. Human dirofilariasis caused by *D. immitis* is common in the warm regions of North and South America, where there are high rates of canine filariasis, Australia and Japan. In the Old World *D. repens* is the most abundant species, with Italy, Spain, France and Greece traditionally endemic; many cases are reported in Europe, Russia and Sri Lanka (fig. 11). In the last decades the number of dirofilariasis in the Mediterranean countries has increased and new cases have been reported in Turkey and in the north Europe (Austria and Hungary) (Simon et al., 2012).

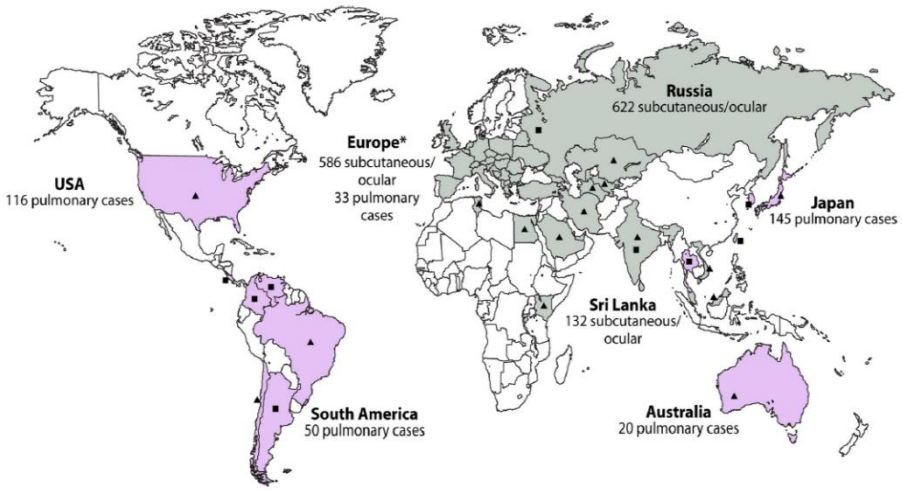

Figure 9. Geographicaly distribution of human dirofilariasis (Simon et al., 2012).

D. immitis are filiform worms with the following dimensions as reported in Simon et al. (2012) :"females measure 250 to 300 mm in length and 1 to 1.3 mm in diameter and males measure 120 to 200 mm in length and 0.7 to 0.9 mm in diameter" […] *D. repens* worms are smaller than *D. immitis* worms: females are 100 to 170 mm in length and 4.6 to 6.3 mm in diameter, and males are 50 to 70 mm in length and 3.7 to 4.5 mm in diameter with a curved tail and rounded cephalic extremity"; adult forms can survival over seven years (Simon et al., 2012).

1.9.1 Life cycle of *D. immitis*

During a blood meal, larvae at the third stage (L3) are transmitted by an infected mosquito to the definitive host where develop into L4 and then in microfilariae-producing adults. The

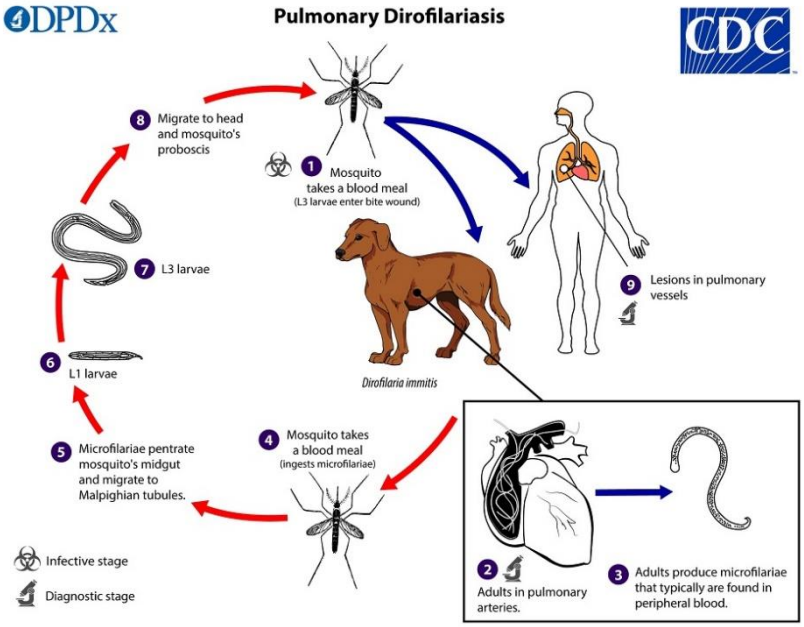

Figure 10. Complete life cycle of *D. immitis* (image from CDC**)**

adult forms are typically found in the pulmonary arteries, where mate producing microfilariae which enter in the peripheral blood and can be taken by a mosquito during a new blood meal. The microfilariae in the mosquito gut migrate after 24h to the Malpighian tubules developing from L1 to L2 in 8-10 days and then to L3, the infective stage, three days after. The L3 larvae (1-1.5 mm in length) migrate to the proboscis, where they can be transmitted to a new host by a new blood meal. If the definitive host is the human, the larvae, instead of migrating to the arteriae, go to the lungs causing infarcts and lesions (www.cdc.gov/parasites/dirofilariasis/biology) (fig.12).

1.9.2 Life cycle of *D. repens*

The life cycle of *D. repens,* reported in figure 13*,* is the same of the previous one, but the only difference is that the larvae inoculated by the mosquito in the definitive host migrate to the subcutaneous tissues, developing in L4 and then in adults. Sometimes they can also be retrieved in the abdominal cavity. In humans *D. repens* can be manifested as wandering worm in the subcutaneous tissue or in the form of a granulomatous nodule. If the infection is caused by *D. tenuis*, the worms tend to accumulate around the eye and for this reason the infection is called subconjunctival dirofilariasis (www.cdc.gov/parasites/dirofilariasis/biology).

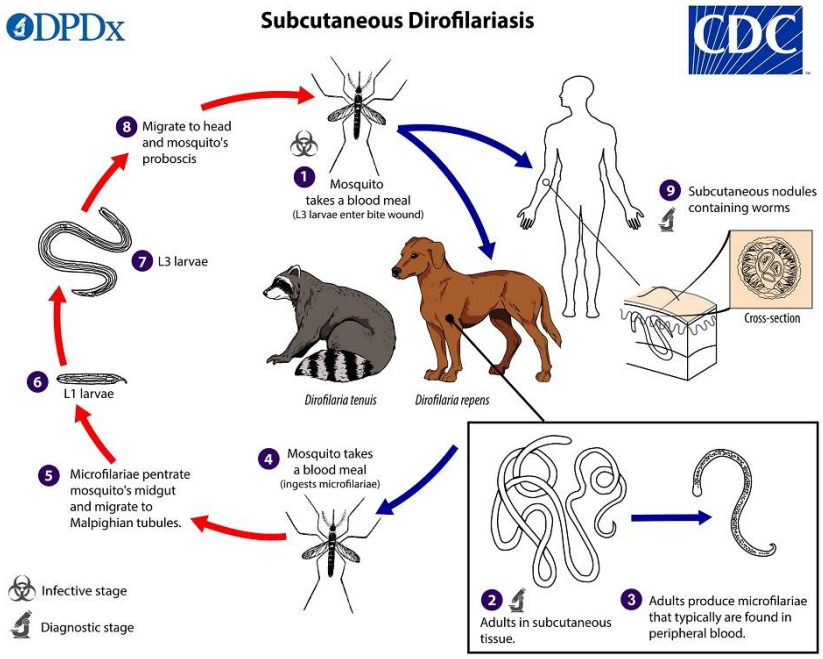

Figure 11. Complete life cycle of *D. repens* (image from CDC)

1.9.3 Vectors of *Dirofilaria* spp.

The following mosquito species are vectors of *D. repens*: *Anopheles maculipennis*, *Aedes aegypti, Mansonia uniformis, Mansonia annulifera, Armigeres obturbans and Aedes albopictus*; while for *D. immitis* the vectors belong to the genera *Culex, Aedes, Anopheles*, and *Culiseta* (Simon et al., 2012).

The mosquito survival is limited by the migration of the larvae to the Malpighian tubules and the mosquito activates defense systems to limit the amount and the progression of larvae L3. For example, the insect can produce antimicrobial peptides and show melanization and encapsulation systems. In addition, there are structures and mechanisms designed to the damage of the larvae such as the buccopharyngeal armature (cibarial armature), the secretion of compounds which can lyse the worm epicuticle, blood coagulation trapping the larvae preventing their transit to the Malpighian tubules (Simon et al., 2012).

The survival of the larvae also depends on the temperature and humidity conditions; as reported in Simon et al. (2012) the following parameters are necessary: “the development of infectious L3 larvae requires 8 to 10 days at 28°C to 30°C, 11 to 12 days at 24°C, and 16 to 20 days at 22°C. Below 14°C, development arrests, although it can be restarted when the temperature increases above this threshold” (Simon et al., 2012).

Due to the climate change, the period of development of the larvae is shortening and the period of activity of the mosquitoes is increasing with huge repercussion on the diffusion of dirofilariases for human and animals (Simon et al., 2012).

1.9.4 Clinical presentation

In humans there are two typical forms of dirofilariases: the pulmonary dirofilariasis caused by *D. immitis* and the subcutaneous dirofilariasis caused by *D. repens, D. tenuis,* and others (www.cdc.gov/parasites/dirofilariasis/biology). The first one causes the pulmonary dirofilariasis with the formation of nodules around the worms, often incorrectly taken for malignant lesions. The worms can occlude the pulmonary vessels provoking embolism and inflammation.
D. repens causes, on the contrary, the subcutaneous dirofilariasis characterized by the formation of nodules in subcutaneous tissues at the level of arms, legs and chest.
In the ocular region dirofilariasis is often caused by *D. tenuis* and is characterized by the formation of nodules in the conjunctive and orbital zone with consequences like retinal detachment, glaucoma or loss of vision and risks associated with the removal of the worms from these sensitive zones (Simon et al., 2012; www.cdc.gov/parasites/dirofilariasis/biology).

1.9.5 Canine dirofilariasis

In American continent canine dirofilariasis is spread in almost all countries (fig. 14); in the USA the prevalence rate is between 1% and 12% and this number increases moving to central and south America. In Italy, *D. immitis* was found in the North with percentages of 2.9% and 1.5% in Veneto and Friuli-Venezia Giulia, respectively.

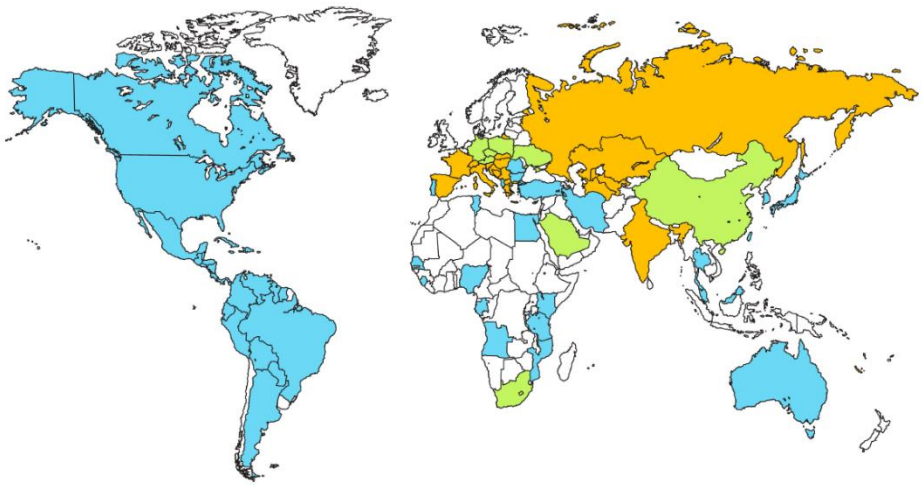

Figure 12. Geographical distribution of canine dirofilariasis. Color blue indicates *D. immitis* infections, the green color the infections by *D. repens* and with orange the presence of both species (Simon et al., 2012).

For *D. repens*, the highest prevalences were recorded in San Pietro island (27.8%) and Abruzzo (18.5%). In general, the rates are decreasing thanks to the actual chemoprevention campaigns. The higher rates of diffusion of *D. repens* are localized in the southern areas of Italy, where there are favorable environmental and climate conditions (Traversa et al., 2019).

In Asia the percentages are around 50-60% with the highest number of cases in Malaysia (70%) (Simon et al., 2012). Acknowledgement of the distribution of canine dirofilariasis in Africa comes from Genchi et al. (2001), who reported cases in Morocco, Tunisia, Egypt, Tanzania, Kenya, Mozambique, Malawi, Senegal, Angola, Gabon, and Nigeria (Genchi et al., 2001).

The cardiopulmonary dirofilariasis (heartworm disease) is the typical disease caused by *D. immitis* in dogs. It represents a serious disease with a chronic progression: first, the damages affect the pulmonary arteries then edema and inflammation occur in lung parenchyma. The death of the worms and/or the treatment with filaricides can provoke thromboembolism and inflammation which can lead to the death of the dog. Sometimes renal problems and the vena cava syndrome are common in dog infected with *D. immitis*.
With the progression of the disease, among many symptoms, the dog can show cough, difficulty in breathing and weakness. The symptoms can occur months or also years after the infection, according to the parasite load and reactivity of the animal. The diagnosis is based on the research of the circulating microfilariae and serological test. Both positive tests can confirm a dirofilariasis. Considering the huge complications and adverse effects of the filaricide treatment, the prevention is very important and is based on the assumption of drugs like ivermectin one month before the infectious period to one month after with the objective of impeding the development of larvae (Simon et al., 2012).

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Sitography

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www.who.int/leishmaniasis/burden/en (visited on February 2020)

2. SELECTED PAPERS

2.1 ARTICLE 1

CHIMERIC SYMBIONTS EXPRESSING A *WOLBACHIA* PROTEIN STIMULATE MOSQUITO IMMUNITY AND INHIBIT FILARIAL PARASITE DEVELOPMENT

Chimeric symbionts expressing a *Wolbachia* protein stimulate mosquito immunity and inhibit filarial parasite development

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ABSTRACT

Wolbachia can reduce the capability of mosquitoes to transmit infectious diseases to humans and is currently exploited in campaigns for the control of arboviruses, like dengue and Zika. Under the assumption that *Wolbachia*-mediated activation of insect immunity plays a role in the reduction of mosquito vectorial capacity, we focused our attention on the *Wolbachia* surface protein (WSP), a potential inductor of innate immunity. We hypothesized that the heterologous expression of this protein in gut- and tissue-associated symbionts may reduce parasite transmission. We thus engineered the mosquito bacterial symbiont *Asaia* to express WSP (*Asaia*^{WSP}). *Asaia*^{WSP} induced activation of the host immune response in *Aedes aegypti* and *Anopheles stephensi* mosquitoes, and inhibited the development of the heartworm parasite *Dirofilaria immitis* in *Ae. aegypti*. These results consolidate previous evidence on the immune-stimulating property of WSP and make *Asaia*^{WSP} worth of further investigations as a potential tool for the control of mosquito-borne diseases.

INTRODUCTION

The microbial communities of insects and mites of medical relevance, such as mosquitoes, sandflies and ticks, have attracted a great deal of attention, and it is now well established that arthropod-associated microbes influence the fitness of the arthropod hosts, as well as their capability to transmit pathogens to humans and animals [1]. Mosquitoes have been in the focus of this research area, with over 150 papers published in the last five years, on their microbiota and, accordingly, on their symbionts. Two symbiotic bacteria found in mosquitoes have emerged for their prominent biological role in these insects, as well for their potential utility for the control of mosquito-borne diseases: *Asaia* spp. and *Wolbachia pipientis.* Representatives of the genus *Asaia* have been detected in different mosquito species; more in general, they have been observed in several insects [2,3]. *Asaia* spp. are extracellular acetic acid bacteria, which can easily be cultured in cell-free media and have already been engineered at both the plasmid and chromosomal level, also for the expression of molecules interfering with the development of malaria parasites [2,4,5,6]. These bacteria colonize the gut, salivary glands and reproductive organs of both male and female mosquitoes. From the reproductive organs, *Asaia* can be transmitted venereally form males to females and vertically from mother to offspring, via egg-smearing [7]. From the salivary glands, *Asaia* can be transmitted horizontally among adults through cofeeding [4,7,8]. The actual capability of *Asaia* to spread into mosquito populations has recently been demonstrated in semi-field conditions [9]. Based on the above characteristics, *Asaia* bacteria have been defined as very promising mosquito symbionts, suitable for the control of vector-borne diseases through paratransgenesis [6]. In vector-borne disease control, paratransgenesis is the use of microbial symbionts manipulated for the expression of molecules that determine, either directly or indirectly, the reduction of pathogen transmission [10,11].

The intracellular bacterium *Wolbachia* is probably the most widespread intracellular symbiont in arthropods [12], found also in filarial nematodes [13], and already used in the field for the control of mosquito-borne viruses [14]. Indeed, through alteration of fatty acid intracellular trafficking, competition for cholesterol, manipulation of miRNAs expression

and/or upregulation of innate immunity responses, *Wolbachia* strains have been shown to interfere with the transmission of human pathogens by mosquitoes (e.g. dengue and Zika viruses, malaria parasites and filarial worms [15-20]). However, the biological effects of *Wolbachia* infection on the insect host and its vector competence are not predictable; for example, Dodson and co-workers reported that *Wolbachia* enhances West Nile viral infection in the mosquito *Culex tarsalis* [21]. Field applications for the control of dengue virus transmission through the release of *Wolbachia*-infected *Aedes aegypti* mosquitoes have been established since 2011, with very effective results [22,23]. The exploitation of *Wolbachia* in paratransgenesis is however impaired by the characteristics of this bacterium: it is an obligate intracellular symbiont and it is not culturable in cell-free media, and thus not easy to be engineered [24].

An alternative approach to exploit *Wolbachia* could be the identification of molecules from this bacterium able to stimulate the immune system of the mosquito, thus potentially interfering with the insect vectorial capacity. The major surface protein (WSP) of the *Wolbachia* hosted by the nematode *Dirofilaria immitis* has been shown to induce an upregulation of immune gene transcription in cells from the mosquito *Anopheles gambiae* [25], which is normally not infected by *Wolbachia* (except for some local populations [26]). WSP has also been shown to activate innate immune responses in mammalian models, supporting the activity of this protein as a general trigger of innate immune activation both in insects and in mammals [27].

According to the above evidence and assumptions, we aimed to combine properties of *Asaia* and *Wolbachia* symbionts, in order to confer an increased immune-activating capability, derived from *Wolbachia*, to the culturable *Asaia* of mosquitoes. To accomplish this aim, we engineered *Asaia* SF2.1 strain [4] for the expression of WSP from the *Wolbachia* infecting the nematode *D. immitis* [25,27]. We then tested the capability of the modified bacterium to colonize mosquito organs, to stimulate the immune system, to induce phagocytosis and to interfere with the development of filarial parasites.

RESULTS

WSP expression by *Asaia* SF2.1 and fitness of the bacteria

A schematic presentation of the *Asaia*-pHM4-WSP (hereafter *Asaia*^{WSP}) construct is shown in Supplementary Fig. 1a,b. Plasmid pHM4-WSP was constructed by inserting the wsp gene cassette flanked by NotI sites in the plasmid pHM4. An E-tag epitope was included for immunodetection purposes; the production of WSP protein by *Escherichia coli* and *Asaia* sp. was evaluated by Western-blot and immunofluorescence assays, with anti-E-tag antibodies. As shown in Supplementary Fig. 1c, *Asaia*^{WSP} is able to express the protein (26 kDa), while, as expected, *Asaia*-pHM4 (hereafter *Asaia*^{pHM4}) does not produce the WSP protein (the same results were observed for *E. coli*). The expression of the *wsp* gene was also verified by RT-qPCR using bacteria grown at different optical densities (ODs) (Supplementary Fig. 1d): no expression was observed for *Asaia*^{pHM4}, while *Asaia*^{WSP} expressed the *wsp* gene, with a substantial increase of the expression from OD 0.5 (6.253 ± 0.385) to OD 1 (9.970 ± 0.391). Based on these results, we decided to use OD 1 for other analyses. In addition to Western blot analysis (see above and Supplementary Fig. 1c), the expression/production of WSP protein was also verified by immunodetection: both

immunofluorescence (Supplementary Fig. 2a-d) and immunogold staining (Supplementary Fig. 1e-g) confirmed the production of the protein by *Asaia*^{WSP} bacteria, while no staining (or a very faint background) was observed in *Asaia*^{pHM4} control bacteria. The anti-Etag immunogold staining on *Asaia*^{WSP} revealed a pattern of colloidal gold deposits associated with the bacterial cells (Supplementary Fig. 1e,f). To verify if the production of the heterologous WSP had negative effects on *Asaia* growth, we analysed growth curves of the bacteria at different pH conditions along a 24 h period. This test was also performed to reproduce the different pH condition in mosquito organs and to test the capability of *Asaia* to survive and grow. In this fitness assay, we compared the strain *Asaia*^{wt} with the engineered strains carrying the plasmids pHM4 (*Asaia*^{pHM4}) or *Asaia*^{WSP}: the mean maximal growth rates (MGRs) of wild type and the two transformed strains were not significantly different in almost all the tested growth conditions, with the exception of the MGRs of *Asaia*^{wt} and *Asaia*^{WSP} at pH 4 ($p=0.038$) (Fig. 1). In conclusion, WSP expression does not significantly affect the fitness of *Asaia*^{wt} in most of the tested pH conditions.

In vitro phagocytosis test and immune-related gene expression

Phagocytosis tests on haemocytes from *Ae. aegypti and An. stephensi* revealed significant differences, after the stimulation with *Asaia*^{pHM4} or *Asaia*^{WSP} for 1 ($p<0.0001$ and $p=0.0089$, respectively) and 2 hours ($p=0.0001$ and $p<0.0001$) (Fig. 2a,b). The expression of the two selected antimicrobial peptides, defensin and cecropin, and the nitric oxide synthase (*NOS*) was investigated on haemocytes from *An. stephensi* and *Ae. aegypti*, after an *in vitro* stimulation with the two engineered bacteria. Stimulation of mosquito haemocytes with *Asaia*^{WSP} induced expression of cecropin, which was significantly different from the control in *Ae. aegypti* at three time points (6, 9, 12 h), and at only one time point in *An. stephensi* (12 h) (Supplementary Fig. 3a,b). In both *An. stephensi* and *Ae. aegypti* haemocytes we detected a significant production of NOS transcripts after 9, 12 and 24 hours of stimulation with *Asaia*^{WSP} (Supplementary Fig. 3a,b). Finally, in both *An. stephensi* and *Ae. aegypti* none of the two bacteria determined a significant up-regulation of defensin gene expression by the haemocytes, considering all the time points.

In vivo immune gene expression

Quantitative real-time PCR assays were used to investigate the capability of *Asaia*^{WSP} to stimulate innate immune responses in *An. stephensi* and *Ae. aegypti* mosquitoes, after a sugar meal containing the engineered bacteria. To determine the dynamics of this immune response, the transcription level of immunity genes was monitored at 6, 12 and 24 hours post “bacterial meal”. Only female mosquitoes with fully- or partially fully-engorged abdomens were selected for these analyses. As reported in Fig. 3 (Supplementary Table 1 and Supplementary Data 1), for *Aedes* mosquitoes, four of the six analysed genes were significantly activated after the *Asaia*^{WSP} bacteria meal (Fig. 3a). On details, cecropin D gene (*CECD*) showed a significantly increased expression after 12 hours compared to pHM4 and sugar control; CLIP-domain serine protease gene (*CLIPB37*) resulted activated after 24 hours compared to the sugar control; thio-ester containing protein 20 gene (*TEP20*) showed a statistically significant upregulation on the first two time points compared to *Asaia*^{pHM4} and sugar control; finally, as for NADPH-oxidases gene (*NOXM*), the gene was upregulated at all the time points, especially after 6 hours. For *Aedes* mosquitoes the expression of the Transferrin gene was also investigated; after feeding with *Asaia*^{WSP} a trend in the overexpression of the gene was observed, even though the differences were not significant (Fig. 3a). This agrees with results obtained on *Ae. aegypti*

mosquitoes transinfected with *Wolbachia*, where the expression of this gene, involved in iron metabolism, immunity and development, is observed [15].

Anopheles mosquitoes that received *Asaia*^{WSP} bacteria showed upregulation of *TEP1*, leucine-rich repeat protein 1 (*APL1C*), NO synthase (*NOS*) and cecropin 1 (*CEC1*) genes, compared to the controls (Fig. 3b, Supplementary Data 1). The degree and the time points of upregulation were different for the different genes: *TEP1* gene for example was significantly upregulated after 6 and 12 hours, compared to the two controls; the expression of *CEC1* gene was enhanced after 12 hours; *APL1C* showed a statistically significant upregulation after all the three analysed time points, while the expression of *NOS* gene was very high after 12 hours post bacterial meal (Fig. 3b). Conversely, no significant expression was detected for defensin gene in both mosquito species, in coherence with the results obtained *in vitro* on haemocytes.

Mosquito colonization by engineered *Asaia*

Asaia bacteria are an important and stable component of the microbiota of *An. stephensi* and *Ae. aegypti*. Here, we investigated if the transgenic bacteria were able to efficiently colonize adult *Ae. aegypti* female mosquitoes, performing an immunofluorescence assays on a total of thirty insects for each of the two different mosquito populations fed with the two engineered strains of *Asaia*. Analyses using a fluorescent confocal microscopy, after secondary staining on anti-E-tag antibodies, showed fluorescence signals inside the crop and the gut of females, indicating that the bacterium efficiently colonized these body organs. Most of the individuals showed fluorescent cells either isolated, aggregated or in microcolonies. Fluorescent cells and microcolonies were detected in the mosquito crop and gut at both 24 h and 48 h after the bacterial-containing meal; colonization of the reproductive system was observed only 48 h after the meal, with very few bacteria. No immunofluorescence staining was detected in organs after the administration of *Asaia*^{pHM4} strain (Fig. 4a), sugar or sugar plus kanamycin (Supplementary Fig. 4a-d and 5a,b).

To quantify *Asaia* colonization, persistence and dynamics in *Ae. aegypti* (in view of the successive challenge with *D. immitis* – see below), bacteria colony-forming units of *Asaia* were assessed at different times after blood feeding, in females previously infected by the bacteria through sugar meal. In general, no statistical difference in the colonization by the two *Asaia* strains was detected (Fig. 4b). As for the pattern of organ colonization, in the midguts bacteria numbers significantly increased 24 hours after the blood meal (T1, p=0.0068, Fig. 4b). In the crops, the numbers decreased with time, in particular after the blood feeding (T2, p=0.0018). As previously reported for *Asaia*-GFP [28], the presence of bacteria was also detected in ovaries, in coherence with the possibility of a transmission to progeny. Indeed, *Asaia*-GFP bacteria have been shown to be transmitted to progeny through and egg-smearing mechanism [4].

Inhibition of *D. immitis* infection by *Asaia*^{WSP} in *Ae. aegypti*

Recombinant *Asaia* were administered to *Ae. aegypti* mosquitoes, Liverpool strain, through a sugar meal, 32 hours before mosquitoes were fed on a *D. immitis*–infected blood meal. Three days after the blood meal we recorded an average survival rate of 35% of the mosquitoes (see Material and Methods and Fig. 5). Figure 5 shows the results of the assay. For each group we determined two parameters: the larval abundance, i.e. the average number of L3 detected in the dissected mosquitoes; the larval prevalence, i.e. the

proportion of mosquitoes that contained at least one larva at the third stage (L3), versus the total number of dissected mosquitoes. *Asaia*^{WSP}, in comparison with mosquitoes fed with sugar or sugar plus kanamycin, determined a significant decrease in L3 abundance, with a reduction of 75.7% ($p<0.0001$) and 66.8% ($p=0.0083$), respectively. In the comparisons of mosquitoes fed on *Asaia*^{WSP} with those fed on *Asaia*^{pHM4}, we observed reduction of 53.8%, that was however not significant ($p=0.17$). Moreover, the feeding on *Asaia*^{WSP} determined a decrease in the prevalence of L3, that was significant in the comparisons with the mosquitoes fed on sugar ($p=0.0006$) or sugar plus kanamycin ($p=0.0243$).

