Symbiosis between arthropods and fungi: the case of *Phlebotomus perniciosus*, the vector of visceral and canine leishmaniasis

Ph.D Thesis of

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ABSTRACT IN ENGLISH

Introduction

Vector borne diseases (VBDs) are one of the greatest public health problems worldwide and their control represents a key global public health challenge. In particular, leishmaniases are parasitic VBDs endemic in 98 countries from five continents distributed in tropical, subtropical and temperate zones. During the last twenty years, their distribution have undergone an expansion, also due to climate and environmental changes, the reason why they are considered re-emerging diseases. Italy is traditionally endemic for cutaneous and visceral leishmaniasis caused by the protozoan parasite *Leishmania infantum*, whose reservoir is the dog. In the Old World, the natural vectors of the protozoa *Leishmania* spp. are several species of sand flies belonging to the genus *Phlebotomus*. To control VBDs, the World Health Organization promotes the rational use of insecticides and integrated strategies for vector control through chemical and biological strategies. The "symbiotic control" is one of the biological control strategies that exploits microorganisms that live in symbiosis with the vector, in order to reduce or interfere with the pathogen transmission.

The aim of my thesis project was to increase the knowledge on killer yeasts, in general, and in the arthropod *Phlebotomus*, in particular, in order to open the way toward their development as tools for the biological/integrated control of vector-borne diseases.

Article 1

My first work, here presented, reports the investigation of the fungal community of the sand fly *Phlebotomus perniciosus*, the main vector of leishmaniasis in the western Mediterranean area and the most widespread sand fly in Italy, with a focus on the dominant yeast species *Meyerozyma guilliermondii*. The yeast displayed higher prevalence in the reared population than in the analyzed wild individuals, letting us hypothesize a contribution of this yeast to the diet of reared sand flies with nutrients that are on the other side easily available in nature. The localization of the yeast, found in the midgut of adults of both sexes and larvae and in Malpighian tubules of female sand flies, in addition to the genome analysis, let us speculate that the yeast may confer the additional advantage of contributing to the removal of the excess of nitrogenous wastes after the blood meal of the insect host.

Article 2

Whole-genome sequencing, *de novo* assemby and phylogenomic analysis of one strain of *M. guilliermondii* isolated from *P. perniciosus* and of other yeast strains of the *M. guilliermondii* complex isolated from arthropods were the goals of another research paper here presented. This work led to publish the first four genomes of isolates of *Meyerozyma caribbica* and to better understand evolutionary relationships within the clade.

Articles 3-5

The other works presented in this document were focused on the yeast *Wickerhamomyces anomalus*, here studied as a yeast candidate for symbiotic VBDs control.

In one of these papers (*Article 3*) the isolation and phylogenetic characterization of a killer strain of *W. anomalus* from the sand fly *P. perniciosus* is reported. The association between the yeast and sand flies seems to be facultative.

The killer toxin produced by the isolated *W. anomalus* strain was purified, characterized and tested against fungal pathogens in another work (*Article 4*). This toxin is a β-1,3-glucanase that possesses *in vitro* activity against *Candida glabrata* isolates, independently from their drug-resistance phenotypes. The obtained results suggest a potential application of this toxin against fungal infections at skin and mucosal membrane levels provoked by *C. glabrata* and may be useful for the design of new therapeutic molecules and the study of yeast resistance mechanisms.

Finally, *W. anomalus* is widely distuributed in nature and is considered by EFSA as a non-pathogenic agent, completely safe for healthy individuals (level of bio-security 1/QPS). Nevertheless, in the last paper I investigated the circulation of *W. anomalus* by qPCR screening examining a large panel of immunocompromised and / or intensive care patients, as well as healthy donors as controls (*Article 5*).
The screening revealed only one qPCR-positive subject, out of over 500 analyzed samples. Considering the few published cases of clinical infection by this yeast, in immunocompromised patients, we can conclude that this microorganism is a rather uncommon opportunistic pathogen.

In conclusion, the study of the mycobiota associated with sand flies could contribute to the understanding of the biology these insects, and offers the potential of discovering novel microrganisms for future application in the symbiotic control of leishmaniasis.