DISCUSSION

Evidence has already been reported on the capability of WSP from the filarial nematode *D. immitis* to determine innate immune responses in both mosquitoes and mammals [25,27,29]. Considering the conservation of the stimuli that induce innate immunity activation across the animal phyla (e.g. [30]) and the abundance of WSP at the surface of *Wolbachia* cells [31], it is likely that this protein represents an important modulator in the interaction between the symbionts and the host in both insects and nematodes, as well as in the tripartite system *Wolbachia*-filaria-mammalian host [32]. For example, it might be a major player of the immune activation determined by *Wolbachia* in mosquitoes, as recently described [33]. Based on the above evidence and considerations, we decided to engineer a mosquito symbiont of the genus *Asaia* for the heterologous expression of WSP, in order to generate a chimeric bacterium capable of inducing immune activation in mosquito hosts and thus potentially interfering with pathogen transmission by the insect.

The first phase of the study consisted in the engineering of *Asaia* strain SF2.1 for the expression of WSP, to determine the production of the protein and to investigate the fitness of the transformed bacteria. The DNA fragment inserted into the plasmid was synthetized optimizing the codon usage and including the signal peptide, in order to allow the delivery of the recombinant protein at the surface of the bacterial cells. Western blotting and immunofluorence tests proved the expression of the protein, and the pattern of immunogold staining was coherent with a localization of the protein at the surface of the bacteria. Genetically modified microorganisms are considered as poor competitors and therefore unable to persist in the environment due to energetic inefficiency. Indeed, several studies support the idea that engineered bacteria are less fit than their native strains, but there are also examples of genetically modified organisms that display an increased fitness [34]. Therefore, the capability of *Asaia*^{WSP} to grow at different pH conditions for 24 h, under continuous observation, was tested. As expected, growth rate of *Asaia*^{WSP} did not surpass either those of *Asaia*^{pHM4} or of the wild type strain. In fact, the growth rate of *Asaia*^{WSP} was slightly slower, but the differences were not significant in all the tested conditions, but one. Thus, WSP expression does not appear to determine a significant reduction of the fitness of *Asaia*, hence a significant energetic load.

The studies published so far on the immune-modulating properties of WSP in humans, dogs, rodents and mosquitoes [25,27,29,35,36,37] have been conducted using a recombinant protein produced in *E. coli*, i.e. using a system that implies a possible contamination by LPS, even after highly accurate purification procedures. In the current study, immunological assays, carried out *in vitro* and *in vivo* in mosquitoes, prove to be an

experimental system in which the control is very sound. The capability of *Asaia*^{WSP} to induce phagocytosis and immune gene activation was significantly higher than that of *Asaia*^{pHM4}*, in vitro* assays*.* Similarly, after *in vivo* tests in mosquitoes, *Asaia*^{WSP} significantly increased the production of antimicrobial peptides and other immune modulators, compared to *Asaia*^{pHM4} (see discussion below).These results rule out the possibility that the observed higher activation of the immune response is due to contamination by LPS or other molecules, since cells and mosquitoes were stimulated with two strains of *Asaia* bacteria, with their load of LPS and other immune-modulating molecules, differing only for WSP expression. In summary, our results provide a further evidence of the capability of WSP to induce innate immune responses in mosquitoes.

It has been proposed that when *Wolbachia* is forced to create a new symbiosis with a mosquito that is naturally non infected by this bacterium, the basal immune response of the insect is enhanced, with negative effects on the mosquito's ability to transmit pathogens [33]. Mosquitoes that normally do not harbour *Wolbachia*, such as An. stephensi and *Ae. aegypti*, can therefore be regarded as good candidates to verify the immune-modulatory effects of *Asaia*^{WSP} and, accordingly, its anti-parasite effects. In *Ae. aegypti* both immune deficiency- and Toll-pathways are activated by *Wolbachia* upon its introduction into the insect [33,38]. As for *Anopheles* mosquitoes, no *Wolbachia* had been detected in the 38 surveyed species (including *An. stephensi*) [39,40], till recent reports that identified *Wolbachia* in *An. gambiae* and *Anopheles arabiensis* [26,41]. In these local mosquito populations, naturally infected by *Wolbachia*, the presence of the bacterium negatively impacts *Plasmodium* sporozoite development [42]. In addition, studies on *An. gambiae* transinfected by *Wolbachia* suggest that *Wolbachia* can confer protection to mosquitoes against the pathogen *Plasmodium*, with an early activation of the immune response [43].

The results of our *in vivo* studies on *An. stephensi* and *Ae. aegypti* proved that *Asaia*^{WSP} is able to induce an immune activation when ingested by mosquitoes. A diverse repertoire of genes coding for immune effector molecules, such as cecropin, thio-ester containing proteins, leucine-rich repeat protein and CLIP-domain serine protease, plus NADPH-oxidases and NO synthase, were significantly upregulated in the presence of *Asaia*^{WSP}; whereas defensin levels remained unchanged. In both *An. stephensi* and *Ae. aegypti*, *TEPs* were among the most upregulated genes, after the bacterial meal with *Asaia*^{WSP}. We emphasize that, in *Anopheles* mosquitoes, TEP induced by *Plasmodium berghei* binds and kills invading *Plasmodium* ookinetes [44]. In transgenic mosquitoes overexpressing *TEP1,* a reduced number of *Plasmodium* parasites has also been observed [45]. *In Drosophila melanogaster* TEPs are required for efficient phagocytosis of both Gram-positive and Gram-negative bacteria [46].

Antimicrobial peptides, that displayed upregulation in both *An. stephensi* and *Ae. aegypti* mosquitoes after feeding *Asaia*^{WSP}, are cecropins, at 6 and 12 hours. Similar results (i.e. the activation of cecropin expression after stimulation with the engineered bacteria) were obtained *in vitro* on haemocytes from both species. It is interesting that cecropins have been shown to inhibit *Plasmodium* development, and also to display antiviral effects, e.g. on HIV-1 [15]. In *An. stephensi*, the *APL1C* gene showed an up-regulation after all the three analysed time points; it is reported that APL1C protein is needed for protection against the rodent malaria parasites *P. berghei* and *Plasmodium yoelli* [47]. Another gene that displayed a strong up-regulation (at 24h), after the *Asaia*^{WSP} stimulus, was CLIPB37;

this result is consistent with the overexpression recorded for this gene after *Wolbachia* infection in *Ae. aegypti* and *An. gambiae* mosquitoes, which determined an inhibition of pathogen transmission [48, 49]. The *NOS* gene expression has been determined both in haemocytes and in mosquitoes: our results recorded a significant expression of NOS gene after the induction by *Asaia*^{WSP}. Mosquito NOS gene, highly homologous to the NOS genes of vertebrates, is known to be expressed during the malaria parasite invasion. In *An. stephensi* mosquitoes, for example, NO production has been shown to limit the development of *Plasmodium* parasites, in particular reducing the release of sporozoites into the hemolymph [50]. In summary, our results show that *Asaia*^{WSP} determines an upregulation of four genes in *An. stephensi*, that have been recorded, in previous studies, to be involved in mosquito defence against *Plasmodium* spp.

A notable characteristic of *Asaia* bacteria is their capability to colonize mosquitoes feeding on sugar meals containing the bacteria, offering a potential tool for their introduction in the field. Our results show that *Asaia*^{WSP} still possess the ability to colonize mosquito organs, with an increase in its abundance after the blood meal (Fig. 4). We emphasize that, in laboratory conditions, cotton pads containing *Asaia* bacteria, either wild type or genetically modified for WSP expression, resulted more attractive to mosquitoes than the sterile ones. Moreover, recent laboratory and field investigations, using sugar feeding as a mean for introducing bacteria different from *Asaia* into adult mosquitoes, highlighted that these insects are attracted by both sterile sugar solutions as well as by solutions containing the bacteria [51].

The evidence that *Asaia*^{WSP} colonizes mosquitoes, and primes the immune response, encouraged us to test the capability of this strain to interfere with the transmission of a parasite by the mosquito themselves. As proof of principle, we focused our attention on a filarial parasite, also considering that paratransgenesis has not yet been applied in the control of insect-borne pathogenic nematodes. The model organisms, that we selected for this test, were the filarial parasite *D. immitis* and the mosquito vector *Ae. aegypti*. Our results show that *Asaia*^{WSP} indeed interferes with *D. immitis* infection in mosquitoes, with significant differences in terms of developed L3 filarial larvae, in comparison with insects fed on sugar meals. We also recorded a difference in L3 numbers in mosquitoes fed on *Asaia*^{WSP} in comparison with those fed on *Asaia*^{pHM4}, but this difference, although evident, was not significant. However, taken together the results on the immune priming by *Asaia*^{WSP} in mosquitoes and the coherence in the results determined by this bacterium on filaria development in *Ae. aegypti* (in terms of both larval abundance and prevalence, Fig. 5), are highly encouraging. In summary, *Asaia* engineered for the expression of WSP can be regarded as an inductor of innate-immune responses in mosquitoes, worth of further investigations for its potential effects on filarial parasite development.

Asaia bacteria have already been investigated for their capacity to interfere with pathogen transmission by mosquitoes. In a study conducted on the murine malaria model *P. berghei*, *Asaia* engineered for the expression of the scorpine antimicrobial peptide determined the inhibition of parasite infection in mosquitoes [5]. More recently, a native *Asaia* strain (SF2.1) has been shown to activate mosquito immunity, with reduction of *P. berghei* development in *An. stephensi* [52]. Our current study shows that the immune stimulating capability of *Asaia* can be boosted through the expression of a protein form *Wolbachia*. The obtained results highlight the plasticity of the *Asaia* system: the engineered bacteria, while expressing a heterologous protein at the surface, preserved

their ability to colonize the insect, determining overexpression for most of the tested mosquito immune effectors. The evidence of the immune-activating capability of *Asaia*, either native [52] or genetically modified, as in the current study, requires to be validated with assays on wild-collected mosquitoes; moreover, future investigations should address the potential of *Asaia* and *Asaia*^{WSP} to interfere with the transmission of arboviruses, such as dengue and Zika. Indeed, a generalized activation of mosquito immunity might imply a generalized protection of the mosquito toward infectious agents, not only filarial nematodes. Finally, safety issues should properly be addressed before proposing any strain of *Asaia* for field release. In this context, interesting investigations have been conducted on *Wolbachia* for the control of dengue virus [53], including a study on the potential transmission of this bacterium to humans [54]. We emphasize that there is strong evidence that *Asaia* colonizes the salivary glands of mosquitoes; mosquitoes might thus inoculate *Asaia* into mammals, including humans. The sole epidemiological investigation, performed so far, has not revealed any evidence for *Asaia* infection in humans, either in serological or PCR-based analyses [55]. However, we can hypothesize that *Asaia*^{WSP} possesses increased immune-stimulating properties also toward humans, hence an increased pathogenic potential (e.g. proinflammatory properties [27, 56]). Therefore, the issue of the potential transmission of *Asaia* to mammals would require further consideration.

METHODS

Bacterial strains and media.

Asaia SF2.1 strain (*Asaia*^{wt}), originally isolated from an *An. stephensi* mosquito [4], was grown at 30°C in GLY medium (25 g L^{-1} glycerol, 10 g L^{-1} yeast extract, pH 5; eventually, GLY medium was solidified adding 20 g L^{-1} agar). *E. coli* XL1Blue (Stratagene), used as the host for construction of plasmids, was grown at 37°C in Luria Broth (LB; LB medium was solidified adding 15 g L^{-1} agar if necessary). If needed, 100 µg/mL kanamycin was added to the media.

Plasmid construction

Plasmid pHM4 (≈5.5 kbp) was obtained by digesting pHM2 [57] with the restriction enzyme *SacI* (Life technologies Italia). Plasmid pHM4-WSP was then constructed by inserting the WSP cassette flanked by *NotI* sites in plasmid pHM4. WSP cassette was synthesized by Eurofins Genomics (Milan) in plasmid pUC57, obtaining the plasmid pUC57-WSP. WSP cassette contains the neomycin phosphotranferase promoter *PnptII*, the coding DNA sequence of WSP from *Dirofilaria immitis* including the signal peptide of the gene [37], the E-TAG epitope (GAPVPYPDPLEPR, [11]) and the transcription terminator *Trrn*. E-TAG epitope was inserted in the 4th loop (L4) of the *wsp* sequence [58] to allow the immunodetection of the expressed protein. Moreover, the *wsp* gene sequence was optimized according to the codon usage of strain SF2.1 as inferred from its genome sequence [59]. WSP cassette was then digested from pUC57-WSP by using the restriction enzyme *NotI*, loaded in 1% agarose gel and purified by using QIAquick Gel Extraction Kit (Qiagen). Plasmid pHM4 was digested with *NotI*, dephosphorylated by using Shrimp Alkaline Phosphatase (SAP, Life technologies Italia) and the *wsp* fragment was ligated to the *NotI*-linearized pHM4 by using T4 DNA ligase (Life technologies Italia). Ligation product was then used to transform *E. coli* XL1Blue electrocompetent cells [4]. Recovery was performed with LB medium for 1 hour at 37°C with shaking before plating on LB plates

added with kanamycin. Putative transformants were selected and successful ligation of WSP cassette was checked by PCR using *wsp*-specific primers (see Supplementary Table 1). The obtained plasmid, named pHM4-WSP (Supplementary Fig. 1a), was then extracted from *E. coli* and electroporated in *Asaia*^{wt} as previously described [4] resulting in the strain *Asaia*^{WSP}. Strain *Asaia*^{pHM4} was also obtained and used as control in the following experiments.

Western-blot detection of WSP produced by *Asaia* strains

For protein secretion, the *Asaia*^{WSP} and *Asaia*^{pHM4} were grown overnight at 30°C in GLY medium supplemented with 100 µg ml^{-1} of kanamycin. Bacterial cultures were centrifuged at 3000 g for 15 min at RT and pellets were resuspended in SDS sample loading buffer 1X; a protease inhibitor cocktail was also added to avoid the protein degradation. Briefly, membranes were blocked in blocking buffer (4% milk in PBS with 0.1% Tween 20) and probed with the primary goat anti-E tag antibody (Novus Biologicals), followed by an HRP-conjugated anti-goat IgG secondary antibody.

RNA extraction and reverse transcription-quantitative PCR

Bacteria were grown at OD 0.5, 1, 1.5 (three pools each) and stored in RNA PROTECT BACTERIA Reagent (Qiagen); RNAs were extracted using RNeasy Mini Kit (Qiagen) including an on-column DNase I treatment to remove residual DNA. RNA was stored at −80°C till further use. RNA purity was checked by determining the 260/280 nm absorbance ratio. cDNAs were synthesized from 250 ng of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen) with random hexamers. The cDNA was used as template in RT-PCR reactions. Quantitative RT-PCRs on *Asaia*^{WSP} and *Asaia*^{pHM4} were performed under the following conditions: 100 ng cDNA; 250 nM of forward and reverse primers (target gene *wsp*; see Supplementary Table 1 for primers sequences); 98°C for 30 sec, 40 cycles of 98°C for 15 sec, 58°C for 30 sec; fluorescence acquisition at the end of each cycle; melting curve analysis after the last cycle. The quantification cycle values were determined, in order to calculate gene expression levels of the target gene relative to 16S rRNA*,* the internal reference gene for *Asaia* [55]. The estimates of the expression level of *wsp* gene has been reported as the means ± standard error (SEM).

***In vitro* growth assays of *Asaia* for fitness measurements**

Growth assays of *Asaia* strains were performed at different pH values (from 3.5 to 8.0 with increases of 0.5). Bacterial cells were grown overnight at 30°C with constant agitation (130 rpm) in GLY broth. For recombinant strains, 100 µg ml^{-1} kanamycin was added to the medium. For each strain, a dilution to 0.1 optical density at 620 nm (OD_{620}) was carried out in GLY medium at different pH (range 3.5 - 8.0) and 200 µl were distributed in 96-well microtiter plates wells (two wells for each condition). Growth was recorded by an EnSight plate reader (Perkin Elmer), measuring the OD_{620} in each well every 10 min for 24 hours at 30°C. Growth was recorded by an EnSight plate reader (Perkin Elmer), measuring the OD_{620} in each well every 10 min for 24 hours at 30°C. As negative control, growth medium without bacteria was used. OD_{620} values were collected and, after baseline correction, the maximal growth rate (MGR) (h^{-1}) was estimated as the slope of the best regression line which fitted to growth curve, for either of the strains during the time interval. Growth assays were repeated three times. MGRs were compared by strain and medium pH using

a Welch test. Student's t-test (two-sides, Welch's correction) was performed by GraphPad Prism 5 software. $P<0.05$ was considered significant.

Immunofluorescence assays on bacteria and on mosquitoes

Recombinant *Asaia* bacteria expressing WSP or with plasmid alone were grown as reported above; 10 µL of a cell suspension at the concentration of 10^8 cells ml^{-1} in PBS were placed on glass slides, air dried, and fixed for 20 min with cold methanol. Bacterial cells were blocked in bovine serum albumin (FBS) and probed with the primary goat anti-E tag antibody (Novus Biologicals), followed by incubation with an anti-goat IgG secondary antibody, FITC Conjugate (Sigma-Aldrich).

As for the detection of transgenic bacteria in mosquito organs, *Asaia*^{WSP} or *Asaia*^{pHM4} were administered to 2-3 day-old *Ae. aegypti* (Liverpool black-eyed strain) females via sugar meal (1×10^8 cells ml^{-1}) plus kanamycin (100 µg ml^{-1}) for 24 hours; 24 hours after the bacterial meal, fed mosquitoes were selected and their organs (crop, midgut and ovaries) dissected and fixed in 4% (wt vol^{-1}) paraformaldehyde at 4°C, washed in PBS, and blocked with 4% (wt vol^{-1}) FBS. The samples were then probed with goat anti-E tag antibody, followed by incubation with a FITC-anti-goat IgG secondary antibody. Observations were recorded with a Leica microscope (LeicaTCSNT) and analysed with ImageJ software. Survival of mosquitoes was also monitored daily. Survival percentages represent the mean survival percentage of three biological replicates of 30 mosquitoes each.

Immunogold staining on bacteria pure culture

Asaia^{WSP} or *Asaia*^{pHM4} samples were fixed by immersion in 4% paraformaldehyde in PBS for 2 h at 4°C and washed in PBS. Free aldehydes were blocked in 0.5 MNHCl in PBS for 45 min at 4°C; samples were washed in PBS, dehydrated through graded concentrations of ethanol and embedded in LR White resin (Electron Microscopy Sciences) overnight, at 4°C. Resin samples were polymerized for 24 h at 60°C. Ultrathin sections were placed on grids coated with a Formvar-carbon layer and then processed for immunocytochemistry. Ultrathin sections were floated for 3 min on normal goat serum (NGS) diluted 1:100 in PBS and then incubated overnight at 4°C with goat anti-E tag antibody diluted with PBS containing 0.1% BSA and 0.05% Tween 20. After rinsing, sections were floated on NGS and then reacted for 20 min at room temperature with secondary 12 nm gold-conjugated antibodies (Jackson Laboratories) diluted 1:20 in PBS. The specimens were observed on a Philips Morgagni transmission electron microscope operating at 80 kV and equipped with a Megaview II camera for digital image acquisition.

Colonization and quantification of *Asaia* in mosquitoes

To investigate colonization of *Asaia* in different tissues of mosquitoes, 2-3 day-old adult mosquitoes were fed for 24 h on a cotton pad moistened with 5% sterile sucrose solution containing 10^8 cells ml^{-1} bacteria (T0). The bacteria-fed mosquitoes were starved for 10 h, and then allowed to feed on a blood meal. Twenty-four (T1) and 48 h (T2) after the blood meal, the individual mosquitoes were surface-sterilized by washing them in 75% ethanol for 3 min and then rinsing them in sterile PBS three times. The crop, midgut and ovaries were dissected under sterile conditions and homogenized in 0.2 ml sterile PBS. The bacterial load was determined by plating ten-fold serial dilutions of the homogenates on GLY plates containing 100 µg ml^{-1} of kanamycin and incubating the plates at 30°C for 48 h. The colonies were counted and the data analysed using RStudio. Briefly, a three-way

ANOVA was used to test the global variance of the data and to assess which of the three categorical independent variables (*Asaia* strain, time and mosquito tissues) influences the *Asaia* load. After having assessed that the *Asaia* strain did not affect the colonization of the mosquito tissues, we performed a two-way ANOVA (using time and mosquito tissues as categorical independent variables) to test the interactions of the variables and one-way ANOVA to analyse the variance of *Asaia* within each of the tissues over time.

Haemocyte primary cultures and phagocytosis test

Mosquito haemocytes were isolated from dissected *An. stephensi* and *Ae. aegypti* adults and maintained 72 hours in Schneider's medium (Sigma-Aldrich), supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin, before further analyses. Antibiotics have been removed before the phagocytosis test by centrifugation of cells and resuspending them in fresh medium without any addition. In the phagocytic tests, haemocyte cultures from both mosquito species were incubated for 6 h in 1 ml of medium containing bacteria. Successively, haemocytes were shortly centrifuged, resuspended in 200 µl of fresh Schneider's medium (without any supplement) and then incubated with 0.1 µl of a FITC-fluorescent beads suspension for 1 and 2 hours in soft oscillation, according to [60]. After incubation, cells were cytocentrifugated onto glass slides, counterstained with a 200 ng ml^{-1} propidium iodide solution and observed with a Zeiss Axioplan epifluorescence microscope. The phagocytosis index was evaluated as the percentage of haemocytes showing inside fluorescent particles. Three phagocytic test replicated experiments were performed. Statistical analysis has been performed using GraphPad Prism 5 utilizing the two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test ($p<0.05$ has been considered significant).

Antimicrobial peptides and nitric oxide synthase expression in hemocytes

An. stephensi and *Ae. aegypti* hemocytes has been incubated with a 10^9 cells ml^{-1} bacterial solution for 0, 3, 6, 9, 12 and 24 hours. After treatments, cells were centrifuged at 800 g for 5 min at room temperature and the supernatant was discarded. Total RNA was extracted from cells using TRI-REAGENT TM (Sigma), following the method described by the supplier. RT-PCR has been performed with the Access RT-PCR System (Promega), according to the supplier's protocols. For *An. stephensi* and *A*e. *aegypti, actin* was used as reference gene; the sequences of the analysed genes and the relative citations were reported in Supplementary Table 1. For both species, PCR amplification gel documentation was collected using a Gel Doc XR, digitally evaluated with Quantity One (Bio-Rad Lab) and normalized to the correspondent signals for cytoplasmic actin. Three replicates were carried out for each induction.

Immune gene expression in mosquitoes fed with bacteria

Asaia^{WSP} or *Asaia*^{pHM4} were administered to 2-3 day-old adult female mosquitoes (*An. stephensi* and *Ae. aegypti*) via sugar meals, bred in small cages containing 50 samples. Mosquitoes were allowed to feed for 6, 12 and 24 h on a sterile cotton pad moistened with 5% sterile sucrose solution containing 10^8 cells ml^{-1} bacteria (plus kanamycin 100 µg ml^{-1}), or 5% sugar plus kanamycin with no bacteria (as control).

After 6, 12 and 24 h, the mosquitoes were collected and stored in RNA later at −80°C for RNA extraction and molecular analysis. The expression profiles of 11 immune-related

genes (see Supplementary Table 1 for primer sequences and details), were analysed by Quantitative RT-PCRs. Briefly, RNA was extracted from pool of three mosquitoes using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. cDNAs were synthesized from 150 ng of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCRs on target genes were performed using a BioRad Real-Time PCR Detection System (Bio-Rad) at the following conditions: 50 ng cDNA; 300 nM of forward and reverse primers; 98°C for 30 s, 40 cycles of 98°C for 15 s, 56-60°C for 30 s; fluorescence acquisition at the end of each cycle; melting curve analysis after the last cycle. In order to calculate the expression of the target genes, quantification cycle (Cq) values were determined for each gene and normalized according to the endogenous reference genes *rps7* or *rps17* (Table S1). The estimates of the expression level of each gene are relative to the control groups and reported as fold change mean ± standard error mean (SEM) of at least three replicates. Statistical analysis has been performed using GraphPad Prism 5 utilizing the analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test ($p<0.05$ has been considered significant).

Mosquito infection with bacteria and microfilariae

Microfilariaemic blood samples from a naturally infected dog and blood from uninfected dog were kindly provided by Prof. Genchi; bloods were anticoagulated with heparin. Since blood was collected for diagnostic purposes and the owners signed an informed consent that authorize the use of residual samples (i.e. the amount of blood remained after diagnostic clinical chemistry) for research purposes, according to the regulations of our Institution (EC decision 02-2016) a formal approval from the Ethical Committee was not required. Vitality and number of *D. immitis* microfilariae in all samples were confirmed by microscopy; briefly, 20 µl of blood were mixed with 40 µl of distilled water, covered with a cover slide, and microfilariae were counted by examination with a microscope (4X). Microfilaraemiae of the dog was determined three times. For the inoculation experiments, *Ae. aegypti* female mosquitoes at an age of 2–3 days were selected, maintained at standard condition in cages of 100 samples [61] and fed on a sterile cotton pad moistened with 5% sucrose solution containing 10^8 cells ml^{-1} bacteria (with kanamycin 100 µg ml^{-1}) for 1 day (four treatments: mosquitoes fed with *Asaia*^{WSP} or *Asaia*^{pHM4} plus microfilariae, sugar solution with/without kanamycin plus microfilariae).

Microfilariaemic counts were adjusted to 3500 mf ml^{-1} with blood from uninfected dog. The microfilaria load in the infecting blood was according to recommended protocols [62], in order to avoid an excess in larval mortality, caused by nematode larvae. Sugar was removed and the mosquitoes were allowed to feed through Parafilm® membranes for at least 1.5 h on 5 ml blood at 37°C in an artificial feeding system. Three to five mosquitoes were immediately dissected to verify mean microfilariae ingested per mosquito. Mosquitoes were kept for up to 14 days in cages with access to 5% glucose and water ad libitum; after this time, mosquitoes were collected and exposed for 2 min in a freezer for immobilization, and the wings and legs were removed. Only the mosquitoes for which a blood meal was completed were collected. These mosquitoes were dissected individually: the abdomen was separated and midgut contents were smeared on a slide; *D. immitis* L3 larvae were thus counted. Statistical analysis was performed by GraphPad Prism 5 software. The mean number of individuals presenting L3 larvae was tested using contingency analysis and the final p values were adjusted using FDR, while the geometric mean number of L3 larvae per infected mosquito was calculated using the William's mean

(Mw) [63], considering the high proportion of mosquitoes not presenting L3 larvae, and Mw were analysed using a ONE-way ANOVA followed by Tukey's post-hoc test.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author (Molecular and Evolutionary Parasitology Lab, Department of Biosciences, University of Milan), on reasonable request.

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Author Contributions

S.E and I.V.B. performed and supervised the majority of experiments. S.E. and E.C. generated transgenic bacteria; I.V.B., L.G. and L.P. conducted fitness experiments; M.B. performed immunogold assays and M.M. conducted the *in vitro* experiments; C.D., P.G. and M.G. performed the cage experiments and transmission-blocking assays; G.F., D.D. and C.B. analyzed the data; S.E., I.V.B. and C.B. wrote the manuscript; all authors revised and provided inputs to the manuscript.

Competing Interests statement

The authors declare no competing interests.

Figures

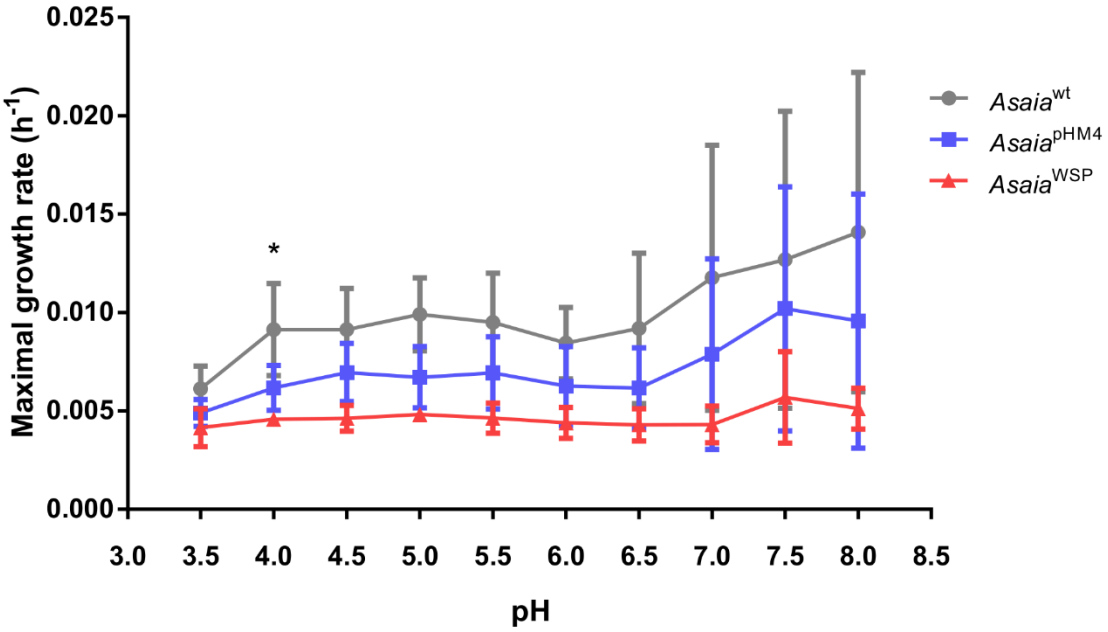


Fig. 1 Grow rates (MGRs) of *Asaia*^{wt} and recombinant strains (*Asaia*^{WSP} and *Asaia*^{pHM4}). MGRs were estimated as the slope of the best regression line which fitted to the 24 h growth curves calculated for either of the strains by measuring OD_{620} at ten different pH values in GLY medium. N=three independent experiments were conducted. Bars represent standard deviations. Statistical analysis was carried out by Welch test with GraphPad Prism 5 software; the 24 h growth curves of wild type and both transformed strains showed differences between the mean MGRs in almost all the tested growth conditions, but these differences were not significant, with the exception of the growth at pH 4 (*$p<0.05$, *Asaia*^{WSP} vs *Asaia*^{wt}).

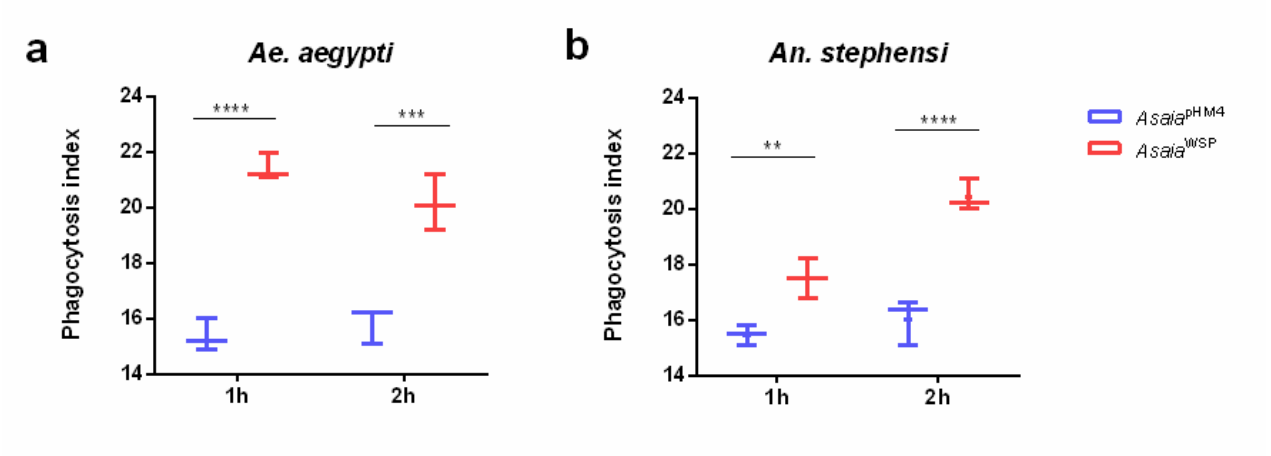


Fig. 2 Phagocytosis tests. Phagocytic activity was evaluated in vitro using cultured (**a**) *Ae. aegypti* and (**b**) *An. stephensi* haemocytes exposed to bacterial cells from strains *Asaia*^{pHM4} and *Asaia*^{WSP} incubated with FITC-fluorescent beads suspension. The percentage of haemocytes showing fluorescent phagocytised bacteria was evaluated after 1 h and 2 h. Values are expressed as median±max and min of n=3 replicates. N=three independent experiments were conducted. Statistical significance for each experiment was determined using the two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test where significance is represented by **p< 0.01, ***p< 0.001 and ****p< 0.0001.

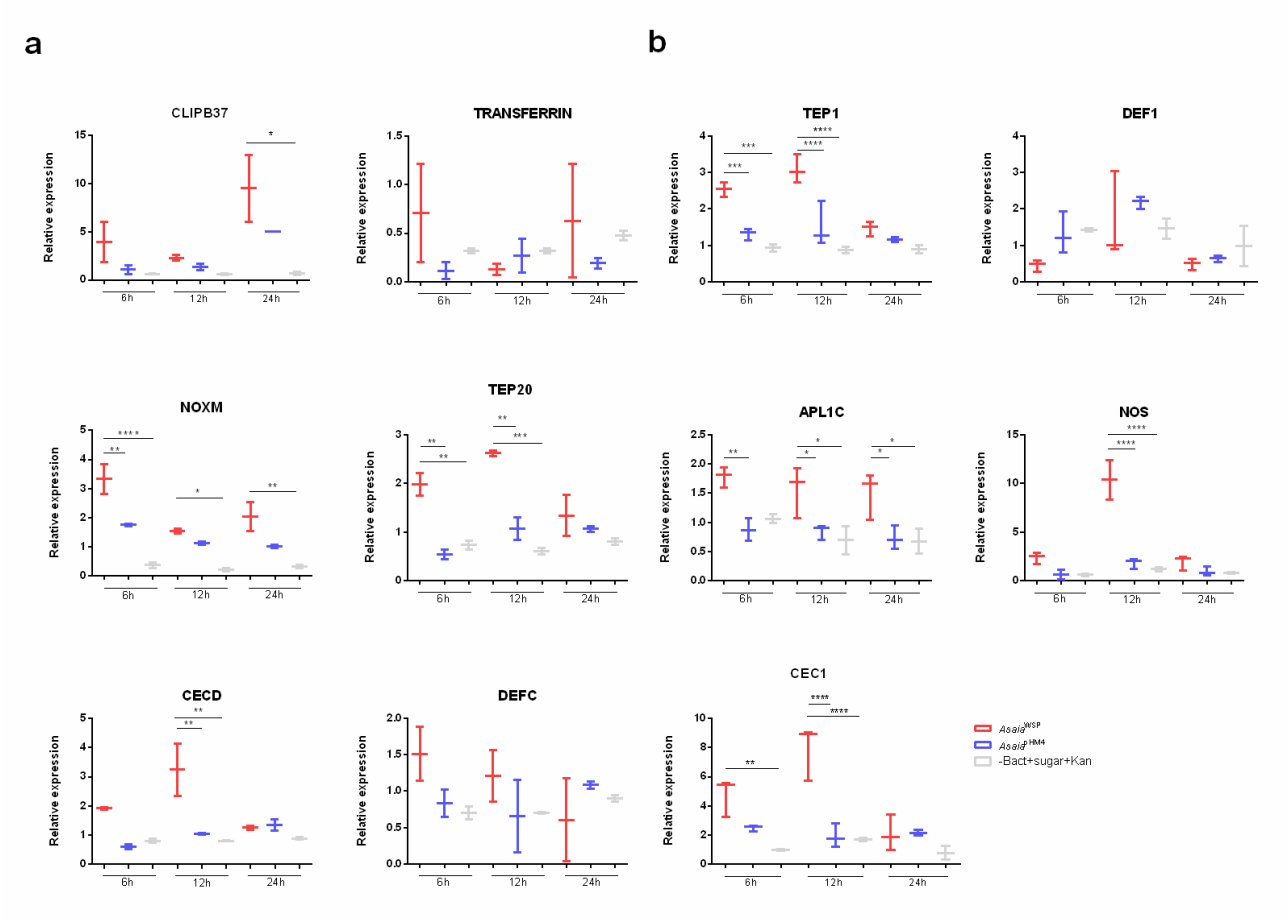


FIG. 3 qRT-PCR analyses of differentially regulated genes. **a**. The differential regulation of transcript levels in *Asaia*^{WSP} infected *Ae. aegypti* versus *Asaia*^{pHM4} infected *Ae. aegypti* or versus mosquito fed only with sugar plus kanamycin (100 µg/mL kanamycin was g ml-1), was examined for six selected genes: cecropin (CECD), a transferrin (TRANSFERRIN), a CLIP-domain serine proteases (CLIPB37), a thioester-containing protein (TEP20), a NADPH oxidase (NOXM) and a defensin (DEFC). **b**. The differential regulation of transcript levels in *Asaia*^{WSP} infected *An. stephensi* versus *Asaia*^{pHM4} infected *An. stephensi* or versus mosquito fed only with sugar plus kanamycin (100 µg/mL kanamycin was g ml-1), was examined for five selected genes: cecropin (CEC1), an *Anopheles Plasmodium*-responsive LRR protein-1C (APL1C), a nitric oxide synthase (NOS), a thioester-containing protein (TEP1) and a defensin (DEF1). The values shown are median±max and min of at least two different qRT-PCR experiments with independent samples. Statistical analysis has been performed utilizing the analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test where significance is represented by *p< 0.05, **p< 0.01, ***p< 0.001 and ****p< 0.0001.

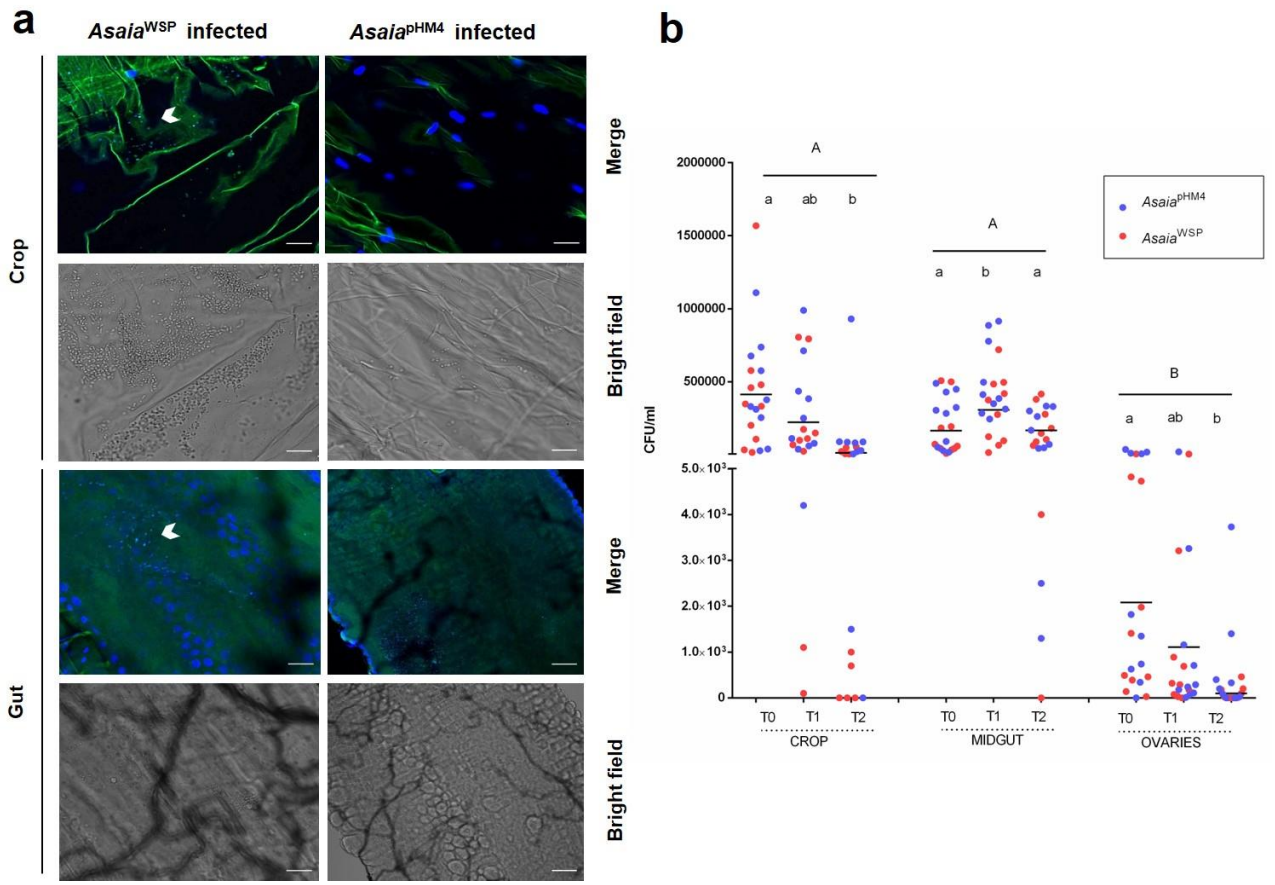


Fig. 4 *Asaia* bacteria colonize mosquito organs and rapidly proliferate after a blood meal. **a.** Immunofluorescence on *Asaia*^{WSP} in *Ae. aegypti* mosquitoes. *Asaia*^{WSP} or *Asaia*^{pHM4} were introduced to 2-3 day-old *Ae. aegypti* females via sugar meal plus kanamycin (100 µg/mL kanamycin was g ml-1) for 24 hours; 24 hours after the bacterial meal, fed mosquitoes were selected and their organs dissected and probed with anti-E tag antibody, followed by incubation with a FITC- anti-goat IgG secondary antibody. Panels show the bright-field of the organs and the staining of the *Asaia*^{WSP} (white arrows indicate group of bacteria) or the staining of *Asaia*^{pHM4}. Bars: 100 µm. **b**. Population dynamics of *Asaia*^{pHM4} and *Asaia*^{WSP}. *Asaia*^{pHM4} and *Asaia*^{WSP} were fed to 2-3-day-old *Ae. aegypti* mosquitoes in a sugar meal for 24 hours (T0), then mosquitoes were allowed to feed on a blood meal and collected after 24 (T1) and 48 hours (T2). Bacteria colony-forming units were determined by plating serially diluted homogenates of organs on GLY plates containing 100 µg/mL kanamycin was g ml^{-1} of kanamycin. The maximum bacteria number is reached when microfilariae would be invading the midgut if the blood was infected with the parasite (T1). Different capital letters represent statistically significant differences between examined organs ($p<0.05$). Different lowercase letters represent statistically significant differences between time points in each organ ($p<0.05$). Bars indicate the means.

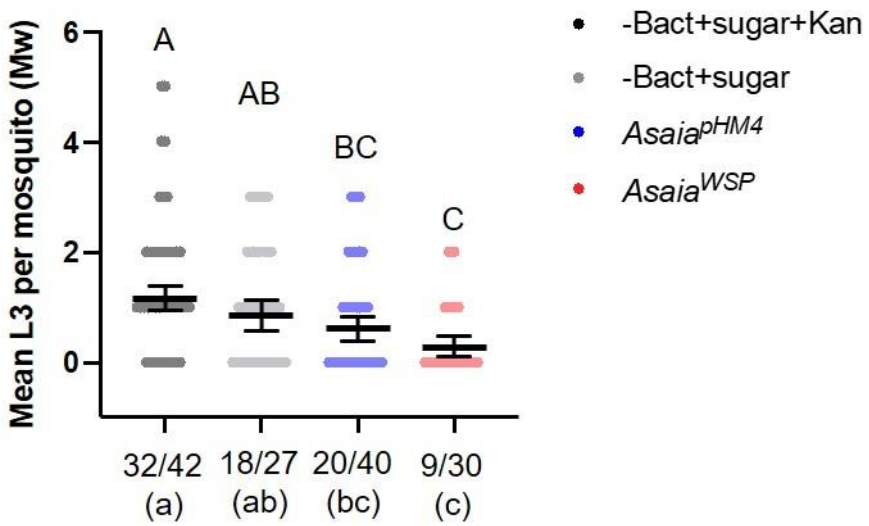
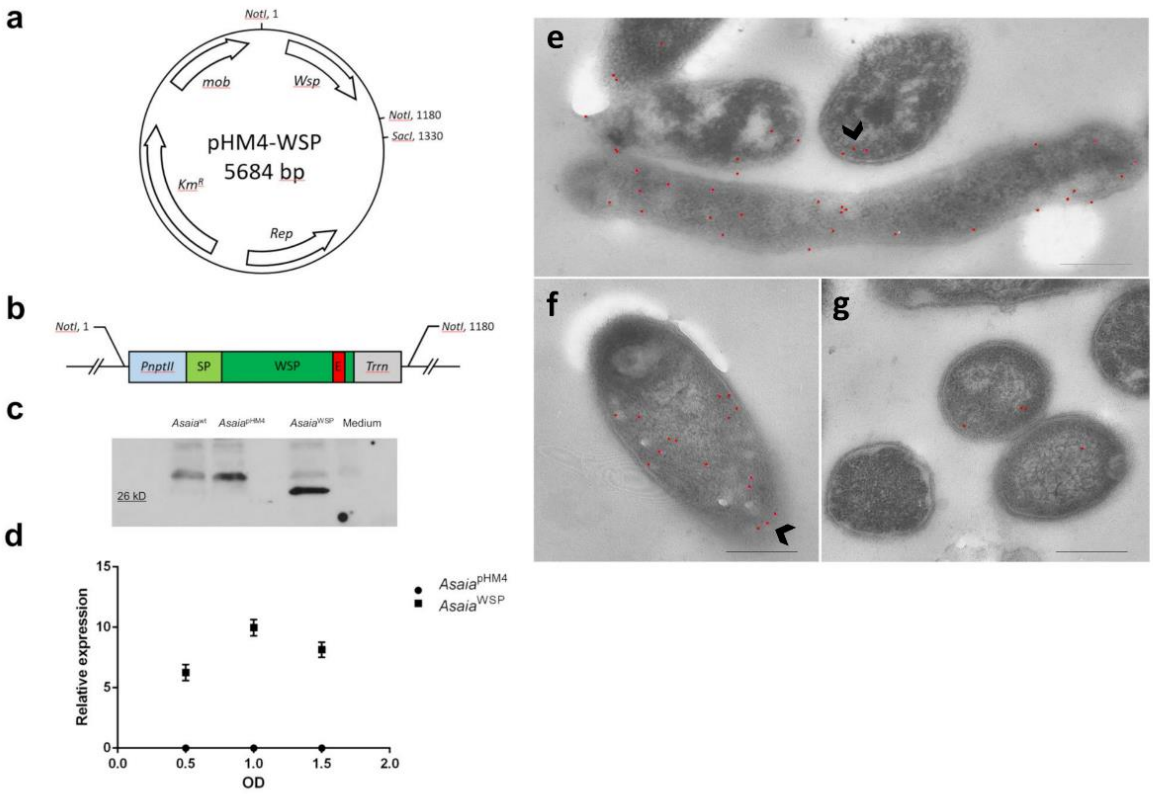


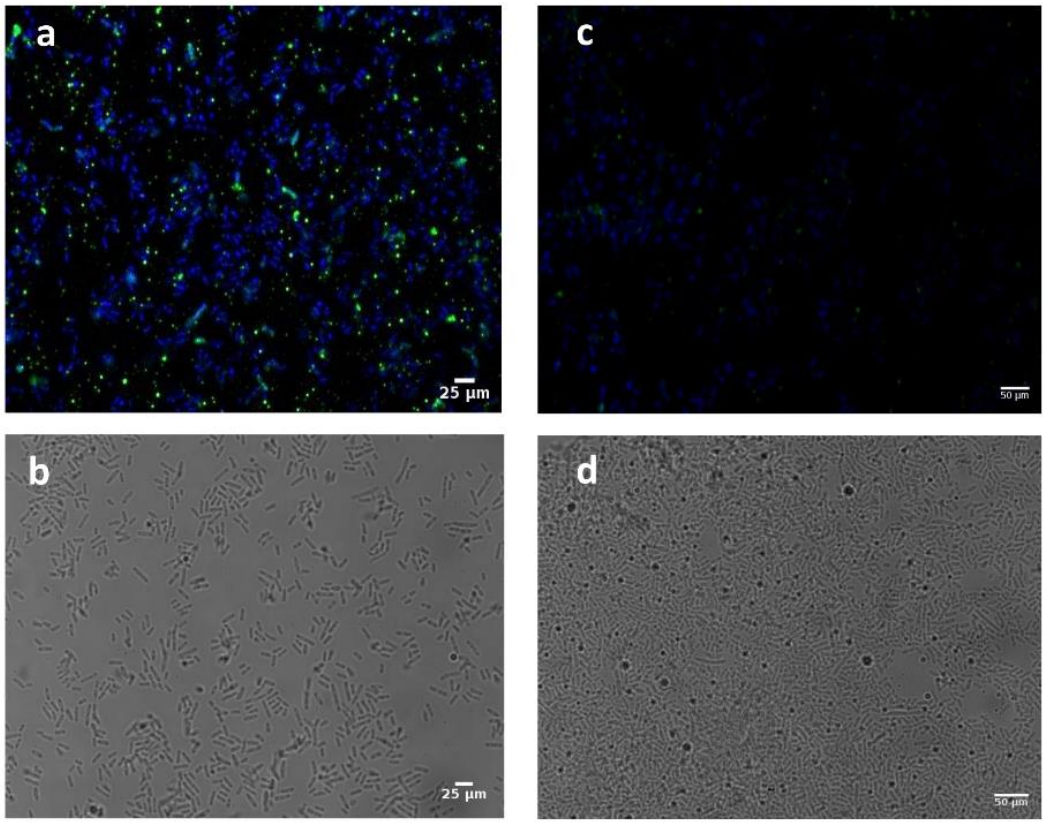
Fig. 5 *Ae. aegypti* infection with transgenic bacteria and *D. immitis* microfilariae. Scatter dot plots show the number of L3 larvae per mosquito. The mean numbers (±SEM) of infective L3 stage of *D. immitis* were determined 14 days post microfilarial challenge in *Ae. aegypti* by microscopical observation. Four treatments have been tested: mosquitoes fed with *Asaia*^{WSP}, *Asaia*^{pHM4}, sugar solution with or without kanamycin, before the infectious blood meal. The graph reports the average number of L3 detected in the dissected mosquitoes (abundance; y axis). The prevalence, i.e. the proportion of mosquitoes that contained at least one L3 larva versus the total number of survived and dissected mosquitoes (starting from n=100 individuals per treatment), is shown on *x* axis. The geometrical mean of L3 larvae per mosquito (William's mean) was tested using a ONE-way ANOVA followed by Tukey's post-hoc test. Different capital letters, on the top of the graph, represent statistically significant differences ($p<0.05$). The mean number of mosquitoes presenting L3 larvae was tested using contingency analysis and the final p values were adjusted using FDR. Different lowercase letters represent statistically significant differences ($p<0.05$).

Supplementary Information

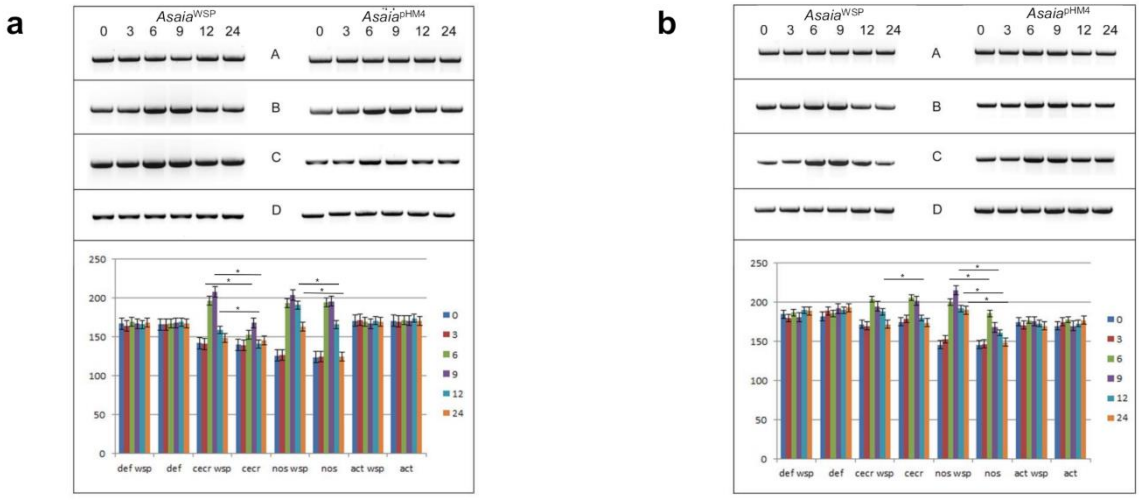
Chimeric symbionts expressing a *Wolbachia* protein stimulate mosquito immunity and inhibit filarial parasite development



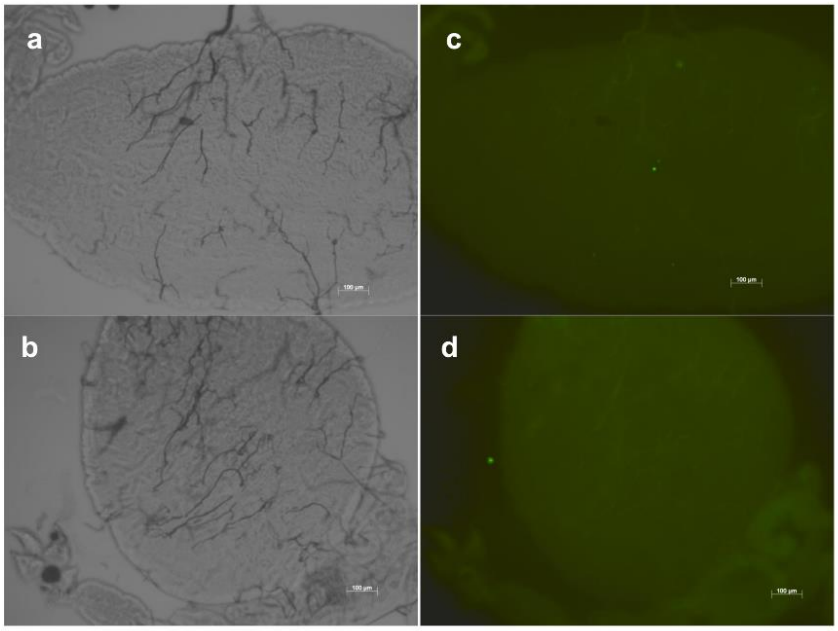
Supplementary Figure 1. WSP protein production. **a**. Map of the plasmid pHM4-WSP. A partial mob gene was present on the final construct that derived from pHM2 one [8]. WSP gene cassette orientation was assessed by PCR. **b**. Details of the WSP cassette. PnptII: neomycin phosphotranferase promoter; SP: signal peptide; WSP: *Wolbachia* surface protein; E: E-TAG epitope; Trrn: transcription terminator. E-TAG epitope was inserted in the 4th loop (L4) of the WSP sequence. Synthetic DNA were synthesized with codons harmonized with the *Asaia* sp. SF2.1 strain preferred codon usage (Accession: NZ_CBLX000000000.1 GI:737464632). **c**. Western blot analysis. The pellet of bacteria was subjected to Western blot analysis using a rabbit anti-E tag antibody. Three strains were tested: the wild strain *Asaia*^{wt}, *Asaia*^{pHM4} and *Asaia*^{WSP}. Images were taken using the same exposure time. 26 kDa represents the molecular weight of WSP protein. Bands with higher molecular weights than the WSP one (26 kDa) are related to nonspecific proteins produced by *Asaia* strains, which cross-reacted with the anti-E tag antibodies. **d**. Expression of wsp gene at different ODs. Bacteria were grown at OD 0.5, 1, 1.5 and analysed for wsp gene expression. *Asaia*^{WSP} showed a maximum expression at OD 1; no expression of wsp was detected for the *Asaia*^{pHM4}. Expression of the WSP protein by the engineered *Asaia*^{WSP} strain. **e-g**. Immunogold staining on the cultured bacteria using anti-E tag antibody. e and f panels show the staining of the *Asaia*^{WSP} (red dots indicate the gold particles present on bacteria and arrows indicate dot groups) while panel g shows the staining of *Asaia*^{pHM4} control. Bar: 1 µm.