RIASSUNTO IN ITALIANO

Introduzione

Le malattie a trasmissione vettoriale (VBDs) rappresentano uno dei più grandi problemi mondiali di salute pubblica e il loro controllo è una sfida cruciale a livello globale. Le leishmaniosi, in particolare, sono malattie parassitarie a trasmissione vettoriale endemiche in 98 nazioni, in cinque continenti, distribuite nella fascia tropicale, subtropicale e temperata. La loro distribuzione ha subito un'espansione nell'ultimo ventennio, anche a causa di cambiamenti climatici ed ambientali e, per tale motivo, sono considerate patologie ri-emergenti. L'Italia è tradizionalmente endemica per le forme cutanee e viscerali di leishmaniosi, il cui serbatoio è il cane. Nel Vecchio Mondo i vettori naturali del protozoo parassita *Leishmania*, agente eziologico delle leishmaniosi, sono le femmine di flebotomo di diverse specie, appartenenti al genere *Phlebotomus*. L'Organizzazione Mondiale della Sanità promuove per la prevenzione e controllo delle VBDs un impiego moderato degli insetticidi e una gestione integrata dei vettori, che combini l'utilizzo di sostanze chimiche e il controllo biologico. Il "controllo simbiotico" è una strategia di controllo biologico che sfrutta microorganismi che vivono in simbiosi con il parassita vettore per ridurre la sua capacità vettoriale o interferire con la trasmissione del patogeno.

Lo scopo della mia tesi è stato quello di aumentare le conoscenze sui lieviti killer in generale e, in particolare, su quelli associati al flebotomo, in modo da porre le basi per lo sviluppo di strategie per il controllo di VBDs incentrate su di essi.

Articolo 1

Il mio articolo qui presentato per primo riporta lo studio della comunità fungina associata al flebotomo *Phlebotomus perniciosus*, il principale vettore di leishmaniosi nel Mediterraneo occidentale e la specie di flebotomo più diffusa in Italia, con un focus sulla specie di lievito dominante *Meyerozyma guilliermondii*. La prevalenza di questo lievito è risultata maggiore negli individui di allevamento analizzati rispetto a quelli raccolti in campo, facendoci ipotizzare un ruolo nutritivo del lievito per i flebotomi di allevamento, ai quali potrebbe fornire nutrienti che sono facilmente reperibili in natura. La sua localizzazione nell'intestino medio di maschi, femmine e larve e nei tubuli di Malpighi degli esemplari femmina del flebotomo ci fa supporre un addizionale ruolo di questo lievito nel contribuire alla rimozione degli scarti azotati dopo il pasto di sangue della femmina.

Articolo 2

Gli scopi del secondo articolo qui presentato sono stati il sequenziamento *whole-genome*, il *de novo assemby* e l'analisi filogenomica di un ceppo di *M. guilliermondii* isolato dal flebotomo *P. perniciosus* e di altri ceppi di lievito del complesso *M. guilliermondii* isolati da artropodi. Questo lavoro ha portato alla pubblicazione dei primi quattro genomi di isolati di *Meyerozyma caribbica* e a capire meglio le relazioni evolutive nel gruppo filogenetico.

Articoli 3-5

Gli altri articoli presentati nella tesi sono focalizzati sul lievito *Wickerhamomyces anomalus*, che ho studiato come candidato per il controllo simbiotico delle malattie trasmesse da vettori. In uno di questi articoli (*Articolo 3*) è riportato l'isolamento e la caratterizzazione di un ceppo a fenotipo killer del lievito *W. anomalus* da flebotomo *P. perniciosus*. L'associazione tra il lievito e il flebotomo pare essere facoltativa.

La tossina killer prodotta dal ceppo di *W. anomalus* isolato è stata purificata, caratterizzata e testata contro patogeni fungini nel lavoro presentato nell'articolo 4. Questa tossina è una β-1,3-glucanasi che possiede attività *in vitro* nei confronti di isolati di *Candida glabrata*, indipendentemente dal loro fenotipo di resistenza agli antimicotici. I risultati ottenuti suggeriscono la potenziale applicazione della tossina per il trattamento di infezioni fungine di cute e mucosa causate da *C. glabrata* e possono essere utili per lo studio dei meccanismi di resistenza fungina e per lo sviluppo di nuove molecole terapeutiche.

Il lievito *W. anomalus* è distribuito ampiamente in natura ed è considerato dall'EFSA un agente non patogeno e sicuro per individui sani (livello di biosicurezza 1/QPS). Ciononostante, nell'ultimo articolo che presento (*Articolo 5*) ho analizzato la circolazione di *W. anomalus* tramite qPCR esaminando campioni di sangue prelevati da individui immunocompromessi, pazienti in terapia intensiva e donatori sani. Lo screening ha rilevato solo un soggetto positivo sui più di 500 campioni analizzati. Considerando che in letteratura sono riportati pochi casi di infezioni causate da *W. anomalus*, tutti in soggetti immunocompromessi, possiamo dedurre che questo lievito sia un agente patogeno opportunista raro.

In conclusione lo studio del micobiota associato ai flebotomi può contribuire alla conoscenza della biologia di questi insetti e offrire la possibilità di scoprire microrganismi utilizzabili per future applicazioni nel controllo simbiotico della leishmaniosi.