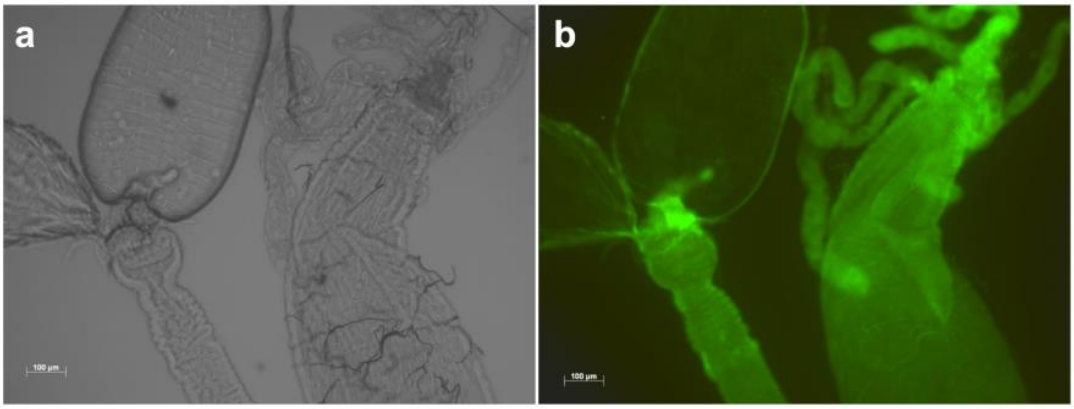
Supplementary Figure 2. Immunofluorescence assay on *Asaia*^{WSP} and *Asaia*^{pHM4} bacteria pure culture. *Asaia*^{WSP} (**a**, **b**) and *Asaia*^{pHM4} (c, d) were blocked in BSA and probed with the primary anti-E tag antibody, followed by incubation with an anti-goat IgG secondary antibody, FITC conjugate. Fluorescent images are shown on a and c panels; top and bright-field images of the same field are shown on b and d panels.



Supplementary Figure 3. Semi-quantitative analysis of the antimicrobial peptides (AMPs) defensin (A) and cecropin (B) and the nitric oxide synthase (NOS) (C) expression in hemocytes. **a**. Gene expression was evaluated in *Ae. aegypti* haemocytes treated with *Asaia*^{pHM4} and *Asaia*^{WSP} at different time points (0, 3, 6, 9, 12, 24 hours). Actin gene (D) was used as a constitutive control gene. **b**. Gene expression was evaluated in *An. stephensi* haemocytes treated with *Asaia*^{pHM4} and *Asaia*^{WSP} at different time points (0, 3, 6, 9, 12, 24 hours). Actin gene (D) was used as a constitutive control gene. The expression of immune genes was evaluated after electrophoresis in 1% agarose gel; the documentation was collected using a “Gel Doc XR” and digitally evaluated with Quantity One as schematized below each panel. Statistical analysis has been performed utilizing the analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons test ($p<0.05$ has been considered significant). One representative set of data is shown.



Supplementary Figure 4. Immunofluorescence controls performed on *Ae. aegypti* mosquitoes. *Ae. aegypti* female mosquitoes were fed with sugar solution for 48 hours until the midgut dissection. Dissected midguts were probed with anti-E-tag antibody, followed by incubation with a FITC-anti-goat IgG secondary antibody. Panels **a** and **b** show the bright-field of two midguts; **c** and **d** pictures display the autofluorescence of the tissues and no staining.



Supplementary Figure 5. Immunofluorescence controls performed on *Ae. aegypti* mosquitoes. *Ae. aegypti* female mosquitoes were fed only with a sugar solution plus kanamycin (100 µg ml^{-1}) for 48 hours until the midgut dissection. Dissected midguts were probed with anti-E-tag antibody, followed by incubation with a FITC-anti-goat IgG secondary antibody. Panel a shows the bright-field of a midgut; b picture displays autofluorescence of the tissues and no staining.

Supplementary Table 1. List of primers used for cloning and real-time PCR analysis here.

TARGET GENE	**FORWARD SEQUENCE 5'-3'**	**REVERSE SEQUENCE 5'-3'**	**REFERENCE**
wsp of *Wolbachia*	TCGATGATGAAGAGACCAG	GCGAGTAAAGACCTTCAATATC	This paper
16S rRNA of *Asaia* sp.	GCGCGTAGGCGGTTTACAC	AGCGTCAGTAATGAGCCAGGTT	[1]
rps7 of *Anopheles stephensi*	AGCAGCAGCAGCACTTGATTTG	TAAACGGCTTTCTGCGTCACCC	[2]
actin of *Anopheles stephensi*	AGCAGGAGATGGCCACC	TCCACATCTGCTGGAAGG	[2]
rps17 of *Aedes aegypti*	TCCGTGGTATCTCCATCAAGCT	CACTTCCGGCACGTAGTTGTC	[3]
actin of *Aedes aegypti*	GATCTGACCGACTACCTGATGA	AGATTCATCGTACTCCTGC	This paper
nitric oxide synthase of *Aedes aegypti*	TGAGTCGTTGCGCTGATTGA	TGGCAACACTGCTGTCTACC	This paper
Thioester-containing protein-1 (*TEP1*) of *Anopheles stephensi*	ACGACGGCTTCAATAACGAT	CCCGAGTTCCAGTTCCACTA	[4]
Anopheles Plasmodium-	CGTGGTAGCGTTCTCCTGAC	GCTGGGACTTCATCACAATC	[4]

responsive LRR protein-1C (APL1C) of *Anopheles stephensi*			
DEF1 of *Anopheles stephensi*	AGGCTGCGGAGAACTATC	ATAGCGACGAGCGATGCAAT	[5]
CEC1 of *Anopheles stephensi*	GCGCCCCGTTGGAAGT	TCAGGTCCGCTCCATTTATCC	[5]
NOS of *Anopheles stephensi*	GGTTCCCATCCGAAGCATT	GCAACACAGGGCAGGTTACAT	[5]
CECD of Aedes aegypti	ATGAACTTCACTAAGCTGTT	TCATTTTCCAATCGCTTTTAT	[6]
DEFC of Aedes aegypti	TTGTTTGCTTCGTTGCTCTTT	ATCTCCTACACCGAACCCACT	[6]
CLIPB37 of Aedes aegypti	TTGGGGGAAAACAGAAACAG	GATCTGCTTCCCAGAGAACG	[6]
NADPH oxidase (*NOXM*) of *Aedes aegypti*	TCCACAATACGGTTTCGCTA	GCCGTCCAACAGAAATTGTA	[6]
Thio-ester containing protein	ATTTTTGACGGCTTTTGTGG	TGGATTACTTGCCCCACTTC	[7]

(*TEP20*) of *Aedes aegypti*			
Transferrin of *Aedes aegypti*	AGCGAACGATGGTTTGAGTT	TATGGCATGCCTTGTACCAC	[7]

Supplementary References

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2.2 ARTICLE 2

BACTERIA EXPRESSING A PROTEIN OF *WOLBACHIA* INDUCE ACTIVATION OF M1 RESPONSE, KILLING *LEISHMANIA* PARASITE

Bacteria expressing a protein of *Wolbachia* induce activation of M1 response, killing *Leishmania* parasite

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ABSTRACT

Leishmaniases are severe vector-borne parasitic diseases affecting humans and dogs, caused by *Leishmania* protozoans. Millions of dogs and over 350 million people are at risk of infection. Immune polarization is thought to play a major role in determining the outcome of *Leishmania* infection: hosts polarized on the Th1 side are protected, while those displaying a Th2 response acquire a chronic infection, eventually developing into an overt and potentially deadly disease. The identification of M1/Th1-biasing factors, protecting mammalian hosts from leishmaniases, would greatly increase the knowledge on the immunology of the disease and would open the way towards the design of a novel generation of prophylactic and therapeutic vaccines. Several lines of evidence indicate that infection by the filarial nematode *Dirofilaria immitis* is one of the factors interfering with dog leishmaniasis. Indeed, filarial nematodes induce a skew of the immune response towards Th1, partially caused by their bacterial endosymbionts, *Wolbachia*. Here we tested the potential of *Asaia*^{WSP}, a bacterium engineered for the expression of the *Wolbachia* surface protein (WSP) from *D. immitis*, as a M1-skewing factor. We also tested the efficacy of *Asaia*^{WSP} as an inductor of macrophage activation and *Leishmania* killing. The experiments were conducted on a macrophage cell lines, using *Leishmania infantum* as the test parasite. Compared to control bacteria, *Asaia*^{WSP} determined the expression of typical markers of M1 macrophage activation, including Th1-type cytokines, ROS and NO, and an increased phagocytosis activity. In addition, macrophages pre-stimulated with *Asaia*^{WSP} showed an increased leishmanicidal action. In summary, our study provides a further evidence on the M1 stimulating capacity of WSP and highlights the potential of *Asaia*^{WSP} as an immunomodulating agents, suitable to be investigated towards the development of novel tools for the control of leishmaniasis.

INTRODUCTION

Naïve macrophages (M0) can differentiate into two major, functionally distinct, subtypes: the classically activated- and the alternatively activated-macrophages (indicates as M1 and M2, respectively). These myeloid cells play crucial roles non only in the immunity towards microbial and parasitic infections, but also in wound healing, tissue repair and in cancer progression or regression (Zhu et al., 2015; Ruytinx et al., 2018). Classically activated macrophages are the pro-inflammatory subtype with microbicidal properties, and the activation of the M1 response is intrinsically associated with increased phagocyte activity and killing of intracellular pathogens, through the production of reactive oxygen species (ROS) and nitric oxide (NO) (Atri et al., 2018); M1 macrophages are also crucial in anti-cancer immunity. The M2 phenotype is an anti-inflammatory/regulatory subtype, that plays a role in the resolution of inflammation and in tissue repair, as well as in tumor progression, and in variety of diseases associated with excessive antibody production (Weagel et al., 2015; Parisi et al., 2018).

A parasitic infection that is paradigmatic in terms of their clinical outcome in relation with the M1 or M2 polarization is leishmaniasis. During *Leishmania* infection the development of a M1/Th1 response is associated with the production of proinflammatory cytokines such as TNF-α, lL-12, and IFN-γ and the release of ROS and NO, with the killing of the parasite, thus with a protective immunity. On the other hand, a M2/Th2 response is associated with anti-inflammatory cytokine production, such as IL-4/IL-13, IL-10, TGF-β, M-CSF, expression of arginase I (with reduced NO production), inhibition of inflammation, parasite survival, and thus disease progression. In summary, while M1 activation is crucial for a successful elimination of *Leishmania* parasites, in some form of leishmaniasis the M2 polarization is not only non-protective, but it is even associated with disease severity, in relation with immune-complex pathology (Rossi & Fasel 2018).

Thus, a major goal in leishmaniasis research is the identification of molecules with immunotherapeutic properties, i.e. molecules capable of modulating the immune response, to be used alone, in combination with drugs, or as vaccine adjuvants. Immunotherapy is already applied for the control of several diseases, e.g. cancer, allergies, and viral infections (Papaioannou et al., 2016; Naran et al., 2018; Roatt et al., 2014). In visceral leishmaniasis, patients non-responding to conventional chemotherapy have been treated with success through combination therapies with various immunomodulators, e.g. MDP13, IFNγ, lL-12, Bacille Calmette-Guérin (BCG) (Roatt et al., 2014; El-On et al., 2009). In addition, also in cutaneous leishmaniasis, BCG has been used in combination with a lysate of *Leishmania* parasites, with positive therapeutic effects (Convit et al., 2003; Mayrink et al., 1992).

Here we suggest that the bacteria of the genus *Wolbachia* represent a promising source of molecules capable of stimulating and modulating innate immunity, to be exploited as immunotherapeutic. In insects, *Wolbachia* has been shown to be a potent activator of innate immunity, able to determine the upregulation of several immune effectors such as antimicrobial peptides, autophagy-related proteins, and ROS (Zug et al., 2015; Epis et al., submitted). Indeed, the successful use of *Wolbachia* to block the transmission of viruses

by mosquitoes has in part been associated with this immune-activating capacity (Rancès et al., 2012). On the other hand, *Wolbachia* from filarial nematodes (or its surface protein, WSP) has been shown to activate macrophages through the stimulation of innate-immunity receptors, determining a M1/Th1-type activation (Brattig et al., 2004; Saint André et al., 2002). In summary, there is strong evidence that *Wolbachia* is an effective inductor of innate immunity, in insects and in mammals, and WSP from the nematode *Wolbachia* represents a promising candidate immunomodulator, with pro-M1 properties.

A strategy to deliver immunomodulators to hosts, for therapeutic or prophylactic purposes, is to engineer non-pathogenic bacteria for their expression; the engineered bacteria are then administered to the host, through different routes (Berlec et al., 2019). For example, Jacouton and colleagues (2019) have modified a *Lactococcus lactis* strain for the expression of the cytokine IL-17A, with tumor prevention in a mouse model, after intranasal delivery of the engineered bacterium. In this context, we recently selected an acetic acid bacterium, *Asaia* sp., as the bacterial vehicle for the expression of the *Wolbachia* surface protein, generating the chimeric symbiont *Asaia*^{WSP} (Epis, et al. submitted). Our hypothesis is that *Asaia*^{WSP} should determine innate immune activation with a M1 bias, thus conferring protection against M1-impaired infections. In order to test its potential utility in immunotherapy, *Asaia*^{WSP} was used to stimulate a macrophage cell line; we then determined the pattern of M1/M2 activation, and its efficacity in the induction of *Leishmania* killing by macrophages.

RESULTS

Uptake and survival of bacteria in macrophage cell line

In order to determine the bacterial uptake and resistance in macrophages, J774A.1 cells were infected with bacteria at different conditions (see Materials and Methods) of co-incubation at a MOI of 100. After 1h of treatment with streptomycin, the bacteria inside the macrophages were quantified and expressed as CFU/ml. *Ad hoc* experiments showed that the uptakes, after 1h or 2h of incubation, were comparable (Fig 2A, p=0.823); for this reason the second time point (2h) was used in all the performed experiments, also considering published protocols (e.g. Migliore et al., 2018). Survival of bacteria after 24h of infection was quantified and expressed as mean number of phagocyted bacteria in 2h and survived until 24h. As reported in Fig 2A, the number of bacteria phagocyted by macrophages infected with *Asaia*^{WSP} is higher than macrophages treated with *Asaia*^{pHM4} (p=0.0007), and comparable with the uptake by macrophages treated with LPS (positive control). In summary, the mean number of bacteria *Asaia*^{WSP} phagocyted is almost double that of *Asaia*^{pHM4} with $6.32x10^5$CFU/ml and $3.52x10^5$ CFU/ml, respectively. As for the survival of bacteria in the macrophage, after 24h of co-infection the vast majority of bacteria phagocyted during 2h of co-incubation is killed by the macrophages with slight differences between the treated groups, indicating a general bactericidal activity of the macrophages (Fig 2B). In particular, as reported in Fig 2B, a slightly higher number of bacteria *Asaia*^{WSP} were counted in macrophages ($1.19x10^4$ CFU/ml), compared both to the

bacterium control and LPS ($6.52x10^3$ CFU/ml and $6.78x10^3$ CFU/ml respectively), but the differences are not statistically significant.

The phagocytosis activity was also evaluated in pre-stimulated macrophages (with *Asaia* bacteria), subsequently infected by the bacteria *Staphylococcus epidermidis.* After 1 hour, the medians related to the number of phagocyted *S. epidermidis* after the pre-treatment with *Asaia* bacteria in general are higher than that of the controls (*Asaia*^{WSP} vs LPS p=0.0012, *Asaia*^{WSP} vs control p=0.0027, *Asaia*^{pHM4} vs LPS p=0.0065, *Asaia*^{pHM4} vs control p=0.0161; S1 Fig). After 2 hours of *S. epidermidis* treatment*,* only the number of phagocyted bacteria in *Asaia*^{WSP}-infected macrophages was significant, compared to the controls, suggestive of a more intense phagocyte activity of these macrophages (*Asaia*^{WSP} vs LPS p=0.0012; *Asaia*^{WSP} vs control p=0.0023). In summary, both after 1 hour and 2 hours, there is an evidence for an *Asaia* bacteria effect on the phagocytosis induction.

TEM analyses of phagocytosis

The intracellular localization of *Asaia*^{WSP} and *Asaia*^{pHM4} after 24h of incubation with macrophages was also investigated by transmission electron microscopy. Once the macrophages were phagocyted, the bacteria formed phagocytic cups in the cytoplasm of the cells. As shown in Fig 3A, when the macrophages were infected with *Asaia*^{WSP}, there were many empty digestive vacuoles or bacteria inside the cell, probably indicating strong digestive activity of the infected macrophage. On the other hand, when the macrophages were treated with the *Asaia*^{pHM4}, the bacteria were mostly outside the macrophage and very few bacteria were detected in the cytoplasm or inside the vacuoles of the cells (Fig 3B).

M1/Th1 cytokines secretion by *Asaia*^{WSP}-infected macrophages

Macrophages of the J774A.1 cell line were infected with *Asaia* bacteria (MOI 100) or with bacteria plus *Leishmania* (MOI 2) promastigotes; after 24h and 48h of co-infection the culture supernatants were collected and analyzed for the presence of the cytokines IL-1β, IL-12p40, TNFα and IL-6 as markers of M1/Th1 polarization by ELISA assay. Fig 4 illustrates the panel of M1/Th1 cytokines tested in this study, after 24h of infection. Twenty-four hours post-infection, the secretion IL-6 cytokine by macrophages treated with *Asaia* bacteria was statistically different compared to controls (means: *Asaia*+= 4934 pg/ml, *Leishmania* = 8.22 pg/ml, untreated = 19.74 pg/ml, LPS = 3543 pg/ml; Fig 4A), indicating a strong effect due to the presence of the bacteria. In particular, Fig 4B showed a higher production of this cytokine by *Asaia*^{WSP} compared to the *Asaia*^{pHM4} treated cells, both in the presence or in the absence of the *Leishmania* (means: *Asaia*^{WSP} not infected= 5722 pg/ml; *Asaia*^{WSP} infected= 4805 pg/ml; *Asaia*^{pHM4} not infected= 4145 pg/ml; *Asaia*^{pHM4} infected=3495 pg/ml). Moreover, the presence of the parasite *Leishmania* determined a general reduction of the IL-6 production that is statistically significant, in particular in *Asaia*^{pHM4} treated cells (p = 0.0058). The same trend was obtained for the expression of the cytokine IL-12p40 (Fig 4C-D), 24h post-infection. The macrophages treated by the bacteria *Asaia* produced a greater amount of cytokine IL-12p40 comparable with the production determined by LPS (means: *Asaia*+= 4157 pg/ml, LPS = 5372 pg/ml), and it

was statistically different from the expression determined by the macrophages untreated or infected only with *Leishmania* (means: untreated= 1 pg/ml, *Leishmania* = 7.5 pg/ml, Fig 4C). Fig 4D reported a higher production of this cytokine by *Asaia*^{WSP} compared to the *Asaia*^{pHM4} treated cells, both in the presence or in the absence of the *Leishmania* (means: *Asaia*^{WSP} not infected= 2301 pg/ml; *Asaia*^{WSP} infected= 1919 pg/ml; *Asaia*^{pHM4} not infected= 986 pg/ml; *Asaia*^{pHM4} infected= 526 pg/ml). Culture supernatants from stimulated macrophages collected at the first time point (24h) were also checked for the production of the cytokines TNFα (Fig 4E-F) and IL-1β (Fig 4G-H). Twenty-four hours post-infection, the secretion TNFα cytokine by macrophages treated with *Asaia* bacteria was statistically significant compared to controls, although lower than macrophages treated with LPS (means: *Asaia*+= 791 pg/ml, *Leishmania* = 6.46 pg/ml, untreated = 5.81 pg/ml, LPS = 2679 pg/ml; Fig 4E). In Fig 4F, when the macrophages were infected by *Asaia*^{WSP} the production of TNFα was significant compared to the *Asaia*^{pHM4} when the parasite was present (means: *Asaia*^{WSP} not infected= 1143 pg/ml; *Asaia*^{WSP} infected= 1491 pg/ml; *Asaia*^{pHM4} not infected= 860 pg/ml; *Asaia*^{pHM4} infected= 879 pg/ml). Finally, the quantification of IL-1β release was obtained after a pre-stimulation with LPS for 12h before the infection. The production of IL-1β cytokine by the bacteria was higher than the LPS treated macrophages (means: *Asaia*+= 334 pg/ml, *Leishmania* = 49.34 pg/ml, untreated = 16.86 pg/ml, LPS = 121.7 pg/ml; Fig 4G); in details, when the macrophages were infected by *Asaia*^{WSP} the production of IL-1β is significant compared to the *Asaia*^{pHM4} , but only when the parasite is absent (means: *Asaia*^{WSP} not infected= 358.6 pg/ml; *Asaia*^{WSP} infected= 304 pg/ml; *Asaia*^{pHM4} not infected= 308.5 pg/ml; *Asaia*^{pHM4} infected= 292.8 pg/ml; Fig 4H). The production of the IL-6 and TNFα was also evaluated after 48h post infections. As for IL-6 there is a positive effect determined by the bacteria, comparable with the production by LPS positive control (means: *Asaia*+= 2168 pg/ml, *Leishmania* = 10.16 pg/ml, untreated = 5.33 pg/ml, LPS = 3162 pg/ml). In particular, Fig 5B showed a higher production of this cytokine by *Asaia*^{WSP} compared to the *Asaia*^{pHM4} treated cells, both in the presence or in the absence of the *Leishmania* (means: *Asaia*^{WSP} not infected= 4247 pg/ml; *Asaia*^{WSP} infected= 6073 pg/ml; *Asaia*^{pHM4} not infected= 1810 pg/ml; *Asaia*^{pHM4} infected= 3783 pg/ml). Moreover, the presence of the parasite *Leishmania* determined a general increase of the IL-6 production (Fig 5B), which is statistically significant, in particular in *Asaia*^{WSP} treated cells (p = 0.0366). The macrophages treated by the bacteria *Asaia* for 48h, produced a greater amount of TNFα as compared to untreated and *Leishmania* treated controls, although it was comparable with production induced by LPS (means: *Asaia*+= 156.4 pg/ml, *Leishmania* = 28.55 pg/ml, untreated = 29.56 pg/ml, LPS = 1735 pg/ml, Fig 5C). No differences were detected in the production of this cytokine by *Asaia*^{WSP} compared to the *Asaia*^{pHM4} treated cells (means: *Asaia*^{WSP} not infected= 152 pg/ml; *Asaia*^{WSP} infected= 171 pg/ml; *Asaia*^{pHM4} not infected= 111 pg/ml; *Asaia*^{pHM4} infected= 136 pg/ml).

M2/Th2 cytokines secretion

Culture supernatants from cells pre-stimulated with LPS and infected with *Asaia* bacteria or *Asaia* bacteria plus *Leishmania* were collected 24h post-infection and tested for the production of IL-10, a typical marker of M2/Th2 polarization. S2A Fig showed that a significant production of IL-10 was obtained when the macrophages were infected with promastigotes (means: *Asaia*+= 576 pg/ml, *Leishmania* = 363 pg/ml, untreated = 2.16 pg/ml, LPS = 491 pg/ml); macrophages infected with *Asaia*^{pHM4} produced more IL-10 compared to the *Asaia*^{WSP} when the cells were infected only with bacteria (means: *Asaia*^{WSP} not infected= 516 pg/ml; *Asaia*^{WSP} infected= 475 pg/ml; *Asaia*^{pHM4} not infected= 694 pg/ml; *Asaia*^{pHM4} infected= 461 pg/ml; S2B Fig).

NO and ROS production by *Asaia*^{WSP}-infected macrophages

The capability of macrophages infected with *Asaia*^{WSP} or *Asaia*^{WSP} plus *Leishmania* to produce NO was investigated, after 24h and 48h. After 24h post-infection, there are not significant differences between bacteria and controls, both in presence and in absence of *Leishmania* (S3 Fig). After 48h post-infection, the secretion of NO in the form of nitrites, as measured by the reduction of nitrates in nitrites, by macrophages treated with *Asaia* bacteria is statistically significant compared to controls (means: *Asaia*+= 84 μM, *Leishmania* = 73 μM, untreated = 80 μM, LPS = 119 μM; Fig 6A). In Fig 6B, when the macrophages were infected by *Asaia*^{WSP} the production of NO is significant compared to the *Asaia*^{pHM4}, when the parasite is absent (means: *Asaia*^{WSP} not infected= 119.9 μM; *Asaia*^{WSP} infected= 131.7 μM; *Asaia*^{pHM4} not infected= 67.7 μM; *Asaia*^{pHM4} infected= 112.1 μM).

In addition to nitrites, the macrophages in response to parasite infection produce the reactive oxygen species as defense mechanism, polarizing the response towards a M1/Th1 phenotype. The production of ROS after 24h in macrophages was investigated by a fluorometric assay using H_2DCF-DA probe. As reported in Fig 7A, cells infected with *Asaia* bacteria showed the same trend of ROS production, reported as fluorescence unites (FU), of the controls, except for the production by macrophages treated with H_2O_2 (means: *Asaia*+= 8346 FU, *Leishmania* = 6841 FU, untreated = 3867 FU, LPS = 101093 FU). However, in Fig 7B, when the macrophages were infected by *Asaia*^{WSP} the production of ROS is significantly different compared to the *Asaia*^{pHM4}, when the parasite is absent (means: *Asaia*^{WSP} not infected= 11294 FU; *Asaia*^{WSP} infected= 9660 FU; *Asaia*^{pHM4} not infected= 5239 FU; *Asaia*^{pHM4} infected= 9660 FU).

Analysis of the expression of iNOS and arginase I genes by macrophages

The cells were collected and analyzed for the expression of iNOS gene, at the first time point (24h) and arginase I gene, at the late time point (48h) by reverse transcription-quantitative PCR. The expression of the two genes *β-Actin* and *cyclophilin* was used to normalize the data. As reported in Fig 8A, iNOS relative expression due to bacteria treatment was significantly higher than the cells infected with *Leishmania* (means: *Asaia*+= 0.772, *Leishmania* = 0.002, untreated = 0.053, LPS = 3.037). The cells infected with *Asaia*^{WSP} showed an up-regulation in iNOS expression compared to the macrophages

incubated with the bacterium *Asaia*^{pHM4}, in presence or absence of *Leishmania,* respectively (Fig 8B). As for arginase I expression, cells were also analyzed at the late time point (48h) and pre-stimulated before the infection. As shown in S4A Fig, no expression was detected when the macrophages were infected with bacteria, while S4B Fig showed that when macrophages were infected with *Asaia*^{WSP} in presence of *Leishmania,* there was a significant downregulation of the gene, compared to the *Asaia*^{pHM4} (means: *Asaia*^{WSP} not infected= 1.025; *Asaia*^{WSP} infected= 0.3694; *Asaia*^{pHM4} not infected=0.976; *Asaia*^{pHM4} infected= 0.7554).

Costimulatory molecules expression by *Asaia*^{WSP}-infected macrophages

To investigate the effect of *Asaia*^{WSP} on the activation of selected cell surface markers (CD80-CD86-CD40) of M1/M2 polarization, the macrophages after 24h of infection with bacteria and parasites were processed for flow cytometry analyses. As shown in Fig 9, both the treatments with *Asaia*^{WSP} and *Asaia*^{pHM4} stimulated a larger number of macrophages to present at least one of the three CD receptors compared to macrophages untreated or stimulated with *Leishmania* alone (*Asaia*^{WSP} = 62%; *Asaia*^{pHM4} = 53%; LPS = 69%; untreated = 16%; *Leishmania* = 26%. p values < 0.0001 for *Asaia*^{WSP} vs untreated, *Asaia*^{pHM4} vs untreated, *Asaia*^{WSP} vs *Leishmania, Asaia*^{pHM4} vs *Leishmania*). Moreover, *Asaia*^{WSP} showed an higher expression of at least one receptors compared to *Asaia*^{pHM4}, comparable to LPS treatment (*Asaia*^{WSP} vs *Asaia*^{pHM4} p= 0.045, *Asaia*^{WSP} vs LPS p=0.198, *Asaia*^{pHM4} vs LPS p=0.0002). Notably, *Leishmania* slightly stimulated macrophages to expose the CD markers. We then analyzed which class of CD are primarily affected by our treatments; we noticed significant differences in the percentage of macrophages presenting CD40 alone or a combination of CD86-CD40 (means CD40+ cells: *Asaia*^{WSP} = 31%; *Asaia*^{pHM4} = 21%; LPS = 8%; untreated = 0.1%; *Leishmania* = 1%; means CD40+ and CD86+ cells: *Asaia*^{WSP} = 34%; *Asaia*^{pHM4} = 22%; LPS = 48%; untreated = 0.5%; *Leishmania* = 2%; p values are reported in S1 Table). Notably, macrophages stimulated with LPS have a higher proportion of CD40+/CD86+ macrophages compared to *Asaia* treated ones, while the opposite was detected for CD40+ cells. When we analyzed the mean fluorescence of the positive cells, we did not detect any differences (apart from a higher levels of CD86 and CD40 in LPS treated cells), probably indicating that positive macrophages expressed the same amount of receptors at single cell level (S5 Fig).

Expression of major histocompatibility complex class II (MHCII) by *Asaia*^{WSP}

We also investigated the expression of MHC class II by macrophages infected with bacteria and *Leishmania* after 48h of infection and pre-stimulated with IFNγ. The priming with IFNγ was necessary to stimulate the expression of MHC class II by this cell line, because the constitute expression on J774A.1 is very low (data not shown) (Kalupahana et al., 2005). As in the case of costimulatory molecules, we noticed that the macrophages infected with both the *Asaia* and treated with PMA (Phorbol 12-Myristate 13-Acetate), positive for MHCII marker, were more abundant than the macrophages infected only with *Leishmania* or untreated; particularly, *Asaia*^{WSP} had the stronger effect compared to *Asaia*^{pHM4} and PMA (*Asaia*^{WSP} = 47%; *Asaia*^{pHM4} = 34%; PMA = 30%; untreated = 5%; *Leishmania* = 25%; all p values are significant except for the combination *Asaia*^{pHM4} vs

PMA) (Fig 10; S1 Table). However, the infection with *Leishmania* had an effect on the proportion of MHC-presenting cells compared to the PMA control. As in the case of CD receptors, when we analyzed the geometric means of fluorescence of the positive cells, we did not detect any differences, again indicating that positive macrophages exposed the same amount of MHC receptor on their surface (S6 Fig).

Killing of *Leishmania* by *Asaia*^{WSP}-infected macrophages

The anti-leishmanial effect of *Asaia*^{WSP} was determined by microscopy observation after 24h and 48h of infection, as reported in Maksouri et al. (2017). J774A.1 cells were pre-infected with the two bacterial strain for 2h and then incubated with *L. infantum* parasites at a ratio of 1:2. The infection rate, the number of amastigotes per macrophage and the parasitic index were determined. By preliminary results, the ratio (1:2) chosen for all the experiments with *Leishmania* resulted the optimal for a sustainable infection (data not shown). After 24h of infection, considering all the three analyzed parameters (see Material and Methods), there were not significant differences between the groups (bacteria plus *Leishmania* and *Leishmania* alone; S7A-C Fig). As for the infection rate (Fig 11A), the percentage of infected macrophages/100 macrophage decreased from 50% (macrophages infected only with *Leishmania*) to 34% when macrophages were treated with *Asaia*^{WSP} or to 37% when treated with *Asaia*^{pHM4}. Interesting, the number of amastigotes in each macrophage decreased from 0.91 to 0.39 (reduction of 57%, p=0.042) when macrophages were infected by *Asaia*^{WSP} compared to *Leishmania* alone. *Asaia*^{WSP} determined a reduction of the number of amastigotes (37%), compared to the macrophages infected by *Asaia*^{pHM4}, though not significant. Finally, the same trend was described in panel C of Fig. 11, which showed the parasitic index (average number of infected macrophages multiplied by the average number of amastigotes per macrophage); shortly, the infection with *Asaia*^{WSP} reduced the parasitic index of 74.3% compared to the infection with *Leishmania* (p=0.043). Moreover, *Asaia*^{WSP} determined a reduction of the parasitic index (37.6%) compared to the macrophages treated with *Asaia*^{pHM4}, though not significant. Fig 11 showed the staining of macrophages co-infected with *Asaia*^{pHM4} or *Asaia*^{WSP} and *Leishmania*, after 48h. In panel E, infected macrophages exhibited several vacuoles and the amastigotes showed morphological changes e.g. loss of membrane integrity and formation of multiple cytoplasmic vacuoles. These cellular modifications are less noticeable in panel D which reported the macrophages pre-infected with *Asaia*^{pHM4}. The panel E (infection only with *Leishmania*) displayed numerous intact amastigotes; part of them are out of macrophages and in proliferation.

DISCUSSION

Intracellular bacteria classically drive the immune response toward the Th1 side, with an effector role of classically activated macrophages. In evolutionary terms, selection is expected to act on pathogenic bacteria to reduce such modulation, as a M1/Th1 response clearly harms them. *Wolbachia* is an intracellular bacterium, but it is hosted by the filarial worm, and its evolutionary success is that of its host. The overall immune modulation it elicits, including the partial Th1 bias, is thought to have a positive effect for the nematode, thus for *Wolbachia* itself, as it protects the worm from an excess of Th2 response. For this reason, we can speculate that selective pressure acts on *Wolbachia* to preserve its "Th1-inductor" phenotype: its survival is closely tied to the survival of the nematode host. Anyhow, while other intracellular bacteria benefit from a reduced Th1 response, *Wolbachia* is for sure not directly harmed by this type of response.

Therefore, we exploited the Th1 immunopolarising protein WSP from *Wolbachia* to engineer the bacterium *Asaia,* obtaining the chimeric bacterium *Asaia*^{WSP}; the aims were to investigate i) the stimulation of the macrophages *in sensu* M1 determined by *Asaia*^{WSP} and ii) the killing activity against the parasite *L. infantum.*

It is known that the outcome of *Leishmania* infection is strongly dependent on the type of macrophage polarization: a M1 activation with the release of microbicidal molecules and pro-inflammatory cytokines led to the parasite killing; instead, a M2 polarization with a down modulation of NO and ROS and the release of anti-inflammatory cytokines led to parasite survival (Rossi et al., 2018).

The immunomodulatory role of the chimeric bacterium was investigated analyzing classical M1 activation markers and the first one was the capability to induce phagocytosis on murine macrophages. The ability of *Asaia*^{WSP} bacteria to induce phagocytosis on mosquito hemocyte cultures was already investigated (Epis et al., 2020). By our experiments on murine macrophage cell line this capability was confirmed: *Asaia*^{WSP} was able to stimulate twice the phagocytic activity compared to the bacterium control *Asaia*^{pHM4} and the number of intracellular bacteria phagocyted by the macrophages was similar to that obtained in LPS treatment. The increase of the phagocytic activity indicates a good competence of *Asaia*^{WSP} bacteria to invade macrophages and make them more activated. The analysis of the survival of bacteria until 24h of infection showed a killing activity of the macrophages against bacteria without differences between the two strains. This data of survival is in accordance with the results reported in Fernandez-Cabezulo (2009) where the same cell line eliminated approximately 90% of phagocyted bacteria.

The induction of phagocytosis was also tested against the bacterium *S. epidermidis* after a pretreatment of the macrophages with *Asaia*^{WSP}. A slight difference between the two strains was observed in terms of number of *S. epidermidis* bacteria internalized after the pre-treatment with *Asaia*^{WSP}, even if not statistically significant. The result was not as strong as that observed with phagocytosis of *Asaia* bacteria only, probably due to the little time elapsed between the treatment with *Asaia* (before) and *Staphylococcus* (after).

Once verified the phagocytic activity, the production of typical M1/Th1 cytokines such as IL-6, IL-12p40, TNFα and IL-1β associated with macrophage activation and pathogen killing was evaluated (Martinez et al., 2014). The presence of *Asaia* bacteria induced an increase in the levels of tested pro-inflammatory cytokines compared to the controls, suggesting an intrinsic effect of the bacteria *Asaia*. The effect of the engineered bacterium *Asaia*^{WSP}, compared to the bacterium control, was clearly visible in the higher levels of IL-6 and IL12p40 cytokines both in presence and in the absence of the parasite. The former cytokine shows a double role being both pro-inflammatory and anti-inflammatory: a protective effect by IL-6 was shown against the visceral *L. donovani* parasite (Stager et al., 2006) and a suppressive mechanism, against *L. major* infection, was highlighted in IL-6 deficient mice (Moskowitz et al., 1997). Considering the general activation of the macrophages with release of M1 cytokines after infection with *Asaia*^{WSP} and the high production of this cytokine, we can hypothesize that in this context IL-6 could be considered as a M1/Th1 marker. IL12 is a key cytokine in the macrophage activation *in sensu* M1 being involved in the induction of nitric oxide production and expression of the enzyme iNOS; during *Leishmania* infection it is required for the maintenance of the resistance (Park et al., 2002). As for TNFα, *Asaia*^{WSP} induced a greater production of this cytokine only in presence of the parasite. During leishmaniasis, TNFα is an important factor involved in the macrophage activation, killing of intracellular parasites and nitrites production; in fact, mice lacking TNFα showed fatal leishmaniasis after *L. major* infection (Wilhelm et al., 2001). The effect of TNFα is increased in presence of IL1-β, a potent inflammatory cytokine involved in the balance between inflammation and immunity which strongly affects the outcome of leishmaniasis (Dajakar et al., 2019). During *L. major* infection the production of IL1-β is downregulated and a treatment with this cytokine induces a slowing down of the disease progression in BALB/c mouse, contrary to what happens during *L. amazoniensis* infection (Ji et al., 2003). In fact, by our experiments the production of this cytokine, as for all the others, in presence of *Leishmania* parasite was very low highlighting the strong Th2 polarization induced by the parasite. In fact, the presence of the parasite decreased the production of these pro-inflammatory cytokines reducing the M1 polarization induced by WSP.

Other two important factors in M1/Th1 activation are NO and ROS, both involved in *Leishmania* killing (Carneiro et al., 2016). They are produced by macrophages as mechanism response to *Leishmania* infection, which in turn downregulates their production (Ball et al., 2014). In particular, while 24h post-infection the production of NO, in form of nitrites was low, after 48h *Asaia*^{WSP} induced a significant increase in the production of nitrites in absence of the parasite, suggesting a delayed production of NO. Similar trends were observed for ROS production by macrophages infected with *Asaia*^{WSP} with a higher production of reactive oxygen species by the chimeric bacterium compared to the bacterium control. The decrease of the levels of ROS in presence of the parasite is a further evidence for the Th2 polarization induced by the pathogen which limits the effect of WSP.

To verify if the differences in NO production could be associated with a change in the expression of the enzyme responsible for its synthesis, the inducible NO synthase (iNOS),

its expression was evaluated in macrophages infected with both bacteria and parasites. The presence of *Asaia* bacteria determined an upregulation of the enzyme and, both in presence and in absence of the engineered bacterium *Asaia*^{WSP}, the expression of iNOS was higher than with bacterium control. As reported in Balestrieri (2002), the cells infected only with the parasites showed a downregulation of the expression of the enzyme. The up regulation of iNOS enzyme reflected the significant production of nitrites obtained after 48h of infection by macrophages infected with bacteria. Since iNOS shares the same substrate with the arginase enzyme, the extracellular stimuli are crucial in determining the pathway activation (Wanasen & Soong, 2008). The production of M1 cytokines as a result of the infection of macrophages with the bacterium *Asaia*^{WSP} created the conditions for the expression of iNOS with a consequent production of nitrites and a downregulation of the arginase enzyme. Our results showed, as expected, that the bacterium *Asaia*^{WSP} did not induce arginase expression and IL10 cytokine production, typical M2 markers, compared with the bacterium *Asaia*^{pHM4}.

The type of immune response and the fate of the infection is also determined by the co-stimulatory molecules which represent the secondary signal; in fact, the presentation of the sole antigen to the T cell naïve is not enough for its activation (Podojil et al., 2009). The analysis of the co-stimulatory molecules (CD80-CD86-CD40) showed that the pre-treatments with *Asaia*^{WSP} and *Asaia*^{pHM4} stimulate a higher number of macrophages to present at least one of the three CD receptors analyzed, compared to controls. As for the geometric mean of fluorescence expressed by the positive cells, there was no differences between the two bacteria, while analyzing the percentage of positive cells, there was a significant difference in the proportion of cells positive for CD40 and for the combination CD86-CD40 between *Asaia*^{WSP} and controls This result can suggest that an increased phagocytosis activity, induced by *Asaia*^{WSP}, activated a greater number of macrophages which expressed a higher number of receptors, though the amount of fluorescence released by the positive cells does not change. The expression of CD40 co-stimulatory molecule during an infection with L. major is associated with macrophage activation and production of IL12, being essential for the clearance of the disease (Tulhadar et al., 2012; Barhoumi et al., 2019). By our experiments, this tendency seems to be confirmed also in presence of *L. infantum* parasites. As for CD86, its role during *Leishmania* infection is critical: it has been reported to both participate in the defense mechanisms (Elloso et l., 1999) and promote the infection with the release of Th2 cytokines (Brown et al., 1996).

As for the expression of MHC class II, the cells pre-treated with *Asaia*^{WSP} and positive for this receptor were in greater numbers than those pre-infected with the bacterium control, underlying again the effect of the *Asaia*^{WSP} in the induction of phagocytosis and therefore in the activation of the macrophages. The geometric mean of the MHCII positive cells and infected with *Asaia*^{WSP} was slightly higher compared to the control bacterium, even if the difference was not statistically significant, suggesting a positive effect of the WSP in the expression of this M1/Th1 activation marker. A downregulation of the MHCII fluorescence by *Leishmania* was highlighted in accordance with Zutshi (2019) where in presence of the parasite the antigen presentation and MHCII conformation were affected.

Once verified that the engineered bacterium *Asaia*^{WSP} could stimulate the macrophage *in sensu* M1/Th1 due to the release and expression of all markers described above, an antileishmanial effect against *L. infantum* parasites by *Asaia*^{WSP} was investigated. After 24h of co-infection, no differences in the parasitic index between the two strains were observed, probability due to the fact that the majority of the parasites were still in the form of promastigotes out of the macrophages. At 48h post-infection, a decrease in the number of macrophages infected with parasites was shown in presence of bacteria compared to the control group (macrophages infected with only *Leishmania*). Since the count was performed only on intact macrophages, the result could be likely underestimated; in fact, the parasite killing process could has already started with the release of parasites from broken macrophages. As for the number of amastigotes per macrophage pre-infected with *Asaia*^{WSP}, a strong reduction of 57% of the intracellular parasite multiplication was obtained compared to the macrophages infected only with *Leishmania;* the result was comparable to the treatment with the anti-parasitic drug Amphothericin B (data not shown). The same trend was confirmed by the third parameter observed, the parasitic index, which reflects the differences obtained in the number of amastigotes between *Asaia*^{WSP} and control (only *Leishmania*). Comparing the results obtained between 24h and 48h of co-infection, the number of amastigotes per macrophages infected with *Asaia*^{WSP} decreased passing from 0.80 to 0.39, contrary to macrophages infected with only *Leishmania* whose number of amastigotes did not change (from 0.96 to 0.91). Therefore, we can assume that *Asaia*^{WSP}-infected macrophages caused the killing of *Leishmania* parasites after 48 h of co-infection. This phenomenon is also visible by microscopic observation: the amastigotes showed degeneration of the membrane and the macrophages appeared with several vacuoles, signs of an intense killing activity compared to the controls. All these results can be suggestive of an anti-*Leishmania* effect induced by the chimeric bacterium *Asaia*^{WSP} in *in vitro* experiments mediated by the production of anti-microbial effector molecules.

In conclusion, the infection of macrophages with the engineered bacterium *Asaia*^{WSP} induced a polarization of the immune response *in sensu* M1; in particular, this chimeric bacterium caused the production of pro-inflammatory cytokines, nitrites and ROS. In addition, an increase of the expression of iNOS enzyme was observed in presence of *Asaia*^{WSP}. On the contrary, typical M2 markers such IL10 and arginase expression were downregulated when the cells were infected with the chimeric bacterium. In addition, *Asaia*^{WSP} induced an increase of the proportion of the positive cells for the co-stimulatory molecules, while a slight increase of the geometric mean of the MHC positive cells and infected with the chimeric bacterium was observed. This type of immunological activation by *Asaia*^{WSP} was suitable to inhibit *L. infantum* development inducing a reduction of the multiplication of the amastigotes. Therefore, *Asaia*^{WSP} acts as a successful immunomodulator which can be used for therapeutic or prophylactic purposes in the treatment of the leishmaniases and other Th1- impaired diseases. Finally, our results further support the evidence that the symbiont *Wolbachia* might be consider as an immunomodulator of the immune system (from insects to mammals) and thus an extraordinary tool for the control of vector-borne diseases, in general.

MATERIALS AND METHODS

Cell and parasite cultures

J774A.1 ATCC® TIB-67 macrophage cell line derived from susceptible BALB/Cn mice was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained under an atmosphere of 5% CO_2 at 37°C in incubator. All reagents for the cell cultures were purchased from ATCC (Manassas, VA, USA).

The *Leishmania infantum* promastigotes (strain MHOM/TN/80/IPT1) were kindly provided by Dott. Marina Gramiccia (Istituto Superiore di Sanità, Rome, Italy). The parasites were grown at 23°C in Schneider's Drosophila medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS and gentamycin (5 µg/ml) within three to four days.

Bacterial strains and growth conditions

The bacterium *Asaia*^{WSP} derived from the engineering of the bacterium *Asaia* SF2.1 strain, originally isolated from the *Anopheles stephensi* mosquito (Favia et al., 2007), with the plasmid pHM4-WSP (Epis et al. 2020). Briefly, the WSP cassette, inserted in the plasmid pHM4, is composed by the *Wolbachia* surface protein sequence of the *Wolbachia* of the nematode *Dirofilaria immitis*, the neomycin phosphotranferase promoter PnptII, the E-TAG epitope and the transcription terminator Trrn. *Asaia*^{pHM4} was also obtained from the strain *Asaia* SF2.1 transformed but with the empty plasmid (without the WSP cassette) and was used as control bacterium (Epis et al. 2020). Both bacteria were grown overnight in GLY medium broth (glycerol 25 g/l and yeast extract 10 g/l, pH 5) supplemented with kanamycin 100 µg/ml, under constant agitation at 30°C overnight.

Phagocytosis Assay

Macrophages were seeded in 24-well plates ($2x10^5$/ml) and allowed to adhere overnight at 37°C in humidified 5% CO_2 atmosphere. Phagocytosis assay was performed applying the gentamicin protection assay as reported in Glasser, 2001, with minor modifications. *Asaia* bacteria, grown overnight, were washed with sterile PBS and resuspended in complete DMEM medium. The macrophages were infected at a multiplicity of infection (MOI) of 100 bacteria per macrophage. As controls, macrophages were infected with *Asaia*^{pHM4} bacteria in presence of *Escherichia coli* lipopolysaccharide (LPS) (0.3 µg/ml) (R&D Systems, Minneapolis, MN). After a 10 min centrifugation at 1,000 rpm, macrophages were incubated 2h at 37°C to allow internalization (Migliore et al., 2018). The choice of time of incubation was defined after preliminary tests. Then, the macrophages were washed with PBS once and treated with complete DMEM containing 100 µg/ml streptomycin for 1h at 37°C to kill extracellular bacteria. After two washes with PBS, macrophages were lysed using deionized water containing 1% (vol/vol) Triton X-100 (Sigma Aldrich, USA) for 15 min at 37°C, to release phagocyted bacteria. The bacterial titer was determined by plating ten-fold serial dilutions of the cell lysates on GLY plates (glycerol 25 g/l and yeast extract 10 g/l, agar 20 g/l with kanamycin 100 µg/ml acidified to pH 5) and CFU/ml were counted after growth for 48h at 30°C. In addition, to determine the bacterial survival inside the cells, the macrophages, after the treatment with streptomycin 100 µg/ml, were left with streptomycin

20 µg/ml until 24h of infection, followed by the final step of the protocol, as described above.

Efficiency of the phagocytosis and killing by activated macrophages with *Asaia*^{pHM4} and *Asaia*^{WSP} bacteria was also evaluated against the bacterium *Staphylococcus epidermidis* following the protocol described above. *S. epidermidis* was grown in LB medium broth (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) buffered to 7.0-7.4 pH with NaOH under constant rotation at 37°C O.N. Briefly, the macrophages were infected first with *Asaia* bacteria and, after a wash with PBS, with *S. epidermidis* at a MOI of 10 bacteria per macrophage and incubated for two time points (1h and 2h) at 37°C to allow internalization. Cell monolayers were washed once in PBS to remove extracellular bacteria and treated with DMEM containing 300 µg/ml gentamycin for 1 h to kill non-internalized bacteria. Then, the macrophages were treated as above for the determination of viable colonies growth on LB agar plates after 24h of incubation at 37°C. Phagocytosis data and the *Staphylococcus* survival data were Log transformed to reach normality, as determined using a Shapiro-Wilk test, and analysed using a two-way ANOVA with repeated measures followed by Sidak's post-hoc test to detect differences between treatments and also taking in account the inoculation period (1 and 2h). Survival data from *Asaia* culture (after 2h of inoculation) were transformed as before and analysed using a ONE-WAY Brown-Forsythe ANOVA. These analyses were performed using GraphPad Prism 8.

Electron microscopy

The bacterial uptake was also evaluated by transmission electron microscopy (TEM). After 24h of infection, the cells were pelleted, washed with PBS and immediately fixed in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 2h at 4°C and postfixed in 1% OsO4 in 0.1 M cacodylate buffer (pH 7.2) for 1.5 h at 4°C. Subsequently, the samples were subject to dehydration in ethanol and then were embedded in Epon 812. Finally, thin sections were stained with uranil acetate and lead citrate and examined under an EM900 transmission electron microscope (Zeiss).

***Leishmania* infection assay**

Macrophages were seeded in 6-well plates ($2x10^5$/ml) and allowed to adhere overnight at 37°C in humidified 5% CO_2 atmosphere. The macrophages were infected with the two strains of *Asaia* at a MOI of 100 bacteria per macrophage, as described above. After the streptomycin treatment for 1h, the cell monolayers were washed once in PBS and then infected with *L. infantum* stationary phase promastigotes at a ratio of 2:1 (2 parasites per 1 macrophage) for 45h at 37°C. Non-internalized promastigotes were removed at 24h post infection by washing with PBS and fresh DMEM was replaced. At designated time points the culture supernatants were collected, centrifugated (14,000 rpm, 15 min at 4°C) and stored at -20°C until cytokines and nitrite determination (see below). For the assessment of leishmanicidal activity, the macrophages after 24h and 48 h of infection were washed with PBS, collected by using a cell scraper, centrifugated at 1,200 rpm for 6 min and washed with PBS. Finally, they were suspended in 200 µl PBS at the final concentration of 10^6cells/ml and cytocentrifugated (Cytospin Hettich) for 5 min at 500 rpm on a slide and

stained with Giemsa solution following the standard protocol (Sigma-Aldrich, USA). As control of the leishmanicidal activity, we treated the macrophages with the anti-*Leishmania* drug Amphotericin B (0.3 µg/ml). At 24h after infection a fresh DMEM medium was replaced in each well until 48h. Then, the cells were collected as above.

The infection rate (percentage of infected macrophages/100 macrophage), the number of parasites in each macrophage and the parasitic index (average number of infected macrophages multiplied by the average number of amastigotes per macrophage) were determined with a microscope at 100X. An average of ten areas of the cover slip was used to determine these indices in duplicate. The experiments were performed in triplicate. Considering that we scored a large proportion of macrophages not containing amastigotes, the William's mean (Mw) was used to calculate a geometric mean. The Mw data were then analyzed using Friedman test for repeated measures followed by a Dunn's post-hoc test. The proportions of macrophages containing at least one amastigote was analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Bonferroni correction to avoid type II errors. The Parasite index, calculated as the product of the first two indexes, was analyzed as Mw data. The Friedman test was performed using GraphPad Prism 8, while the CMH test was performed in RStudio from the "dplyr" package.

Determination of M1/Th1 and M2/Th2 cytokines and NO production

All the cytokines were determined by ELISA kits: IL12p40, IL10 (Biolegend, USA), IL-1β, IL-6, TNFα (Thermo Fisher, USA), according to manufacturer's instructions. Only for IL-10 and IL-1β quantification, the cells were pre-stimulated with LPS 1 µg/ml for 12h, before the infection.

Simultaneous evaluation of nitrate and nitrite concentrations induced by the bacteria was measured by Vanadium assay with the reduction of nitrate to nitrites by Vanadium (III) combined with detection by the acidic Griess reaction (Sigma-Aldrich, USA), as reported in Miranda, 2001. In brief, saturated solutions of Vanadium (III) chloride (VCl_3) were prepared in 1M HCl. Hundred µl of culture supernatants, collected at 48h of co-infection, were mixed with the same volume of VCl_3 and reacted with an equal volume of the Griess reagents (1% sulfanilamide and 0.1% naphthylethylenediamine-HCl in 2.5% phosphoric acid). The absorbance at 540 nm was measured using a plate reader following the incubation (usually 30-45 min at 37°C). The data (pg/ml) were first checked for normality using a Shapiro-Wilk test and Log transformed if needed and analyzed in two groups: a first test was performed comparing the samples containing *Asaia* (independently from the strain), LPS, the Medium alone or *Leishmania* only. This set was meant to detect if the bacteria have a comparable effect to LPS and differ from other controls. A second set was meant to detect the effect of *Asaia* strain during *Leishmania* infection. For the first set, data were analysed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet's T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn's test. For the second set, data were analysed using a two-WAY Mixed-effects model with repeated measures matching followed by Sidak's post-hoc test. The tests were performed using GraphPad Prism 8.