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1. INTRODUCTION

1.1. Vector borne diseases.

Vector borne diseases (VBDs) are infectious pathologies that are characterized by a vector mediated transmission of the pathogen. The great majority of vectors of VBDs are bloodsucking arthropods, such as mosquitoes, sand flies and ticks, that acquire the pathogenic microorganism during a blood meal on an infected vertebrate host and transmit it to another host during a subsequent blood meal (Romi, 2010). Nowadays VBDs still remain a severe global health problem: they account for more than 17% of all infectious diseases and cause more than 700.000 deaths annually (WHO, 2017b). In the last decades their diffusion has shown an expansion, due to demographic, environmental and social factors (Gould and Higgs, 2009; Vora, 2008). Climate changes, such as variation of rainfall and temperatures, can lead to an expansion or a shift of the geographic distribution of vector populations and make the transmission season longer (Alekseev, 2006). Urbanization, combined with new urban slums creation, can reduce the application of good hygienic and sanitary practices, such as the avoidance of stagnant water and the use of sanitation, so promoting vector reproduction; furthermore the increment of population density can lead to epidemics. Finally, travels and global trades have led to mobility of vector populations (Vora, 2008). In this scenario, VBDs are spreading in countries that were unaffected or in which the parasitic infections were eradicated, so diseases that were common in tropical and sub-tropical regions, are now emerging or re-emerging in temperate countries (Takken and Knols, 2007; Hotez, 2018).

1.2. The study model *Phlebotomus perniciosus*.

Sand flies are holometabulum insects with a complete metamorphosis (Fig. 1). Their pre-imago phase presents an embryonic stage as an egg (~10 gg), four larval stages (~25-35 gg) and one pupal stage (~10 gg) (Maroli, 1983). The average duration of the development cycle, from the blood meal of the female to the emergence of the first adult, depends on the species and on climate factors, in *P. perniciosus* is averagely of 42 days (Maroli, 1986). In temperate Countries, sand flies complete at least two development cycles each reproductive season (June-September) (Busani et al., 2012). Eggs are deposited in ecological niches recognized by the females in order to guarantee optimal conditions for the development of pre-imago stages. The factors that drive the females in the choice of the place of ovodeposition are both the chemical and physical constituents of the substrate both the pheromones on eggs of the same species already laid in the place (Romi et al., 2012).

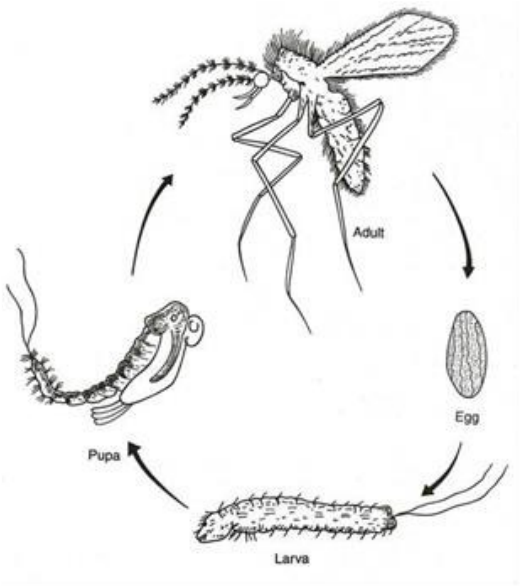

Figure 1. Life cycle of a sand fly

The development of their terrestrial larvae requires a constant temperature (24-30°C), an almost complete darkness, a high humidity (~90%) and a nutritive medium composed of dried leaves, the remains of other insects and rodent feces (Busani et al., 2012). Larval stages can be distinguished thanks to their different size and the presence of two caudal setae in the first stage and four in the later stages. The pupa remains attached to the substrate by the exuvium of the fourth instar larva. Before hatching the pupae performs energetic movements until its back breaks and the adult emerges (Romi et al., 2012). Adult sand flies of both sexes are melliphagous, but the female needs blood meals to acquire proteins to complete the oogenesis. The mating takes place preferably in the presence of a host on which the female can take the blood meal and she lays from 50 to 100 eggs each time (Busani et al., 2012).

According to the classification proposed by Lewis (1982), sand flies are grouped into five genera: *Phlebotomus*, *Sergentomyia*, *Warileya*, *Lutzomyia* and *Brumptomyia*. The last three genera include sand flies of the New World (South and Central America), while the species of the Old World belong to the genera *Phlebotomus* and *Sergentomyia*. Females of the genera *Phlebotomus* and *Lutzomyia* are medically important as they are the only proven vectors of some species of *Leishmania* pathogenic for humans (Killick-Kendrick, 1999). Sand flies of the genus *Phlebotomus* are insects belonging to the Phylum Artropoda, Order Diptera, Family Psycodidae, Subfamily Phlebotominae. The Phlebotominae subfamily contains more than 700 species including 80 proven vectors of the pathogen *Leishmania* spp. In Italy, the protozoa *Leishmania infantum*, the prevailing species of *Leishmania* in the Country, is transmitted by the sand flies *Phlebotomus perniciosus* and *Phlebotomus perfiliewi*; between the two, *P. perniciosus* is the most important from the epidemiological point of view being the vector of human and canine visceral leishmaniasis and the most widespread sand fly species in Italy (Rossi et al., 2008). In fact, it is very sensitive to *L. infantum* infections (Bettini et al., 1986; Maroli et al., 1980) and in Italy its distribution coincides with the disease's one (Busani et al., 2012). *P. perniciosus* is present in 18 of the 20 Italian regions, with high density in the

Tyrrhenian and Ionian coastal area, in Sicily and Sardinia (Romi et al., 2012). In Italy the altitude limit of this species is 1.070 m (Maroli et al., 1991).

1.3. Leishmaniases.

Leishmaniases are a group of re-emerging parasitic VBDs that are endemic in 98 countries from five continents in tropical, sub-tropical and temperate zones (Alvar et al., 2012) (Fig. 2). The etiological agents of leishmaniases are protozoan parasites of the genus *Leishmania* that are transmitted by the bite of infected female sand flies of different species belonging to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Leishmaniases are among the most dangerous (but neglected) tropical diseases: they are, after malaria, one of the main parasitic cause of death (Ghorbani and Farhoudi, 2017). World Health Organization (WHO) estimates that there are over one billion people living in endemic areas, 1,2 million of new cases and 20.000-30.000 deaths that occur annually due to leishmaniases (Lucius et al., 2017). In 2015, almost 200.000 new cutaneous leishmaniasis cases and 25.000 new visceral leishmaniasis cases were reported to WHO; however, we have to take into account that only 64% of the countries endemic for cutaneous leishmaniasis and 72% of the countries endemic for visceral leishmaniasis reported their incidence data to WHO in 2015 (WHO, 2017).

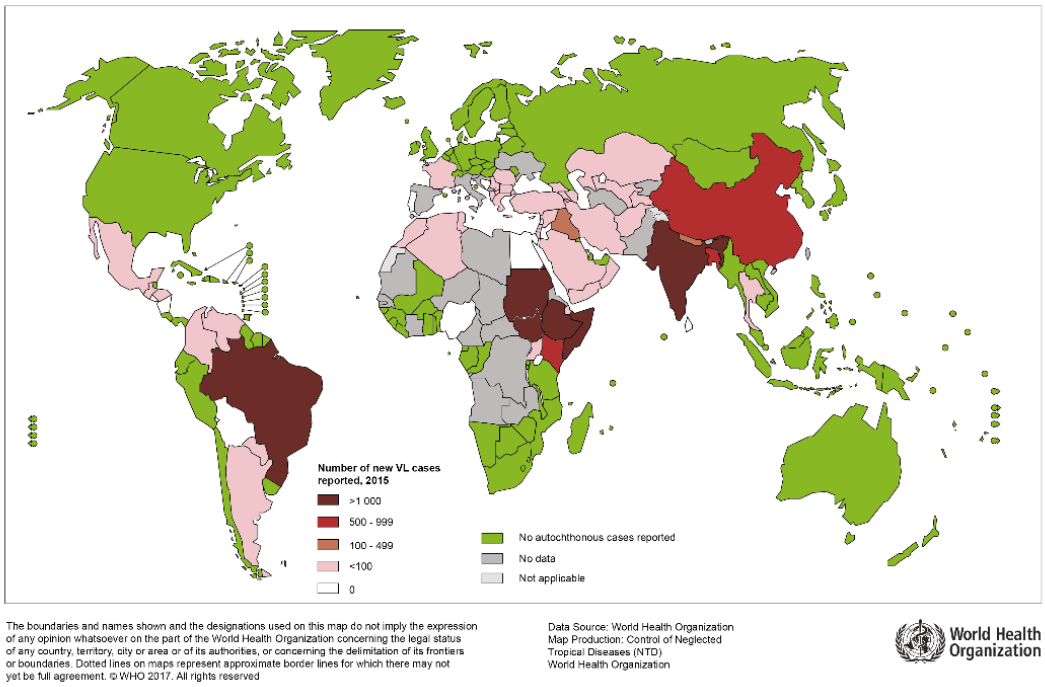

Figure 2. Status of endemicity of visceral leishmaniasis worldwide, 2015 (WHO, 2017)

Based on the natural mammalian reservoir of the parasite, these diseases can be classified in zoonotic and anthroponotic. Zoonotic leishmaniases have the principal vertebrate host in animals like dogs, foxes, lagomorphs and rodents, but can also affect humans, especially children and immunocompromised individuals; meanwhile, anthroponotic leishmaniases affect primarily humans and can cause overwhelming epidemics. There are three main forms of the disease: visceral, cutaneous and mucocutaneous (Herwaldt, 1999).