Arginase and iNOS expression by real time quantitative PCR

To evaluate arginase expression, the cells were pre-treated with IL4 (200 U/ml) (R&D Systems, Minneapolis, MN) for 12h at 37°C and then infected as above, while for iNOS (inducible nitric oxide synthase) expression no priming was performed. After 24h of infection for iNOS and 48h for arginase, the cells were collected and stored in RNA later (Qiagen, Germany) at -80°C till further use. Total RNA of infected cells was extracted using the ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI, USA) following manufacturer's instructions. RNA purity was checked by determining the 260/280 nm absorbance ratio. cDNAs were synthesized from 200 ng of total RNA using the LunaScript™ RT SuperMix Kit from New England BioLabs (NEB, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using a BioRad CFX Real-Time PCR Detection System (Bio-Rad, USA) using *β-Actin* and *cyclophilin* genes for normalization (S2 Table) at the following conditions in a final volume of 20 µl: 10 µl of Supermix SsoAdvanced SYBR Green, 10 µM of primers, 2.4 µl of cDNA. After initial denaturation for 95°C for 30 sec, the amplification was performed at 95°C (15 s), 58°C (30 s) for 45 cycles then 65°C (5 s). The data (relative expression) were first checked for normality using a Shapiro-Wilk test and Log transformed if needed and analyzed in two groups as described before. For the first set, data were analysed using a Kruskal-Wallis test followed by a Dunn's test. For the second set, data were analysed using a two-WAY ANOVA followed by Sidak's post-hoc test.

ROS determination

Intracellular reactive oxygen species (ROS) were measured by a fluorometric assay using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) as probe. In brief, macrophages were seeded (35,000/well) in a final volume of 200 µl/well in 96well microplates and allowed to adhere 24h at 37°C in humidified 5% CO_2 atmosphere. After an overnight incubation, the supernatants were discarded, and the cells were washed with PBS. Two hundred µl/well of H_2DCF-DA (5 µM) were added to the macrophages, and incubated 1h a 37°C. Subsequently, the cells were washed and infected with the two strains of bacteria first and then with *Leishmania,* as reported above. The cells were incubated in humidified 5% CO_2 atmosphere at 37°C, protected from light, for approximately 14h. Half an hour before ending of incubation, a group of cells were treated with 1mM H_2O_2 and the fluorescence at 485 nm (Ex) / 535 nm (Em) was measured. Data were analysed as described for the secreted cytokines.

Cell surface markers analysis by flow cytometry

Expression of CD40, CD80, CD86 and MHC class II was evaluated with a FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ). For the evaluation of co-stimulatory molecules, the cells were harvested 24h post infection with bacteria and *Leishmania* as indicated previously, washed with PBS and stained with appropriate dilutions of the following fluorochrome-conjugated antibodies: CD40-PE, CD80-Alexa Fluor488 and CD86-PE/Cy7 (all purchased from Biolegend San Diego, CA) for 15 min at 37°C. The cells were washed, resuspended in the Facs washing buffer (PBS/FCS 1%) and finally analyzed.

For the evaluation of MHC class II molecules, the cells were pre-stimulated with INFγ (1ng/ml) 12h before the infection. After 48h of co-infection the cells were harvested and processed as above using MHC class II-FITC (Biolegend San Diego, CA) as antibody. Fluorescence-activated cell sorting (FACS) data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

The proportions of macrophages presenting the studied markers were analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Holm correction to avoid type II errors, performed in RStudio from the “dplyr” package. The geometric means of fluorescence of the fluorescent cells in each set was compared using one-way ANOVA followed by Sidak’s post-hoc test for multiple comparisons performed in GraphPad Prism 8.

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Supporting information captions

S1 Table. Statistical analyses

S2 Table. List of primers used in this study.

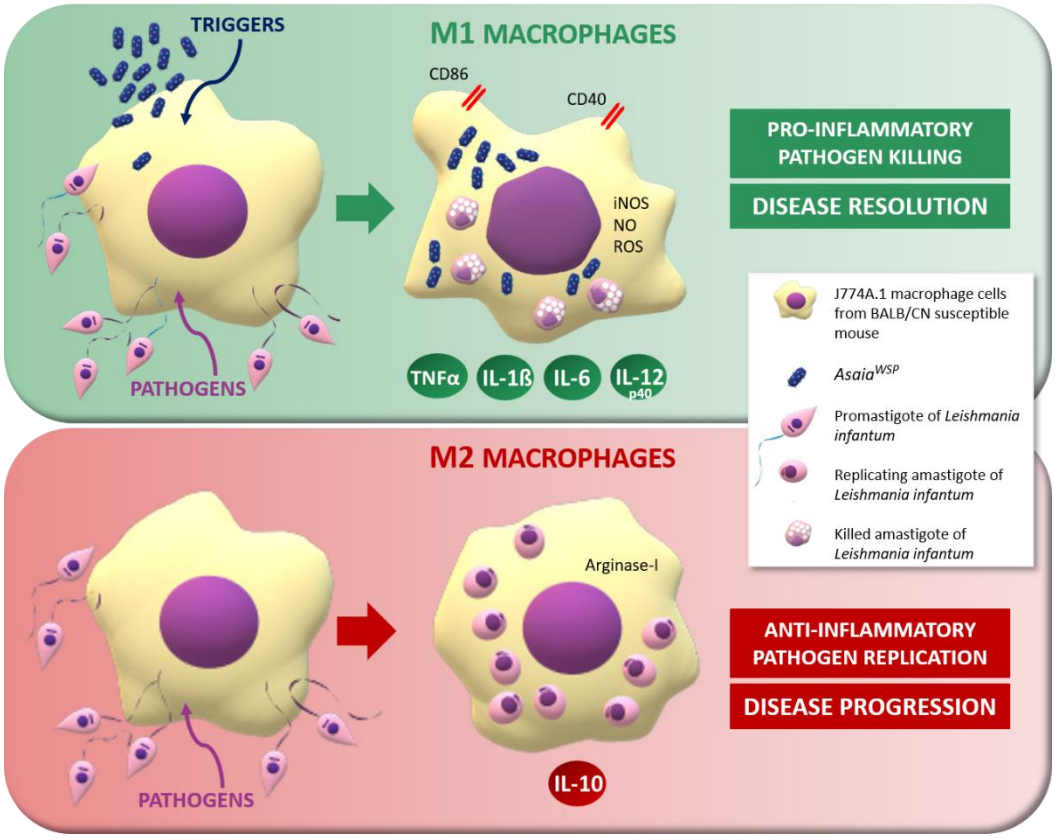


Fig 1. Experimental design. M1/M2 polarization of macrophage cells induced by *Asaia*^{WSP} and markers analysed. TNF: tumor necrosis factor; IL: interleukin; ROS: reactive oxygen species; iNOS: inducible nitric oxide synthase; NO: nitric oxide.

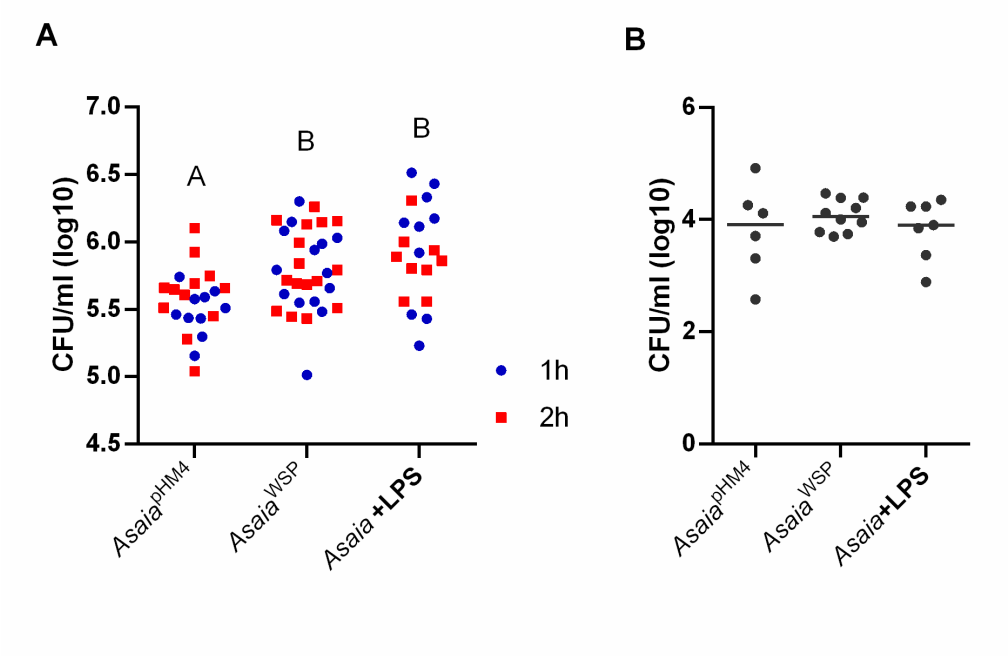


Fig 2. Bacteria uptake and survival in macrophages.

Murine macrophages were infected with *Asaia*^{WSP} and *Asaia*^{pHM4} or bacteria plus LPS. A) The number of bacteria internalized within macrophages was determined at 1h and 2h post-infection and expressed as CFU/ml. The mean number of bacteria phagocyted was Log transformed to reach normality and analysed using a two-way ANOVA followed by Sidak's post-hoc test. Different capital letters represent statistically significant differences ($p<0.05$). B) The number of bacteria phagocyted after 2h of co-incubation and survived until 24h was expressed as CFU/ml. The mean number of bacteria phagocyted was Log transformed to reach normality and analysed using a ONE-WAY Brown-Forsythe ANOVA.

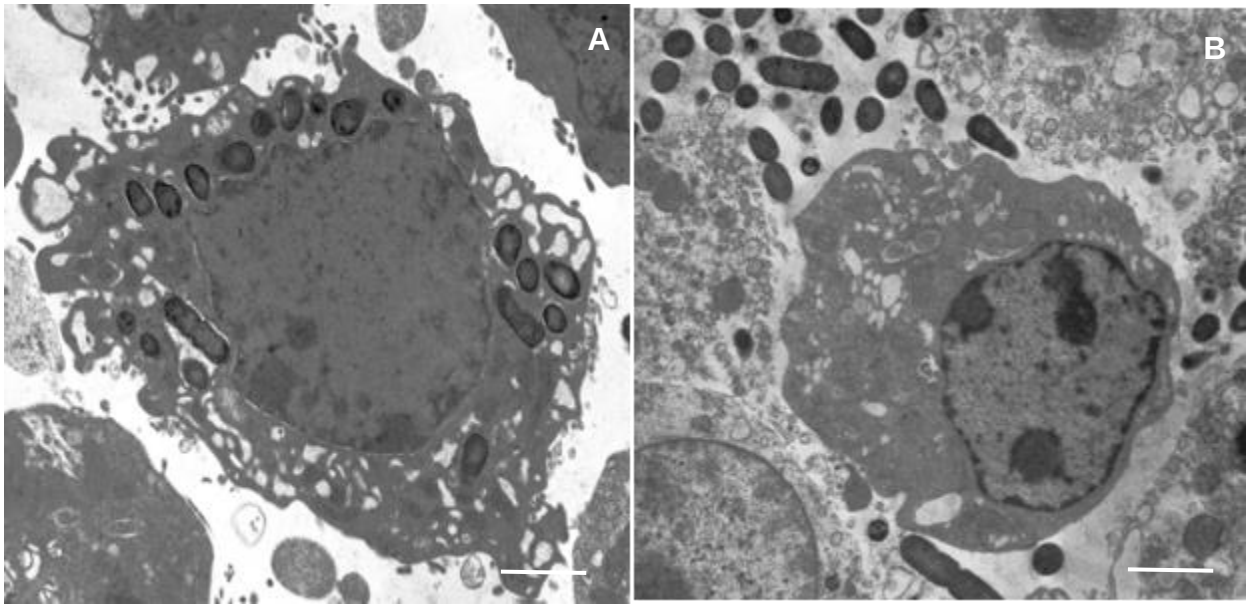


Fig 3. TEM analysis of macrophage phagocytosis.

A murine macrophage infected with *Asaia*^{WSP} (A) and by *Asaia*^{pHM4} (B) after 24h of co-infection. In panel A, note the very high number of bacteria in vacuoles and the intense phagocytic activity. In panel B, bacteria are mostly outside the macrophage. Bar: 5 μm.

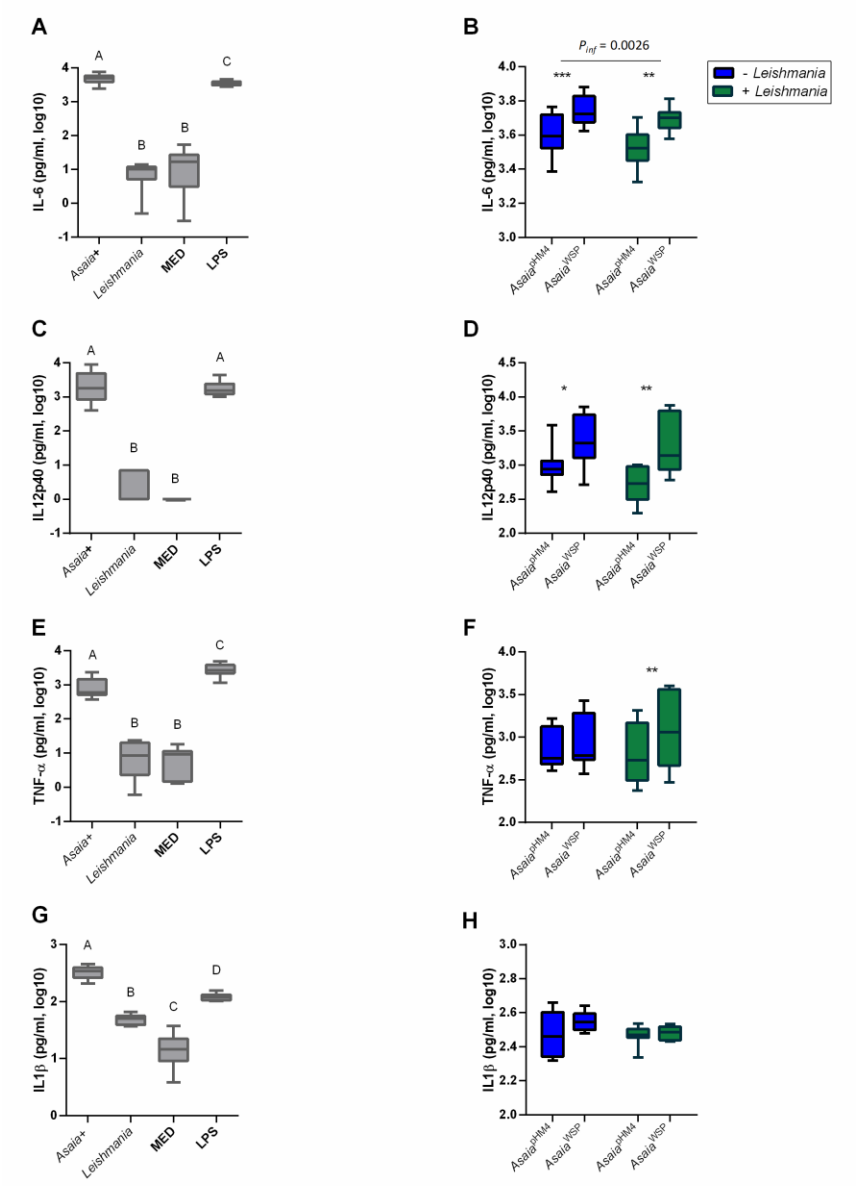


Fig 4. IL6, IL12p40, TNFα, IL1β cytokines produced by macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} after 24h of infection.

A, C, E, G) Levels of cytokines produced by macrophages treated with *Asaia* bacteria (*Asaia*+), *Leishmania*, LPS or untreated (MED) were represented as mean ± SD of three independent experiments (ELISA assay). The data (pg/ml) were first checked for normality using a Shapiro-Wilk test and Log transformed. Data were analysed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet's T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn's test. Different capital letters represent statistically significant differences ($p<0.05$). B, D, F, H) Levels of cytokines produced by macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} in presence or in absence of *Leishmania* were reported. Data were analysed using a two-WAY Mixed-effects model with repeated measures matching followed by Sidak's post-hoc test. Asterisks indicate statistically significant differences ($p<0.05$; $p<0.01$; $p<0.001$).

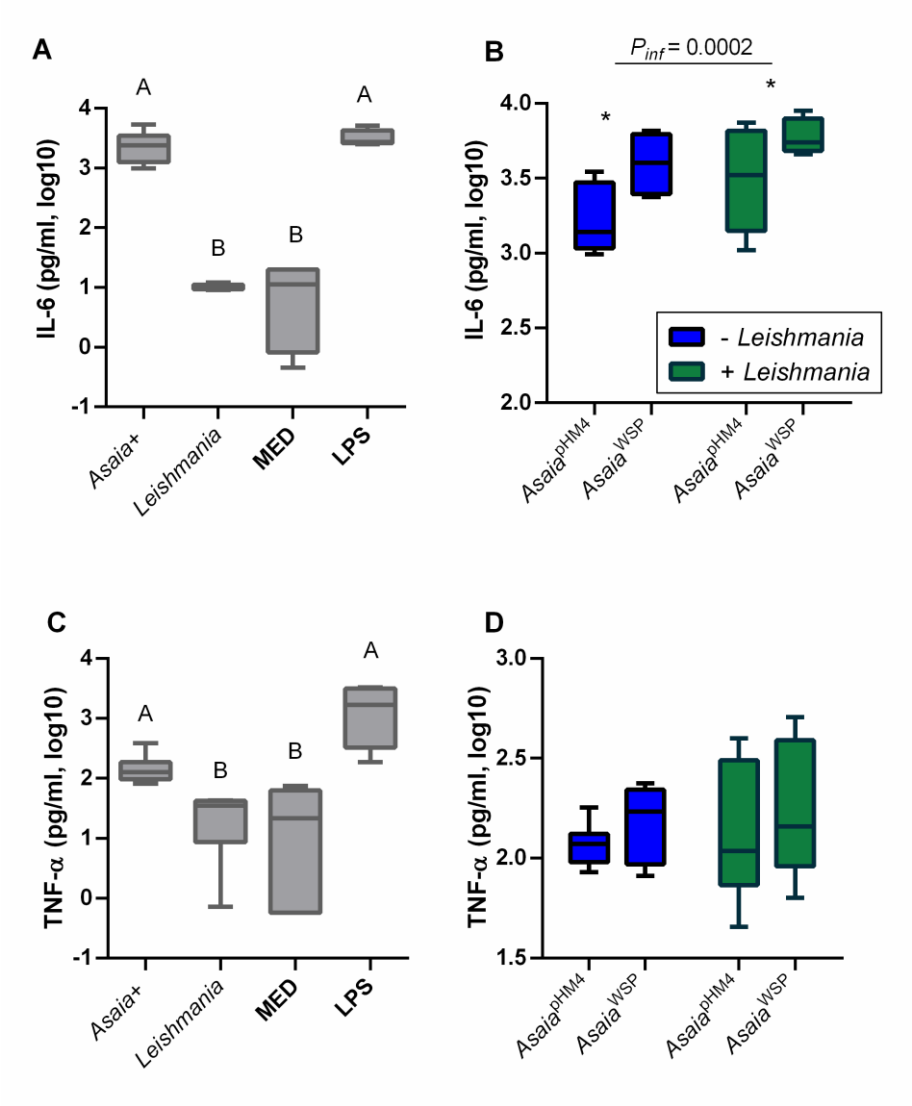


Fig 5. IL6, TNFα cytokines produced by macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} after 48h of infection.

A, C) Levels of cytokines produced by macrophages treated with *Asaia* bacteria (*Asaia*+), *Leishmania*, LPS or untreated (MED) were represented as mean ± SD of three independent experiments (ELISA assay). The data (pg/ml) were first checked for normality using a Shapiro-Wilk test and Log transformed. Data were analysed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet's T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn's test. Different capital letters represent statistically significant differences ($p<0.05$). B, D) Levels of cytokines produced by macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} in presence or in absence of *Leishmania* were reported. Data were analyzed using a two-WAY Mixed-effects model with repeated measures matching followed by Sidak's post-hoc test. Asterisks indicate statistically significant differences ($p<0.05$)

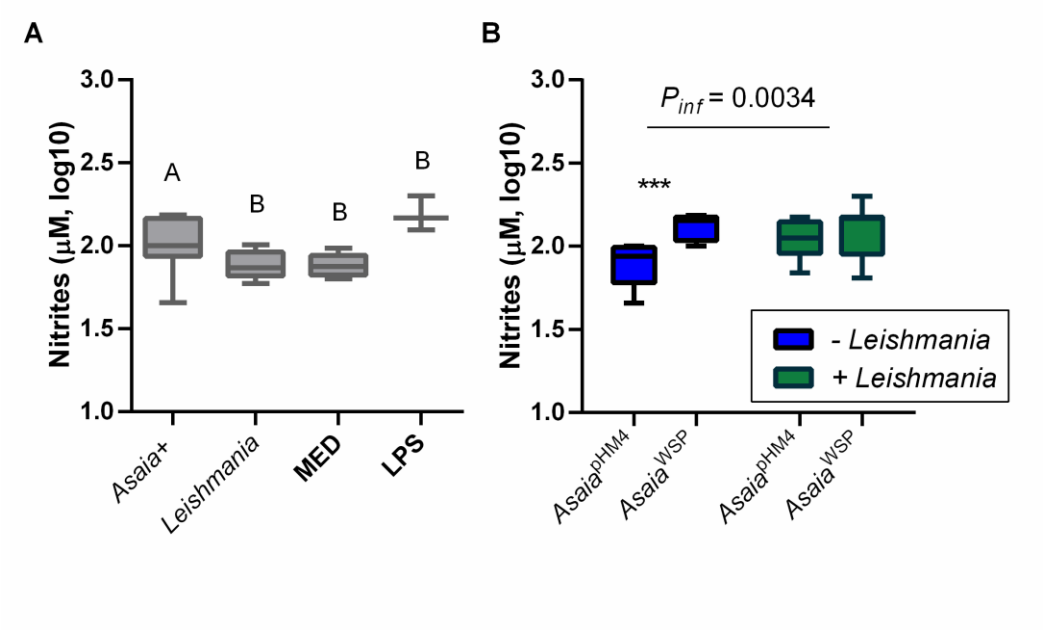


Fig 6. Nitrites production by macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} after 48h of infection.

A) Nitrites production by macrophages treated with *Asaia* bacteria (*Asaia*+), *Leishmania*, LPS or untreated (MED) was represented as mean ± SD of three independent experiments (Vanadium chloride assay followed by Griess reaction). The data were first checked for normality using a Shapiro-Wilk test and Log transformed. Data were analysed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet's T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn's test. Different capital letters represent statistically significant differences ($p<0.05$). B) Nitrites production by macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} in presence or in absence of *Leishmania* was reported. Data were analyzed using a TWO-WAY Mixed-effects model with repeated measures matching followed by Sidak's post-hoc test. Asterisks indicate statistically significant differences ($p<0.001$).

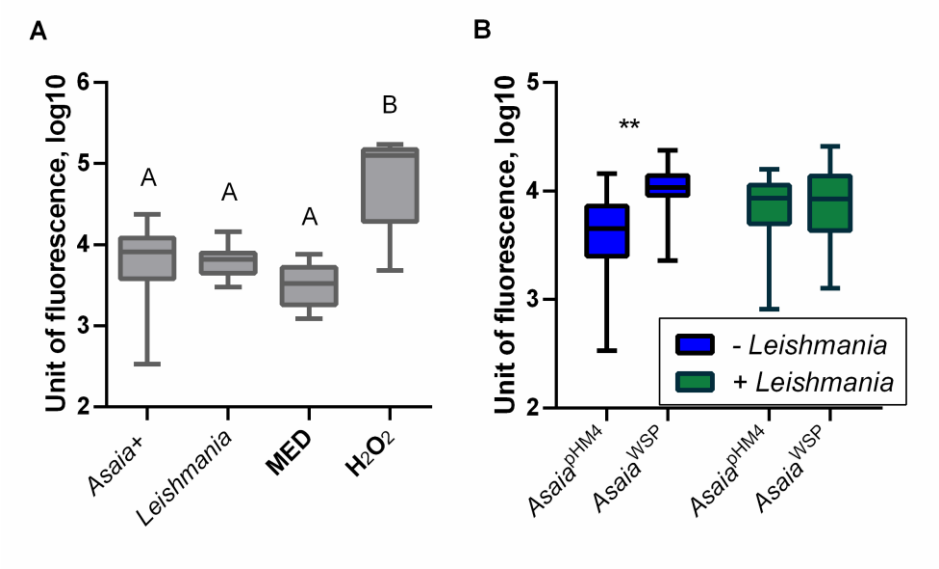


Fig 7. ROS production by macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4}.

A) ROS production by macrophages treated with *Asaia* bacteria (*Asaia*+), *Leishmania*, H_2O_2 or untreated (MED) was represented as mean ± SD of three independent experiments. Data (units of fluorescence) were first checked for normality using a Shapiro-Wilk test and Log transformed. Data were analysed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet's T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn's test. Different capital letters represent statistically significant differences ($p<0.05$). B) ROS production by macrophages infected with *Asaia*^{WSP} or with *Asaia*^{pHM4} in presence or in absence of *Leishmania* was reported. Data were analyzed using a TWO-WAY Mixed-effects model with repeated measures matching followed by Sidak's post-hoc test. Asterisks indicate statistically significant differences ($p<0.01$).

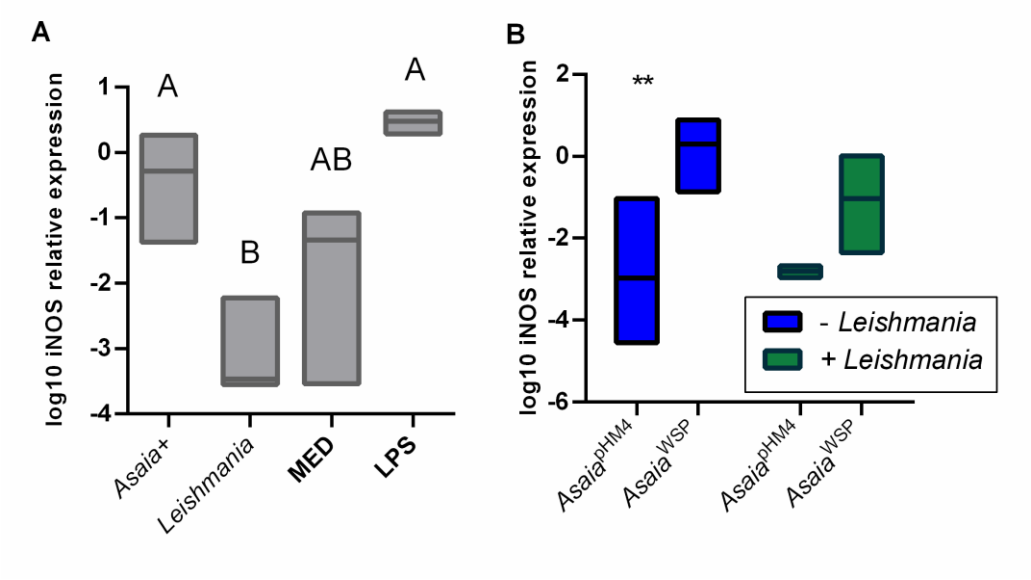


Fig 8. qRT-PCR analyses of iNOS expression.

A) Relative expression of *iNOS* gene of murine macrophages treated with *Asaia* bacteria (*Asaia*+), *Leishmania*, LPS or untreated (MED) was determined after 24h of infection. The data (relative expression) was first checked for normality using a Shapiro-Wilk test and Log transformed. Data were analyzed using a Kruskal-Wallis test followed by a Dunn's test. Different capital letters represent statistically significant differences ($p<0.05$). B) Relative expression of iNOS gene of murine macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} in presence or in absence of *Leishmania* was reported. Data were analysed using a TWO-WAY ANOVA followed by Sidak's post-hoc test. Asterisks indicate statistically significant differences ($p<0.01$).

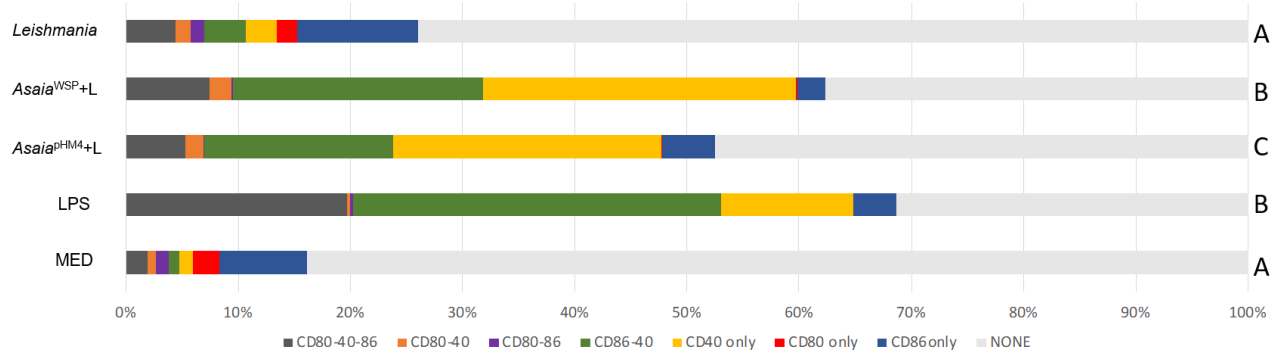


Fig 9. **Percentages of macrophages positive for the co-stimulatory molecules CD80-CD40-CD86 and for the combinations CD40-86, CD80-40, CD86-40.**

Percentages of macrophages infected with *Asaia*^{WSP} or *Asaia*^{pHM4} (both in presence of *Leishmania),* with *Leishmania* alone, stimulated with LPS or untreated (MED) were determined after 24h by flow cytometry using the following fluorochrome-conjugated antibodies: CD40-PE, CD80-Alexa Fluor488 and CD86-PE/Cy7. The proportions of macrophages presenting the studied markers were analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Holm correction, performed in RStudio from the “dplyr” package. Different capital letters represent statistically significant differences among experimental groups expressing at least one co-stimulatory molecule ($p<0.05$).

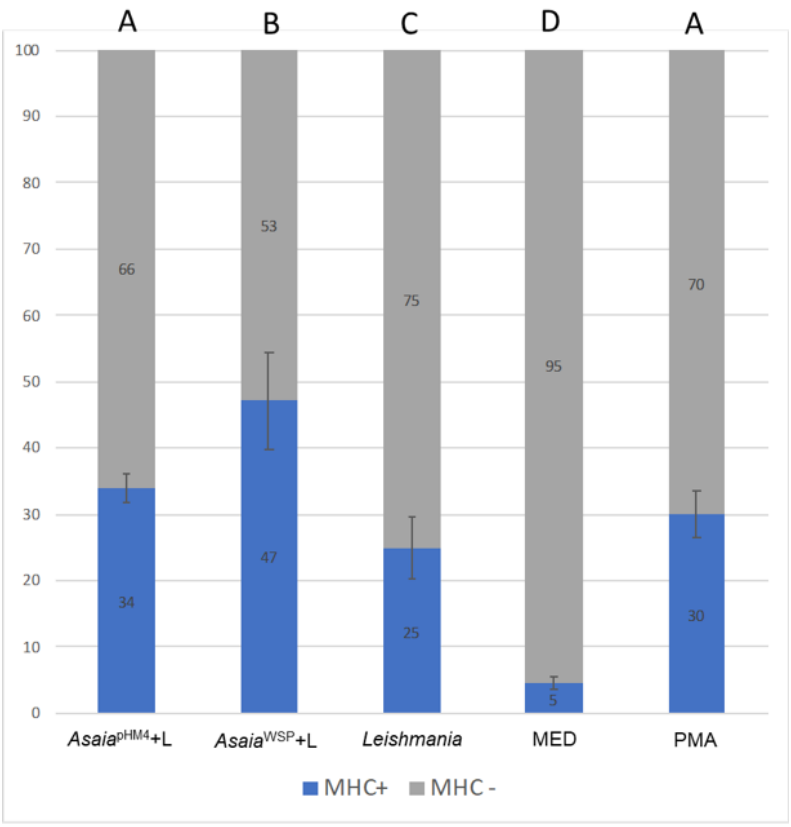


Fig 10. **Percentages of macrophages positive for MHC class II marker**.

Percentages of macrophages infected with *Asaia*^{WSP} or *Asaia*^{pHM4} (both in presence of *Leishmania*), with *Leishmania* alone, stimulated with PMA or untreated (MED), were determined after 48h by flow cytometry using an MHC class II-FITC antibody. The proportions of macrophages presenting the selected marker were analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Holm correction, performed in RStudio from the “dplyr” package. Different capital letters represent statistically significant differences ($p<0.05$).

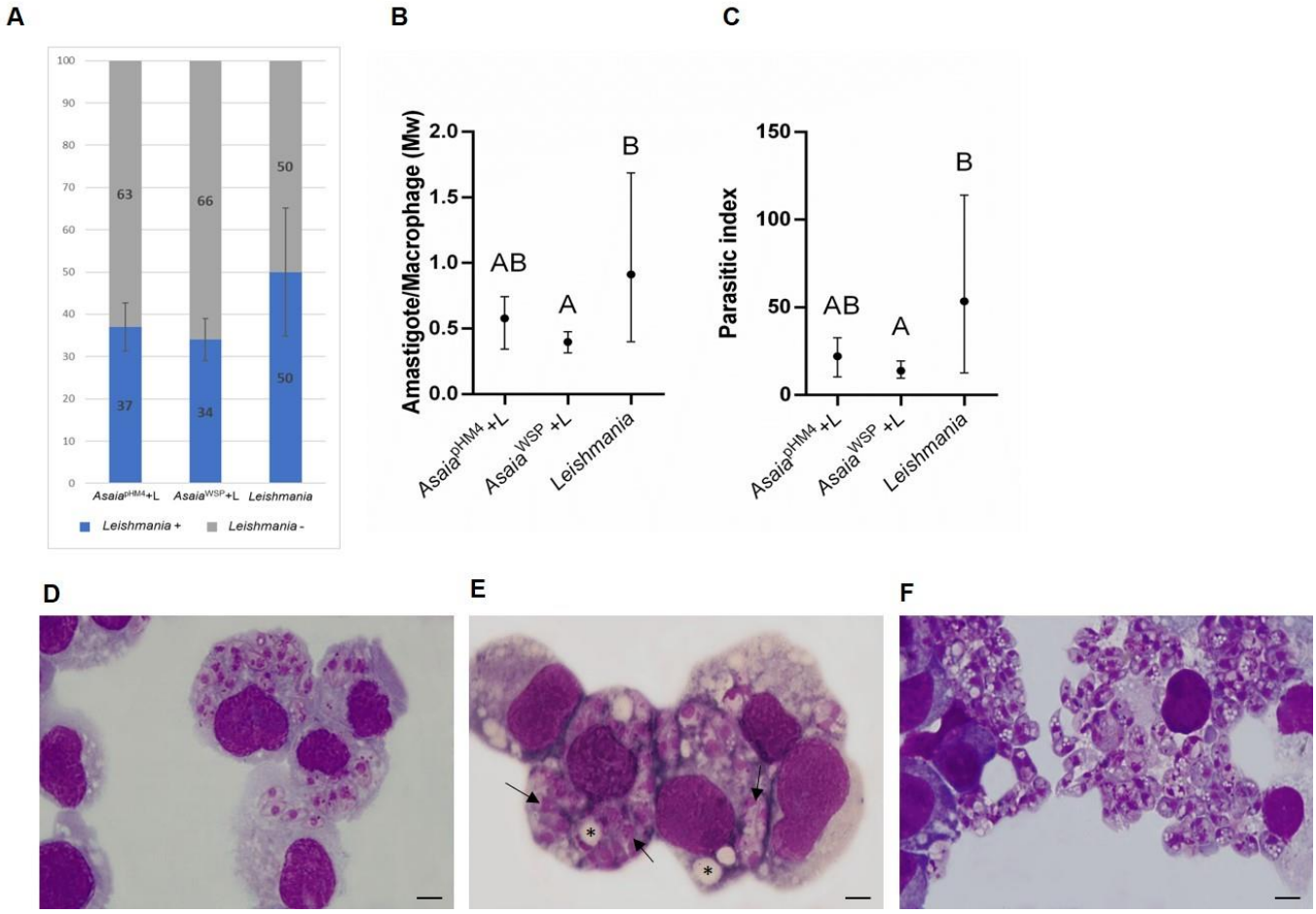
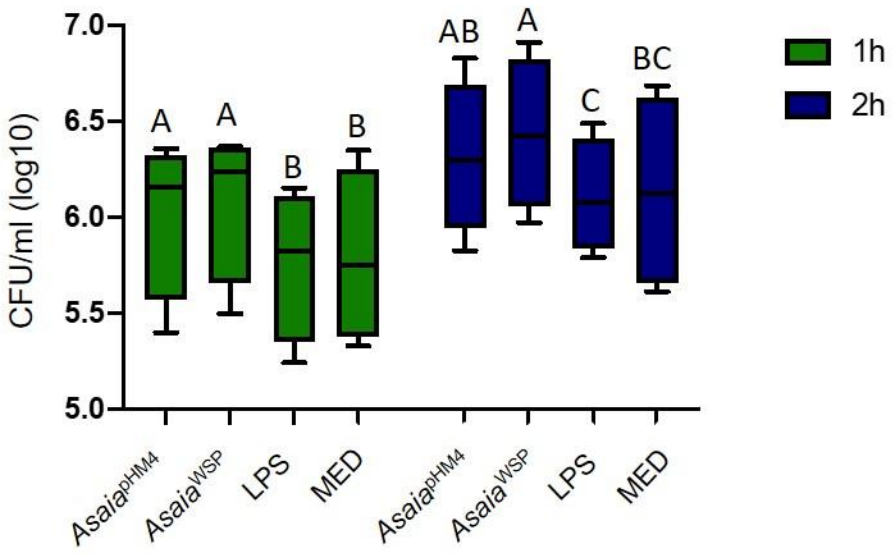


Fig 11. Anti-leishmanial effect determinated by *Asaia*^{WSP}- infected macrophages after 48h of co-infection.

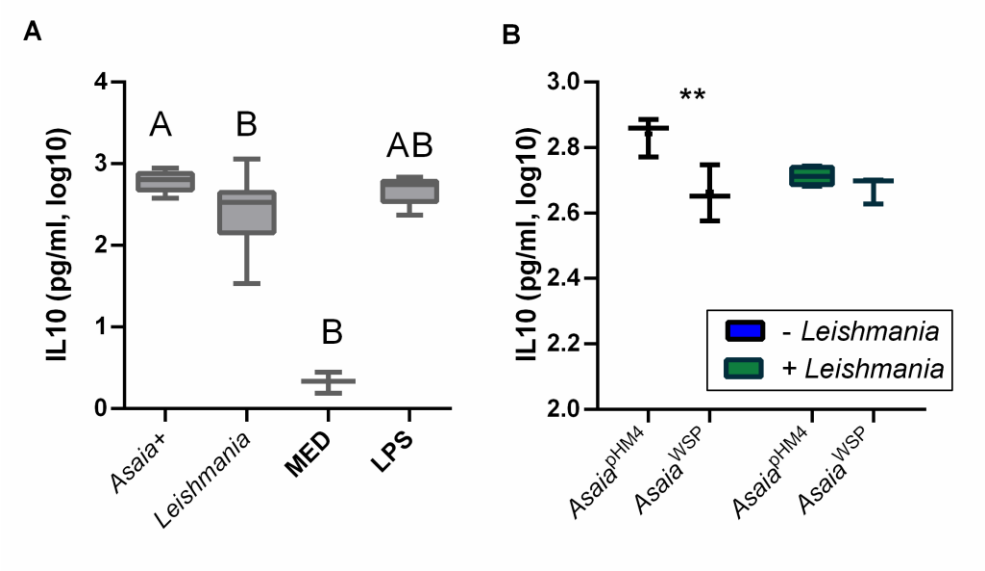
Macrophages were pre-stimulated with *Asaia*^{WSP}, *Asaia*^{pHM4} and then infected with *Leishmania* promastigotes (*Asaia*$^{WSP+L}$, *Asaia*$^{pHM4+L}$ and *Leishmania* alone, respectively). (A) The percentage of infected macrophages/100 macrophage (infection rate), (B) the number of parasites in each macrophage expressed as William's mean (Mw) and (C) the parasitic index (average number of infected macrophages multiplied by the average number of amastigotes per macrophage) were determined by microscopic observation. D-F) Giemsa staining of macrophages infected with *Leishmania* and *Asaia*^{pHM4} (D), *Leishmania* and *Asaia*^{WSP} (E) and *Leishmania* alone (F). Arrows indicate groups of killed amastigotes and asterisks indicate the presence of vacuoles in macrophages infected with *Asaia*^{WSP}, signs of a high leishmanicidal activity. Bar: 5 μm. The Mw data were analyzed using Friedman test followed by a Dunn's post-hoc test. The proportions of macrophages containing at least one amastigote was analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Bonferroni correction. The parasitic index, calculated as the product of the first two indexes, was analyzed as Mw data. Different capital letters represent statistically significant differences between the treated groups ($p<0.05$).

SUPPLEMENTARY FIGURES



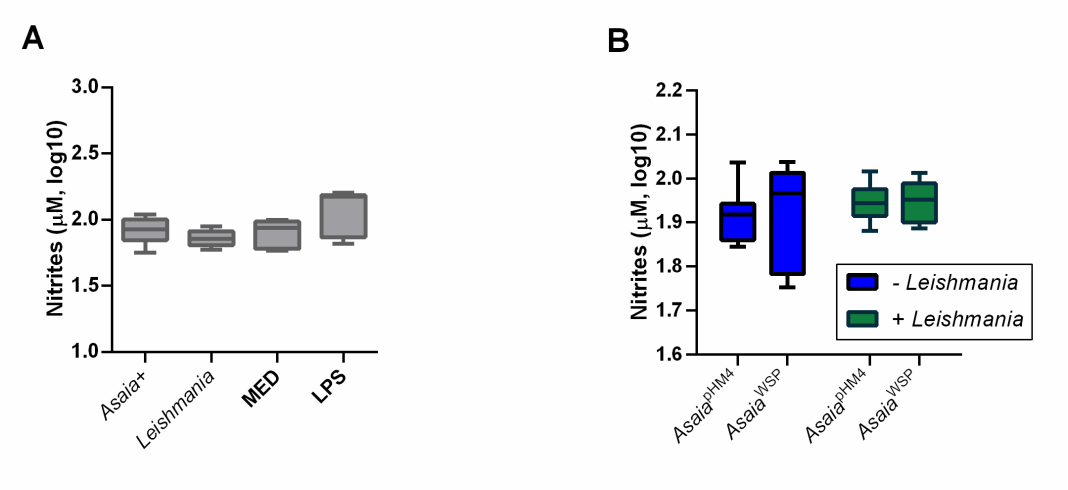
S1 Fig. ***Staphylococcus epidermidis*** **uptake by macrophages pre-infected with** ***Asaia*** **bacteria.**

Murine macrophages were pre-stimulated with *Asaia*^{WSP}, *Asaia*^{pHM4}, LPS or unstimulated and then infected with *S. epidermidis* bacteria. The number of *S. epidermidis* bacteria internalized within macrophages was determined 1h and 2h post-infection and expressed as Log CFU/ml. The data were analyzed using a two-way ANOVA with repeated measures followed by Sidak's post-hoc test. Different capital letters represent statistically significant differences ($p<0.05$).



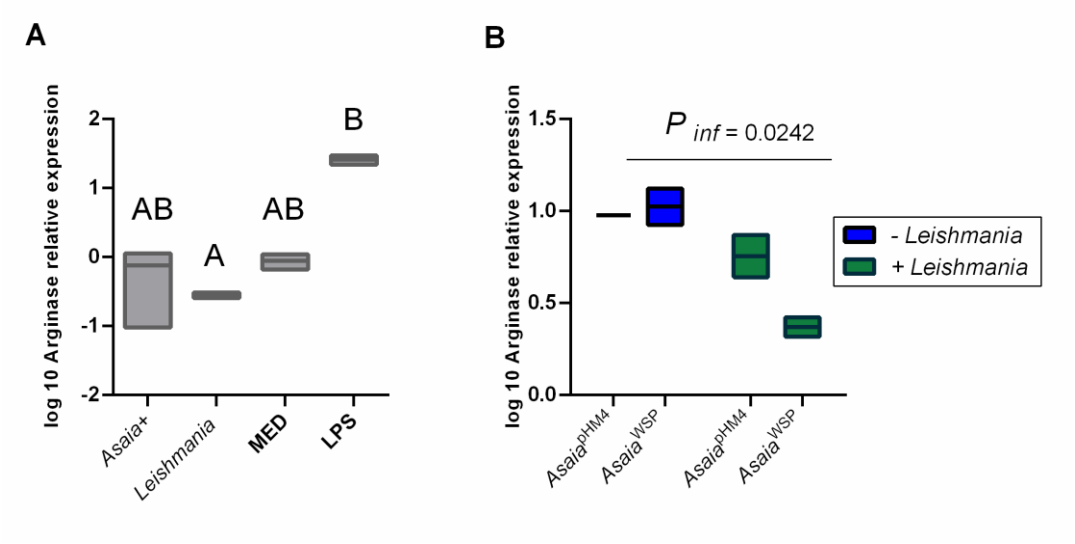
S2 Fig. IL10 cytokine production by macrophages infected with *Asaia*^{WSP} or *Asaia*^{pHM4} after 24h of infection.

A) The levels of IL10 cytokine produced by macrophages treated with *Asaia* bacteria (*Asaia*+), *Leishmania*, LPS or untreated (MED) were representative of two independent experiments. The data (pg/ml) were first checked for normality using a Shapiro-Wilk test and Log transformed. Data were analysed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet's T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn's test. Different capital letters represent statistically significant differences ($p<0.05$). B) The levels of cytokine produced by macrophages infected with *Asaia*^{WSP} or with *Asaia*^{pHM4} in presence or in absence of *Leishmania* were reported. Data were analyzed using a two-WAY Mixed-effects model with repeated measures matching followed by Sidak's post-hoc test. Asterisks indicate statistically significant differences ($p<0.01$).



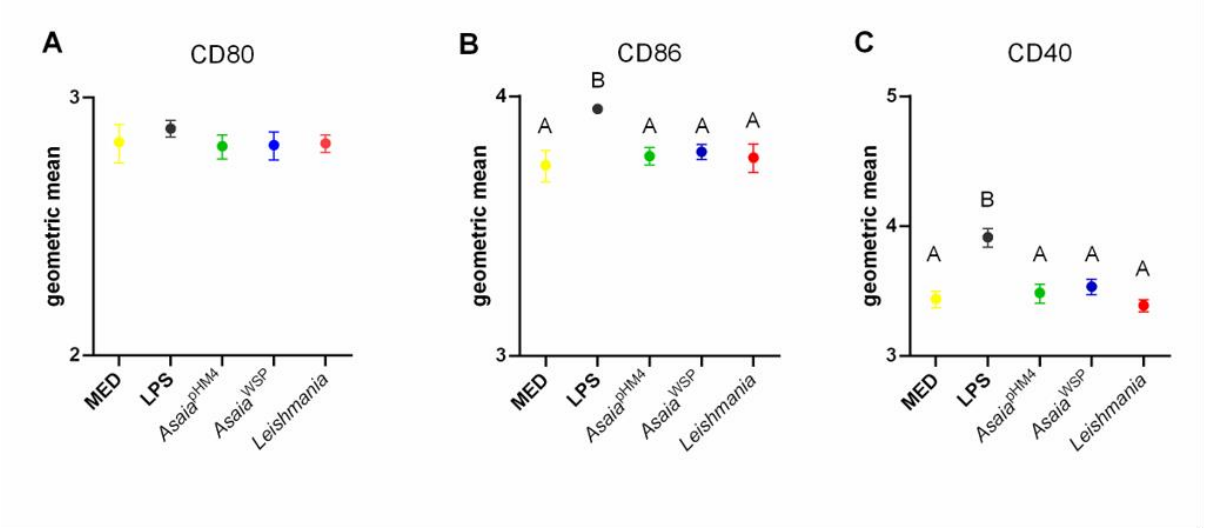
S3 Fig. Nitrites production by macrophages infected with *Asaia*^{WSP} and *Asaia*^{pHM4} after 24h of infection.

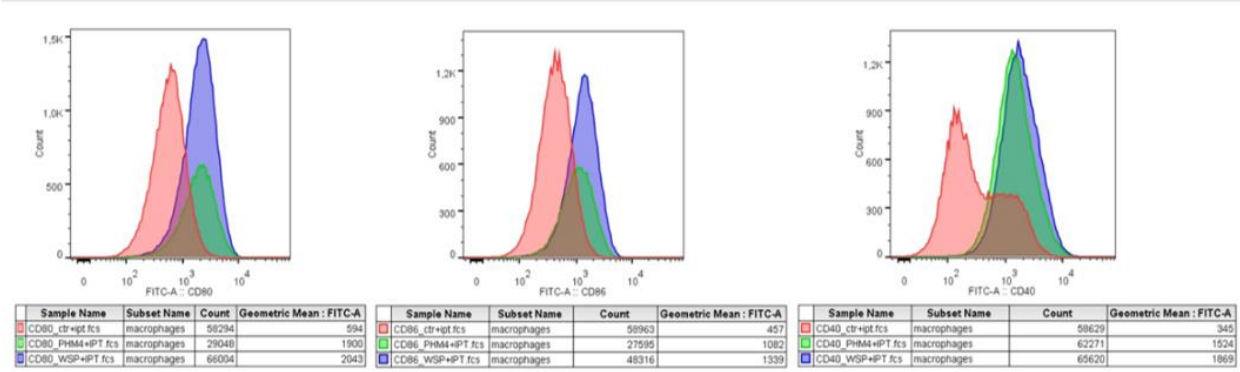
A) Levels of nitrites, expressed in µM, produced by macrophages infected with *Asaia* bacteria, *Leishmania*, stimulated with LPS or untreated (MED) were reported. Data were Log transformed and analyzed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet's T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn's test. B) Nitrites produced by macrophages infected with *Asaia*^{WSP} or with *Asaia*^{pHM4} in presence or in absence of *Leishmania* were Log transformed and then analysed using a two-WAY Mixed-effects model with repeated measures matching followed by Sidak's post-hoc test.



S4 Fig. qRT-PCR analyses of iNOS expression

A) Relative expression of *arginase* gene of murine macrophages infected with *Asaia* bacteria (*Asaia*+), *Leishmania*, stimulated with LPS or untreated (MED) was determined after 24 h of infection. The data was first checked for normality using a Shapiro-Wilk test and Log transformed. Data were analysed using a Kruskal-Wallis test followed by a Dunn's test. Different capital letters represent statistically significant differences ($p<0.05$). B) Relative expression of *arginase* gene of murine macrophages infected with *Asaia*^{WSP} and with *Asaia*^{pHM4} in presence or in absence of *Leishmania* was reported. Data were analysed using a two-WAY ANOVA followed by Sidak's post-hoc test.





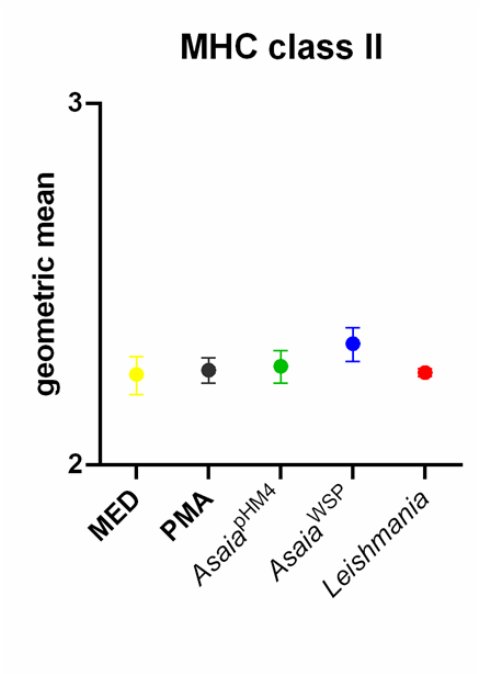
Sample Name	Subset Name	Count	Geometric Mean : FITC-A
CD80_ctr+ipt.fcs	macrophages	58294	594
CD80_PHM4+IPT.fcs	macrophages	29048	1900
CD80_WSP+IPT.fcs	macrophages	66004	2043

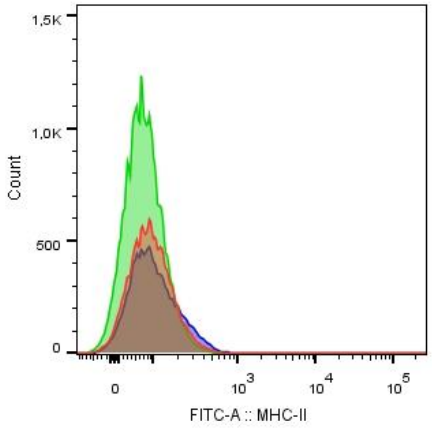
Sample Name	Subset Name	Count	Geometric Mean : FITC-A
CD86_ctr+ipt.fcs	macrophages	58963	457
CD86_PHM4+IPT.fcs	macrophages	27595	1082
CD86_WSP+IPT.fcs	macrophages	48316	1339

Sample Name	Subset Name	Count	Geometric Mean : FITC-A
CD40_ctr+ipt.fcs	macrophages	58629	345
CD40_PHM4+IPT.fcs	macrophages	62271	1524
CD40_WSP+IPT.fcs	macrophages	65620	1869

S5 Fig. **Expression of co-stimulatory molecules CD80-CD86-CD40 by macrophages co-infected with the two strains of bacteria and *Leishmania* after 24h**.

The geometric mean of fluorescence of CD80 (A) CD86 (B) CD40 (C) positive cells co-infected with *Asaia*^{WSP} or *Asaia*^{pHM4}, both in presence of *Leishmania, Leishmania* only, LPS or untreated (MED) was determined by flow cytometry using the following fluorochrome-conjugated antibodies: CD40-PE, CD80-Alexa Fluor488 and CD86-PE/Cy7. The geometric means of fluorescence of the positive cells was compared using one-way ANOVA followed by Sidak's post-hoc test for multiple comparisons. Different capital letters represent statistically significant differences ($p<0.05$).