Anthroponotic visceral leishmaniasis (AVL, also known as kala-azar) is the more severe form causing lethality in 95% of the untreated patients. AVL is caused by the protozoa *Leishmania donovani* and it is endemic in Indian subcontinent and in East Africa. The symptoms include bouts of fever, anemia, swelling of spleen and liver and weight loss. In 2015, more than 90% of new cases reported by WHO occurred in 7 countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan (WHO, 2017).

Zoonotic visceral leishmaniasis (ZVL), whose only confirmed reservoir is the dog (Quinnell et al., 2009), is a disease of medical and veterinary relevance in South America, Asia and the Mediterranean area (Alvar et al., 2012). The etiological agent of ZVL is the pathogen *L. infantum*. Dogs are the main source of infection, but the disease occurs also in humans, especially in children and immunocompromised adults (Gradoni et al., 2008), and there were reported cases involving foxes, domestic cats, opossum and rodents.

Anthroponotic cutaneous leishmaniasis (ACL) is caused by *Leishmania tropica* and is diffused in Americas, the Mediterranean area and Asia. Its epidemiology is characterized by major epidemics related to high-density population settings, war, famine and income of refugee, as in the recent outbreaks in Afghanistan and the Syrian Arab Republic. ACL develops as a papular rash, beginning with one papule in the site of the inoculation of parasites, then more papules (sometimes up to 200) can be formed during parasite dissemination and the papules became lesions with scabs and ulcers. When the ulcers heal, they leave permanent scars in exposed parts of the body, typically on face, arms and legs, that can be cause of discrimination and prejudice (Bennis et al., 2017).

Zoonotic cutaneous leishmaniasis (ZCL) is caused by *L. infantum, Leishmania major* and *Leishmania aetiopica* and is diffused in the Mediterranean basin and in the East Mediterraneum. The reservoir of this disease are dogs for *L. infantum* (Gradoni et al., 2015), rodents for *L. major* (Mascari et al., 2013), and hyraxes and rodents for *L. aetiopica* (Mutinga et al., 1989). It typically affects people living in rural areas and its clinical aspects can differentiate it from

ACL. In fact dry lesions characterize urban ACL; wet lesions, often superinfected and disseminating, characterize rural ZCL (Faulde et al., 2006).

If ZCL and ACL are considered together, CL is the most common form of leishmaniasis (Aronson et al., 2016). Over two thirds of the new CL cases reported by WHO in 2015 occurred in 6 countries: Afghanistan, Algeria, Brazil, Colombia, Islamic Republic of Iran and the Syrian Arab Republic (WHO, 2017).

Mucocutaneous leishmaniasis is diffused in the New World and in Africa and is caused by several species of *Leishmania*. It is a zoonotic form of leishmaniasis, with wild animals as reservoir. It is considered a sequel of CL, in which the parasites disseminate from skin lesions to naso-oropharyngeal mucosa and lead to lesions with partial or total destruction of nose, mouth and throat (Amato et al., 2008).

In addition to these main forms, there is a sequel of visceral leishmaniasis diffused in East Africa and in the Indian subcontinent known as Post-kala-azar dermal leishmaniasis (PKDL). PKDL is characterized by macular, papular or nodular rashes that appear from six months to one or more years after the treatment for VL and can became permanent. Humans with PKDL can serve as reservoir host of VL (Bern et al., 2008).

1.4. Leishmaniasis in Italy.

Italy is traditionally endemic for cutaneous and visceral leishmaniasis, whose reservoir is the dog; all of them are caused by the parasite *L. infantum* (Romi et al., 2012). Central-south regions, the isles and Tyrrhenian coastal regions of the North-Central Italy are endemic. In Italy leishmaniasis is a re-emerging disease, in fact, from 2000, around 200 new human cases are reported every year and the prevalence in dogs in rural areas of central-south regions is estimated between 15% and 20% (Busani et al., 2012).

1.5. The pathogen *Leishmania*.

More than 20 species of *Leishmania* infect humans and are responsible for one or more forms of the disease (WHO, 2017). *Leishmania* is a parasite belonging to the family Trypanosomatidae, that presents a polymorphism linked to life cycle stages. *Leishmania* is a dixenic pathogen with a vertebrate host and an invertebrate host that is the vector. It is dimorphic: in the mammalian host *Leishmania* is found as non-motile intracellular amastigote,

meanwhile, in the sand fly vector it is found as extracellular motile flagellated promastigote (Fig. 3).

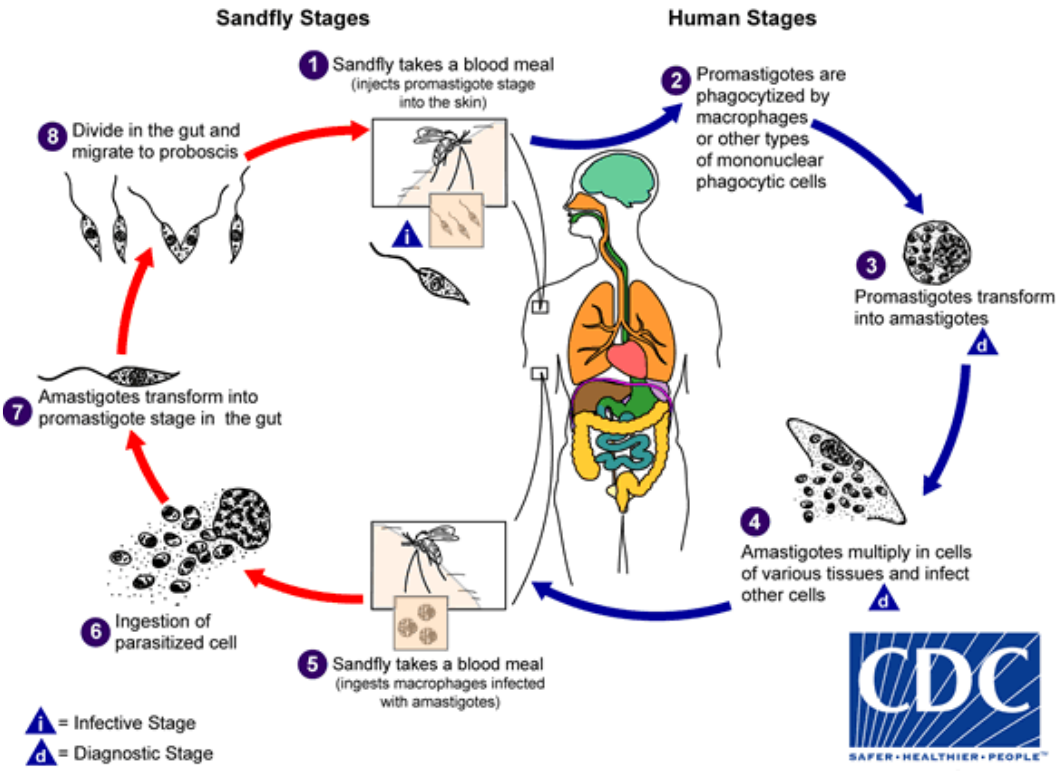

Figure 3. Life cycle of *Leishmania* spp. (www.cdc.gov)

Based on their localization in the gut of sand fly, the species of the genus *Leishmania* are divided in the subgenera *Leishmania* and *Viannia*. Species of the subgenus *Leishmania* (like *L. infantum*) are suprapylarian parasites and develop in the midgut and foregut of their vectors, whereas members of the subgenus *Viannia* are peripylarian parasites and have a phase of development in the hindgut before migrating in the midgut (Gossage et al., 2003). Infection of sand flies starts when the arthropod, taking the blood meal on an infected mammal, ingest macrophages containing amastigotes. In suprapylarian species the blood meal in the posterior abdominal midgut is surrounded by the peritrophic matrix, a chitinous matrix produced by the epithelium of the gut of the sand fly during the blood ingestion, amastigotes are released during the blood meal digestion (Kamhawi, 2006) and parasites then undergo a morphological modification process, called metacyclogenesis (Kbaier-Hachemi et al., 2012), while they migrate anteriorly from abdominal midgut to the stomodeal valve (Fig. 4).

After the blood meal, amastigotes differentiate in procyclic promastigotes, that start the first multiplication cycle in the sand fly and develop into nectomonads (Gossage et al., 2003). Nectomonads are a non-dividing motile stage that migrate forward towards the anterior thoracic midgut. Here parasites differentiate into leptomonads, that undergo the second multiplication cycle (Bates and Rogers, 2004). Anteriorly, haptomonads and metacyclic promastigotes are observed in the first part of the sand fly digestive apparatus, in correspondence of the stomodeal valve. Haptomonads form a parasite plug at the stomodeal valve and produce a chitinase that is responsible of valve degeneration, impairing the feeding dynamics of the sand fly (Schlein et al., 1992). Metacyclic promastigotes, derived from leptomonads, are the highly motile and highly specialized form that infects the mammalian host. The timing and peak of presence in the sand fly gut of each developmental form depends on the parasite species (Gossage et al., 2003), but the entire cycle of development in the arthropod host last 6–9 days; in general, sand flies can transmit *Leishmania* to a mammalian host after 6-9 days of the bloodmeal on the infected mammal (Kamhawi, 2006). In the sand fly *P. perniciosus* the cycle last 6 days (Gradoni and Gramiccia, 2004).