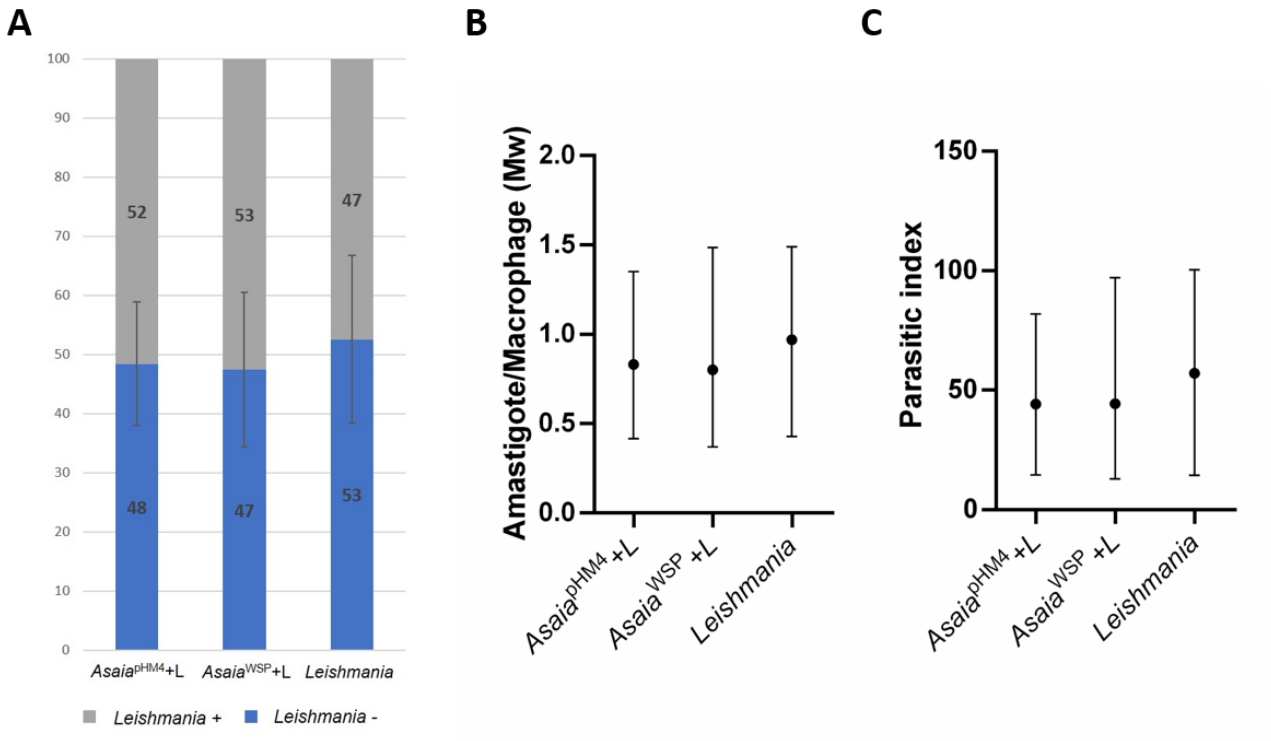




	Sample Name	Subset Name	Count	Geometric Mean : FITC-A
	MHC-II_ctr+ipt.fcs	Macrophages	21937	114
	MHC-II_PHM4+IPT.fcs	Macrophages	38320	89,8
	MHC-II_WSP+IPT.fcs	Macrophages	19239	129

S6 Fig. Expression of MHC class II by macrophages co-infected with *Asaia*^{WSP} and *Leishmania*.

The geometric mean of fluorescence of MHC class II positive cells co-infected with *Asaia*^{WSP} or *Asaia*^{pHM4}, both in presence of *Leishmania, Leishmania* only, treated with PMA or untreated (MED) was determined after 48h of infection by flow cytometry using a MHC class II-FITC antibody. The geometric means of fluorescence of the positive cells was compared using one-way ANOVA followed by Sidak's post-hoc test for multiple comparisons.



S7 Fig. Determination of the anti-leishmanial effect after 24h of bacteria and *Leishmania* co-infection.

The macrophages were pre-infected with *Asaia*^{WSP}, *Asaia*^{pHM4} or unstimulated and then infected with *Leishmania* promastigotes (*Asaia*$^{WSP+L}$, *Asaia*$^{pHM4+L}$ and *Leishmania,* respectively). The infection rate (percentage of infected macrophages/100 macrophage) (a), the number of parasites in each macrophage (b) and the parasitic index (average number of infected macrophages multiplied by the average number of amastigotes per macrophage) (c) were determined by microscopic observation. The William's mean (Mw) was used to calculate a geometric mean. The Mw data were then analyzed using Friedman test for repeated measures followed by a Dunn's post-hoc test. The proportions of macrophages containing at least one amastigote was analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Bonferroni correction to avoid type II errors. The parasite index, calculated as the product of the first two indexes, was analyzed as Mw data.

3. CONCLUSIONS

According to WHO, vector-borne diseases represent one-sixth of all infectious diseases and cause more than 1.000,000 deaths every year. In addition to the clinical consequences, they also have a strong socioeconomical impact on the populations (Campbell-Lendrum et al., 2015). Moreover, insecticides resistance development and climate change, together with many other factors, are threatening the control of vector-borne diseases (Campbell-Lendrum et al., 2015; Vonesch et al., 2016; Githeko et al., 2000). All these conditions urge us to find new methods to fight the vector-borne diseases. In this scenario the symbiotic control, based on the use of microorganism symbionts of insects, is attracting attention as it is particularly promising (Coutinho-Abreu et al., 2010).

An approach that is a part of the symbiotic control is the paratransgenesis: the manipulation of the symbionts of a vector to produce anti-parasitic molecules (Beard et al., 1998). In the recent years, many researches have performed experiments of paratransgenesis using different symbionts, mostly bacteria, (e.g. *Serratia, E. coli, Rhodococcus, Pantoea*) to express molecules which could interfere with the pathogen development (Hurwitz et al., 2011; Riehle et al., 2007; Wang et al., 2017).

A promising candidate for the mosquito paratransgenic approach is the bacterium *Asaia bogorensis* (Ricci et al., 2012). *Asaia* spp. are acetic acid bacteria, symbionts of the mosquitoes *A. stephensi*, *Ae. aegypti* (Crotti et al., 2009), *Ae. albopictus* and *Culex quinquefasciatus* (De Freece et al., 2004). This bacterium has already been transformed for the expression of antiplasmodial molecules, making it a suitable candidate for the control of malaria through paratransgenesis (Bongio et al., 2015). *Asaia* sp. is also a symbiont of the sandfly, the vector of *Leishmania* parasite. It is known the presence of *Asaia* spp. in the species *Lu. Longipalpis*, vector of *L. mexicana* in the new world (Sant'Anna et al., 2014), *Phlebotomus papatasi, P. tobbi, P. argentipes, P. duboscqi* and *Sergentomyia* spp. (Akhoundi et al., 2012).

Another symbiotic bacterium whose interest in the control of the vector borne diseases is well documented is *Wolbachia pipientis*. *Wolbachia* is an intracellular bacterium widely diffuse in the arthropods, about 65% of the insects are infected, and in filarial nematodes. In the arthropods *Wolbachia* can induce feminization, male killing, parthenogenesis, and cytoplasmic incompatibility (Zabalou et al., 2004); in addition, it can stimulate the innate immune system of the insect with the expression of AMPs and the production of ROS interfering with pathogen transmission (Brennan et al., 2008). In fact, great results have been obtained trans-infecting *Ae. aegypti* mosquitoes with *Wolbachia* for the control of dengue virus (Walker et al., 2011).

Moreover, *Wolbachia* is reported to infect filarial nematodes of the family of Onchocercidae with which it establishes a mutualistic relationship testified by the fact that the use of an antibiotic to which *Wolbachia* is sensitive leads to the death of the parasite (Bandi et al., 1999). Furthermore, *Wolbachia* has a critical role in the pathogenesis of filariases: the pro-inflammatory response that occurs in presence of nematode extracts is induced by the bacterium *Wolbachia* and it is TLR dependence (Daehnel et al., 2007).

However, *Wolbachia* cannot be used in the paratransgenesis, since it cannot be cultivated in cell-free media and for these reasons it is not easily engineered. A solution to overcome this impediment and exploit the capacities of *Wolbachia* is the use of the molecules derived from this bacterium able to stimulate the immune system of the mosquito. The most abundant molecule produced by this bacterium is the major surface protein of

Wolbachia (WSP). Not only it is known the role of this molecule to induce the innate immune system of the mosquitoes (Pinto et al., 2012), but it can also be considered a Th1 promoting factor in mammalian cells where elicits the release of pro-inflammatory cytokines via TLR2 and TLR4 (Brattig et al., 2004).

In order to join both qualities of these bacteria, the bacterium *Asaia* was engineered for the expression of the protein WSP from *Wolbachia* of the nematode *D. immitis* obtaining a chimeric bacterium *Asaia*^{WSP}. In this way, the engineered bacterium had both the immune-activating capacities of *Wolbachia* and the culturable characteristics of *Asaia*. The engineered bacterium *Asaia*^{WSP} was tested against the heartworm *Dirofilaria immitis*, *in vivo* (see article 1), and against *Leishmania* parasite (see article 2) in *in vitro* assays.

In the first article, the engineered bacterium *Asaia*^{WSP} has been tested for its capability to stimulate the immune system of the mosquitoes *An. stephensi* and *Ae. aegypti* and the potential to interfere with *D. immitis* development after infection. In these mosquitoes *Asaia* is a part of their microbiota, while *Wolbachia* normally is not present. The capability of *Wolbachia* to stimulate the immune system of mosquitoes naturally not infected by this bacterium is well described (Kambris et al., 2010), suggesting that when the bacterium infects a mosquito, that naturally does not harbor *Wolbachia,* the basal immune response of the insect is enhanced with the consequent inhibition of pathogen transmission. For these reasons we used these two study “models” as candidates to investigate the immune-modulatory effects of *Asaia*^{WSP}.

The activation of the innate immune system of these mosquitoes by the chimeric bacterium was investigated in terms of expression of immune genes coding for effector molecules such as cecropin*,* thio-ester containing proteins (TEP) and NO synthase (NOS). In presence of *Asaia*^{WSP} the genes involved in the activation of innate immune response were up regulated, with TEP the most expressed gene. In particular, the up regulation of the TEP gene is very important since its role in *Plasmodium* infection (Volohonsky et al., 2017).

This is a further prove that WSP is able to stimulate the immune system of the insect, also considering that the previous studies were performed with the recombinant protein WSP with the possibility of a contamination by LPS, even though the purification procedures. In this study, the cells were stimulated with the engineered bacterium *Asaia*^{WSP} and the control bacterium *Asaia*^{pHM4}, in this way both bacteria had the same proportion of LPS with the only difference represented by WSP.

Once verified the stimulation of the immune system of the mosquitoes, the capability to inhibit the development of the nematode *D. immitis* in the vector *Ae. aegypti* was investigated. The mosquitoes fed on *Asaia*^{WSP} showed a significant decrease in the number of L3 larvae detected compared with insects fed on sugar meals. Also, the larval abundance was recorded with differences statistically significant between mosquitoes fed on sugar meal and the engineered bacterium *Asaia*^{WSP}. Therefore, a first evidence of inhibition of larval development by the bacterium *Asaia*^{WSP} was highlighted. This study represents the first test on the potential of paratransgenesis for the control of a filarial parasite; in fact, up to now paratransgenesis had not yet been applied in the control of insect-borne pathogenic nematodes.

Taking into account all the results obtained, the engineered bacterium *Asaia*^{WSP} could be considered a promising tool for the vector-borne diseases control. In the mosquito model, based on the intrinsic features of *Asaia* combined with the immune activation by WSP, the chimeric bacterium represents a good candidate for paratransgenesis-based control strategies not only against filariases. Even if concerns about environmental compatibility and biosecurity impede the real application of paratransgenesis now, this approach could surely represent a further arm for integrated approaches.

In the second article we investigated the capability of the chimeric bacterium *Asaia*^{WSP} to polarize the immune response towards the M1/Th1 phenotype, which is protective for the host, and to determine an anti-*Leishmania* effect. In fact, the Th1/Th2 dichotomy is fundamental for the resolution of the disease: Th1 differentiation is linked to host protection, while Th2 with parasite survival (Awasthi et al., 2004).

The stimulation of the host immune response to fight a disease is on the basis of the immunotherapy, which actually is used for the treatment of cancer and allergies (Naran et al., 2018). The boosting of the immune response could be obtained with cytokines (IFNγ, IL-12) or by exploiting the intrinsic features of bacteria. For example, Bacillus Calmette-Guérin (BCG), the bacterium responsible of tuberculosis, is used in the weakened form to fight different types of cancer (www.cancer.gov/about-cancer/treatment/types/immunotherapy) and in the form of adjuvant, in combination with a lysate of *Leishmania,* it is used for the immunotherapy applied for the control of leishmaniasis. The immunotherapy for the treatment of visceral leishmaniasis has led to good results, especially for patients who do not respond to conventional chemotherapy (El-On et al., 2009).

In this second study, our first aim was to evaluate the production of IL-6, IL-12p40, TNF, IL-1β cytokines, nitrites, ROS, the expression of iNOS and the phagocytosis as markers of M1/Th1 polarization by macrophages activated with *Asaia*^{WSP} bacterium. The engineered bacterium *Asaia*^{WSP} induced the production of all these pro-inflammatory cytokines, whose roles during *Leishmania* infection are well known (Dayakar et al., 2019). In addition, high levels of ROS and nitrites were detected after the macrophage infection. ROS and nitrites are important molecules produced by the macrophages, following *Leishmania* infection, as mechanism response and their elevated production is a further evidence of the polarization of the macrophage *in sensu* M1/Th1 (Carneiro et al., 2016). Moreover, the expression of iNOS was evaluated in macrophages infected with both parasites and bacteria. Th1 cytokines and chemokines can stimulate iNOS activity, while typical cytokines of a Th2 response inhibit its expression and, on the contrary, activate the arginase metabolism (Ibiza et al. 2008). The enzymes iNOS and arginase compete for the same substrate: L-arginine. Based on the extracellular stimuli, if the expression of iNOS prevails, the production of NO is activated promoting the parasite killing; on the other hand, if the pathway of arginase is activated, the substrates necessary for the parasite growth are produced (Wanasen & Soong, 2008). The production of Th1 cytokines as a result of the infection of macrophages with the bacterium has created the conditions for the expression of iNOS with a consequent production of nitrites and a downregulation of the arginase enzyme.

In addition, the phagocytosis was also stimulated by the *Asaia*^{WSP} bacterium, with a higher number of bacteria phagocyted after 2h of infection compared with macrophages treated with the bacterium control. This tendency was confirmed testing the phagocytosis of

Staphylococcus epidermidis by macrophages pre-treated with *Asaia*^{WSP} and *Asaia*^{pHM4}, highlighting, also in this case, an effect of the engineered bacterium expressing WSP to stimulate the phagocytic activity.

The type of immune response and the fate of the infection is also determined by the co-stimulatory molecules which represent the secondary signal; in fact, the presentation of the sole antigen to the T cell naïve is not enough for its activation. The different expression of the surface markers is modulated by *Leishmania* parasites and it is also known that different *Leishmania* species activate different co-stimulatory molecules (Costa et al., 2018). In studies with *L. major*, the CD40 expression was associated to an activation *in sensu* Th1, with IL-12 production by murine dendritic cells. A similar result was seen in *L. mexicana* infection (Tuladhar et al., 2011; Kamanada et al., 1996). If human macrophages infected by *L. chagasi* downregulated CD86, the same molecule was upregulated when the cells were infected with *L. major* (Costa et al., 2018). By our experiments, the macrophages infected with *L. infantum* and the two bacteria expressed at least one of the three molecules analyzed (CD80-CD86-CD40) compared to untreated cells and infected with *Leishmania* alone, suggesting an effect of the bacterium itself. In particular, there was a difference between the two bacteria analyzing the percentage of cells positive for CD40 marker.

As for MHC class II, the macrophages infected with the bacterium *Asaia*^{WSP} expressed a higher mean of fluorescence compared to the controls. Also in this case, *Leishmania* did not induce the expression of the MHC class II marker. The increased presence of cells preinfected with *Asaia*^{WSP} and positive for all these surface markers may be due to the greater phagocytosis activity of the *Asaia*^{WSP}-activated macrophages; compared to the bacterium control, the macrophages infected with *Asaia*^{WSP} are in greater number and consequently more activated.

Then, the anti-leishmanial effect of *Asaia*^{WSP} was determined analyzing three parameters: the infection rate (percentage of infected macrophages/100 macrophage), the number of amastigotes in each macrophage and the parasitic index (average number of infected macrophages multiplied by the average number of amastigotes per macrophage). Interesting, in presence of *Asaia*^{WSP} after 48h of co-infection there was a decreasing of all these parameters compared with the infection only with *Leishmania*: i) the infection rate showed a decrease of 32%; ii) the number of amastigotes per macrophage decreased from 0.90 to 0.40; iii) the parasitic index was reduced of 73%. All these results highlighted the potentiality of the engineered bacterium *Asaia*^{WSP} to activate the macrophages determining an anti-*Leishmania* effect.

The capability of the bacterium *Asaia*^{WSP} to stimulate an efficient M1/Th1 response make it a successful immunomodulator, which could be used in combination with the classical chemotherapeutic agents in the treatment of the leishmaniases. Moreover, the bacterium *Asaia*^{WSP} could be used in the future as a scaffold for a further engineering with antigens of *Leishmania* obtaining the so-called live bacterial vaccines for a therapeutic or prophylactic approach.

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Sitography

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4. OTHER PUBLISHED PAPERS OF MICROBIOLOGICAL AND PARASITOLOGICAL INTEREST

PLoS ONE Volume 12, Issue 8, August 2017, Article number e0182533

A survey of the mycobiota associated with larvae of the black soldier fly (*Hermetia illucens*) reared for feed production

Boccazzi, I.V., Ottoboni, M., Martin, E., Comandatore, F., Vallone, L., Spranghers, T., Eeckhout, M., Mereghetti, V., Pinotti, L., Epis, S.

Abstract

Feed security, feed quality and issues surrounding the safety of raw materials are always of interest to all livestock farmers, feed manufacturers and competent authorities. These concerns are even more important when alternative feed ingredients, new product developments and innovative feeding trends, like insect-meals, are considered. The black soldier fly (*Hermetia illucens*) is considered a good candidate to be used as feed ingredient for aquaculture and other farm animals, mainly as an alternative protein source. Data on transfer of contaminants from different substrates to the insects, as well as the possible occurrence of toxin-producing fungi in the gut of non-processed insects are very limited. Accordingly, we investigated the impact of the substrate/diet on the intestinal mycobiota of *H. illucens* larvae using culture-dependent approaches (microbiological analyses, molecular identification through the typing of isolates and the sequencing of the 26S rRNA D1/D2 domain) and amplicon-based next-generation sequencing (454 pyrosequencing). We fed five groups of *H. illucens* larvae at the third growing stage on two substrates: chicken feed and/or vegetable waste, provided at different timings. The obtained results indicated that Pichia was the most abundant genus associated with the larvae fed on vegetable waste, whereas *Trichosporon, Rhodotorula* and *Geotrichum* were the most abundant genera in the larvae fed on chicken feed only. Differences in the fungal communities were highlighted, suggesting that the type of substrate selects diverse yeast and mold genera, in particular vegetable waste is associated with a greater diversity of fungal species compared to chicken feed only. A further confirmation of the significant influence of diet on the mycobiota is the fact that no operational taxonomic unit common to all groups of larvae was detected. Finally, the killer phenotype of isolated yeasts was tested, showing the inhibitory activity of just one species against sensitive strains, out of the 11 tested species.

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Folia Parasitologica

Folia Parasitologica Volume 65, 2018, Article number 008

Molecular screening for bacterial pathogens in ticks (*Ixodes ricinus*) collected on migratory birds captured in northern Italy

Pajoro, M., Pistone, D., Boccazzi, I.V., Mereghetti, V., Bandi, C., Fabbi, M., Scattorin, F., Sassera, D., Montagna, M.

Abstract

Migratory birds have an important role in transporting ticks and associated tick-borne pathogens over long distances. In this study, 2,793 migratory birds were captured by nets in a ringing station, located in northern Italy, and checked for the presence of ticks. Two-hundred and fifty-one ticks were identified as nymphs and larvae of *Ixodes ricinus* (Linnaeus, 1758) and they were PCR-screened for the presence of bacteria belonging to *Borrelia burgdorferi sensu lato, Rickettsia spp., Francisella tularensis* and *Coxiella burnetii*. Four species of *Borrelia* (*B. garinii, B. afzelii, B. valaisiana* and *B. lusitaniae)* and three species of *Rickettsia* (*R. monacensis, R. helvetica* and *Candidatus Rickettsia mendelii*) were detected in 74 (30%) and 25 (10%) respectively out of 251 ticks examined. Co-infection with Borrelia spp. and Rickettsia spp. in the same tick sample was encountered in 7 (7%) out of the 99 infected ticks. We report for the first time the presence of *Candidatus Rickettsia mendelii* in *I. ricinus* collected on birds in Italy. This study, besides confirming the role of birds in dispersal of *I. ricinus*, highlights an important route by which tick-borne pathogens might spread across different countries and from natural environments towards urbanised areas.

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environmental microbiology
Environmental Microbiology (2018) **20**(3), 1064–1077 doi:10.1111/1462-2920.14044

Environmental Microbiology Volume 20, Issue 3, March 2018, Pages 1064-1077

The mycobiota of the sand fly *Phlebotomus perniciosus*: Involvement of yeast symbionts in uric acid metabolism

Martin, E., Varotto Boccazzi, I., De Marco, L., Bongiorno, G., Montagna, M., Sacchi, L., Mensah, P., Ricci, I., Gradoni, L., Bandi, C., Epis, S.

Abstract

The knowledge of the fungal mycobiota of arthropods, including the vectors of human and animal diseases, is still limited. Here, the mycobiota associated with the sand fly *Phlebotomus perniciosus*, the main vector of leishmaniasis in the western Mediterranean area, by a culture-dependent approach (microbiological analyses and sequencing of the 26S rRNA gene), internal transcribed spacer (ITS) rRNA amplicon-based next-generation sequencing, fluorescence in situ hybridisation (FISH), and genome sequencing of the dominant yeast species was investigated. The dominant species was *Meyerozyma guilliermondii*, known for its biotechnological applications. The focus was on this yeast and its prevalence in adults, pupae and larvae of reared sand flies (overall prevalence: 57.5%) and of field-collected individuals (overall prevalence: 9%) was investigated. Using whole-mount FISH and microscopic examination, it was further showed that *M. guilliermondii* colonizes the midgut of females, males and larvae and the distal part of Malpighian tubules of female sand flies, suggesting a possible role in urate degradation. Finally, the sequencing and analysis of the genome of *M. guilliermondii* allowed predicting the complete uric acid degradation pathway, suggesting that the yeast could contribute to the removal of the excess of nitrogenous wastes after the blood meal of the insect host.

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Journal of the American Mosquito Control Association Volume 34, Issue 4, December 2018, Pages 311-314

Adenosine triphosphate-binding cassette transporters are not involved in the detoxification of azadirachta indica extracts in *Anopheles stephensi* larvae

Ferrari, M., Negri, A., Romeo, C., Boccazzi, I.V., Nodari, R., Habluetzel, A., Molteni, G., Corbett, Y.

Abstract

Detoxifying pathways of mosquitoes against the neem (*Azadirachta indica*) extracts are still unclear. The aim of the present study was to investigate the role of adenosine triphosphate-binding cassette (ABC) transporters in this process in *Anopheles stephensi*, one of the main malaria vectors in southern Asia. Third-stage larvae of *An. stephensi* were fed with fish food alone or in combination with neem extract at 0.5%, 1%, 5%, and 10%. Six ABC-transporter genes from 3 different subfamilies (B, C, and G) were analyzed to assess their relative expression compared with controls. A bioassay was also performed to assess larval mortality rate at different concentrations and in combination with verapamil, an ABC-transporter inhibitor. No significant variation in the expression levels of any transporter belonging to the B, C, and G subfamilies was detected. Furthermore, the use of verapamil did not induce an increase in mortality at any of the tested neem extract concentrations, indicating that ABC transporters are not involved in the detoxification of neem extracts in *An. stephensi* larvae.

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Ticks and Tick-borne Diseases 2019, Article number 101299

Rickettsia buchneri, symbiont of the deer tick *Ixodes scapularis*, can colonise the salivary glands of its host

Al-Khafaji, A.M., Armstrong, S.D., Varotto Boccazzi, I., Gaiarsa, S., Sinha, A., Li, Z., Sassera, D., Carlow, C.K.S., Epis, S., Makepeace, B.L.

Abstract

Vertically-transmitted bacterial symbionts are widespread in ticks and have manifold impacts on the epidemiology of tick-borne diseases. For instance, they may provide essential nutrients to ticks, affect vector competence, induce immune responses in vertebrate hosts, or even evolve to become vertebrate pathogens. The deer or blacklegged tick *Ixodes scapularis* harbours the symbiont *Rickettsia buchneri* in its ovarian tissues. Here we show by molecular, proteomic and imaging methods that *R. buchneri* is also capable of colonising the salivary glands of wild I. scapularis. This finding has important implications for the diagnosis of rickettsial infections and for pathogen-symbiont interactions in this notorious vector of Lyme borreliosis.

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Vector-Borne and Zoonotic Diseases Volume 19, Issue 5, May 2019, Pages 328-332

Denaturing gradient gel electrophoresis analysis of bacteria in Italian ticks and first detection of *Streptococcus equi* in *Rhipicephalus bursa* from the lazio region

Cappelli, A., Capone, A., Valzano, M., Bozic, J., Preziuso, S., Mensah, P., Varotto Boccazzi, I., Rinaldi, L., Favia, G., Ricci, I.

Abstract

Tick-borne diseases are an increasing problem for the community. Ticks harbor a complex microbial population acquired while feeding on a variety of animals. Profiling the bacterial population by 16S rDNA amplification and denaturing gradient gel electrophoresis enables detection of the broad spectrum of bacteria that settles in the ticks. This study identified known and unknown tick-infecting bacteria in samples from Italy. Seven adult ticks from different hosts and origins were analyzed: two *Rhipicephalus sanguineus* ticks from dogs (Lombardia), two *Rhipicephalus bursa* ticks from bovines (Lazio), and three *Ixodes ricinus* ticks from humans (Marche). The major result was the first report of the zoonotic agent *Streptococcus equi* in ticks. *S. equi* is a species complex of highly contagious pathogens. Subsequent to *S. equi* detection in a *R. bursa* tick removed from a bovine of Lazio in 2012, we studied 95 *R. bursa* samples collected from 3 bovines, 3 ponies, and 1 sheep grazing in the same area in 2012 and from 6 ponies grazing there in 2017. The results of a specific PCR assay indicated a not sporadic occurrence of *S. equi* in ticks. This finding provides a basis for assessing the potential of ticks to harbor and disperse *S. equi*.

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Ticks and Tick-borne Diseases Volume 10, Issue 5, August 2019, Pages 1070-1077

Tissue tropism and metabolic pathways of *Midichloria mitochondrii* suggest tissue-specific functions in the symbiosis with Ixodes ricinus

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Abstract

A wide range of arthropod species harbour bacterial endosymbionts in various tissues, many of them playing important roles in the fitness and biology of their hosts. In several cases, many different symbionts have been reported to coexist simultaneously within the same host and synergistic or antagonistic interactions can occur between them. While the associations with endosymbiotic bacteria have been widely studied in many insect species, in ticks such interactions are less investigated. The females and immatures of *Ixodes ricinus* (Ixodidae), the most common hard tick in Europe, harbour the intracellular endosymbiont "*Candidatus Midichloria mitochondrii*" with a prevalence up to 100%, suggesting a mutualistic relationship. Considering that the tissue distribution of a symbiont might be indicative of its functional role in the physiology of the host, we investigated *M. mitochondrii* specific localization pattern and the corresponding abundance in selected organs of *I. ricinus* females. We paired these experiments with in silico analysis of the metabolic pathways of *M. mitochondrii*, inferred from the available genome sequence, and additionally compared the presence of these pathways in seven other symbionts commonly harboured by ticks to try to obtain a comparative understanding of their biological effects on the tick hosts. *M. mitochondrii* was found to be abundant in ovaries and tracheae of unfed *I. ricinus*, and in ovaries, Malpighian tubules and salivary glands of semi-engorged females. These results, together with the in silico metabolic reconstruction allow to hypothesize that the bacterium could play multiple tissue-specific roles in the host, both enhancing the host fitness (supplying essential nutrients, enhancing the reproductive fitness, helping in the anti-oxidative defence, in the energy production and in the maintenance of homeostasis and water balance) and/or for ensuring its presence in the host population (nutrients acquisition, vertical and horizontal transmission). The ability of *M. mitochondrii* to colonize different tissues allows to speculate that distinctive sub-populations may display different specializations in accordance with tissue tropism. Our hypotheses should be corroborated with future nutritional and physiological experiments for a better understanding of the mechanisms underlying this symbiotic interaction.

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