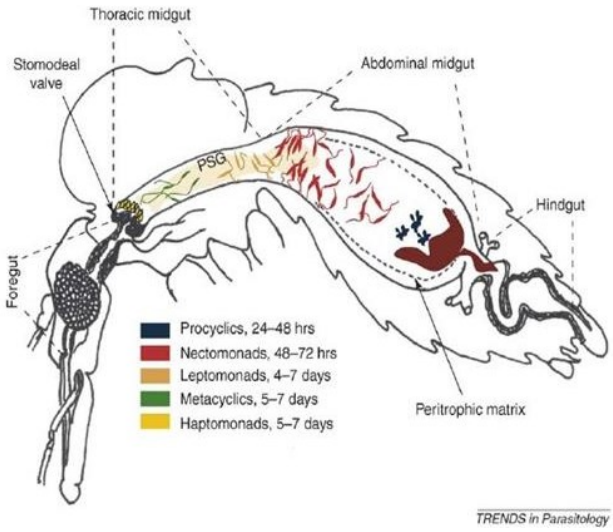

Figure 4. *Leishmania* spp. development in the body of sand fly (Kamhawi, 2006)

During the subsequent bloodmeal, the infected sand fly regurgitates metacyclic promastigotes in the mammalian host. Metacyclic promastigotes are taken up by phagocytic cells, including macrophages and neutrophils, and inside the macrophages develop into amastigotes, the intracellular, non-motile form of the parasite. Amastigotes multiply by binary division inside macrophages, the parasite-laden macrophages undergo apoptosis and released amastigotes infect other phagocytic cells (Liu and Uzonna, 2012).

The outcome of the mammalian infection depends on the species of the parasite, its tropism for the host, the immunity of the host itself and the interaction between them. In susceptible host the infection can became symptomatic due to parasite-laden macrophages (*Liu and Uzonna, 2012)*. In uncomplicated CL and MCL, the large number of parasite-laden macrophages in the site of the bite produces the dermal lesion and amastigotes continue to invade lymphocytes and plasma cells as the lesion evolves, provoking papules and nodules. After infection by *L. donovani* and *L. infantum,* the parasitized macrophages are disseminated in reticuloendothelial tissues and produce a reticuloendothelial hyperplasia that affects the spleen, the liver, the mucosa of the small intestine, the bone marrow, the lymph nodes and the other lymphoid tissues, so the mammalian host develops the symptoms of VL (WHO, 2010).

1.6. Prevention and control of leishmaniasis.

A complex system of factors such as the mammalian host, the sand fly vector, the parasite and the animal reservoir impacts on the distribution and prevalence of leishmaniasis. In such complicated situation, a combination of case management strategies, animal reservoir control and integrated vector control is needed to archive the control of leishmaniasis (WHO, 2010). No human vaccines against leishmaniasis has demonstrated to produce long-term immunization. As concern drugs for treatment of VL and CL in humans, the available ones showed toxicity and have high cost, so no one has been useful to eradicate this disease (Ghorbani and Farhoudi, 2017). Furthermore, different strains of *Leishmania* spp. are developing resistance to antileishmanial drugs (Jain and Jain, 2015).

In order to control the domestic animal reservoir, topical treatments of dogs with synthetic pyrethroids have been applied obtaining a significant reduction of incidence of zoonotic leishmaniasis thanks to their anti-feeding (individual protection) and lethal-by-contact activity (mass protection) (Maroli et al., 2001; Mazloumi-Gavgani et al., 2002; Otranto et al., 2013). However, they can't prevent all sand fly bites, therefor canine vaccines are needed. An effective canine vaccine should induce long-lasting Th1-dominated immunity in order to prevent the establishment of the infection and control its progression (Gradoni, 2015). There are three canine vaccines commercially available: Leish-tec® and Leishmune®, registered in Brasil, and CaniLeish®, registered in Europe. The vaccine Leish-tec® is a recombinant A2 antigen plus saponin that resulted in only 40% protection from infection in a small study trial (Fernandes et al., 2008); meanwhile, both Leishmune®, constituted by a fucose-mannose ligand (FML) antigen purified from *L. donovani* with QuilA saponin as adjuvant (Borja-Cabrera et al., 2002), and CaniLeish®, constituted by purified excreted-secreted proteins of *L. infantum* (LiESP) with

QA-21 saponin as adjuvants (Bongiorno et al., 2013; Moreno et al., 2014; Martin et al., 2014), are highly immunogenic for dogs and can induce a long-lasting protective effect against the disease. In regions where reservoir hosts are wild animals, their control includes burrow destruction and animal poising (WHO, 2010).

Finally, leishmaniasis control involves the control of sand fly vector of *Leishmania* with the aim of interrupt or reduce the transmission of the pathogen. The available methods for controlling sand fly vectors include personal protection from arthropods (for example with insecticide impregnated bed-nets), the use of insecticides and environmental management (such as forest cleaning and sanitation programs) (WHO, 2010).

1.7. Symbiotic control.

For the prevention and control of VBDs, WHO promotes the judicious use of insecticides and an integrated vector management (IVM) that combines chemical- and biological-control. An increasing number of Member States are now implementing vector control through the IVM approach (WHO, 2015). The framework of IVM proposed by WHO is: i) Advocacy, social mobilization and legislation; ii) Collaboration within the health sector and with other sectors; iii) Integrated approach; iiii) Evidence-based decision-making; iiiii) Capacity-building (WHO, 2004). The "integrated approach" is described as "*the integration of non-chemical and chemical vector control methods"*. Following WHO guidelines and due to the proven inefficacy of the use of a single method for VBDs control, it is a common thought that the control of arthropod vectors and of diseases they transmit, could be archived only using an integrated approach in which traditional methods, based on insecticides, are combined with biological control strategies (Beier et al., 2008). The "symbiotic control" is a biological control strategy that exploits microorganisms that live in symbiosis with vectors of parasites to reduce their vectorial capacity or interfere with the transmission of the pathogen. Three appealing approaches of symbiotic control are: i) the killing of symbionts essential to the host survival; ii) the manipulation of symbionts in order to make them produce anti-pathogens molecules in their host (paratransgenesis); iii) the introduction of microorganisms that influence the life-span of vectors and their vectorial capacity (Ricci et al., 2012). A well developed strategy of symbiotic control based on elimination of essential symbionts in order to impair the parasite host, is the antibiotic administration to treat filarial diseases. In fact, the antibiotics directed again the alphaproteobacterium *Wolbachia*, primary symbiont of nematods as *Dirofilaria immitis*, *Brugia malayi*, *Wuchereria bancrofti* and *Oncocherca volvulus* (agents of lymphatic filariasis and river blindness), kill the larvae of the nematodes and a proportion of adult worms (Bandi et al., 1998;

1999; Taylor et al., 2005; Bazzocchi et al., 2008; Wanji et al., 2009; Gyapong et al., 2005). Among innovative symbiotic control techniques, paratransgenesis is one of the most studied in the last years. This strategy is based on genetic manipulation of symbiotic or commensal microorganisms of arthropod vectors in order to make them produce substances that interfere with pathogen transmission (Hurwitz et al., 2011). Examples of this approach are the exploitation of genetically modified *Rodhococcus rhodnii* bacteria to control the transmission of *Trypanosoma cruzi* by triatomine, vectors of Chagas disease (Beard et al., 1998; Beard et al., 2002; Durvasula et al., 1997) and the modification of the fungus *Metarhizium anisopliae* to express molecules whose targets are *Plasmodium* sporozoites to control malaria-transmitting anophelines (Fang et al., 2011; Bukhari et al., 2011). Another symbiotic control strategy is the introduction of exogenous microorganisms in vector populations. In this approach, the microorganisms that are introduced are selected in order to reduce the life-span and fitness of vectors or interfere with the pathogen transmission. The best candidate for this technique is the bacterium *Wolbachia,* of which a life-shortening strain was studied to reduce the life-span of the mosquito *Aedes aegypti* (McMeniman et al., 2009). Furthermore, *Wolbachia* can inhibit the pathogen development in the vector, also activating its immunity (Kambris et al., 2009; Hughes et al., 2011).

The first essential step to develop symbiotic control strategies is the study of the microbial community associated to the arthropod and the identification of the symbionts. After this, it is important to study them to evaluate if the candidates have all the requisites to be further studied: stable association with the host, dominance in the microbial community, localization in the same organs of the pathogen, easiness of cultivation and *in vitro* manipulation and vertical/horizontal transmission routes (Alma et al., 2010).

1.8. Symbiosis between arthropods and yeasts.

While symbiosis between bacteria and insects has been the subject of numerous studies in the last two decades, yeasts associated with arthropods are scarcely investigated (Ricci et al., 2011). In the last years, fungal diversity associated with insect hosts, the role of mutualistic fungi and their transmission route are attracting increasing attention. Yeast mutualism seems to be predominant in insect-fungi associations, indeed, until 2005, 143 insect species in eight orders were reported infected by asymptomatic or beneficial yeasts (Vega and Dowd, 2005). Furthermore, most of the described fungi associated with insects are true yeasts in the Saccharomycotina (Suh et al., 2005). Based on current knowledge, yeasts associated with arthropods are primarily located among host cells (intercellularly), facultative and horizontally

transmitted (Gibson and Hanter, 2010). Exceptions to the main fungal transmission route are transovarially transmitted yeast-like symbionts (YLS, i.e. unicellular fungi not in the subphylum Saccharomycotina) of planthoppers, aphids and beetles, but, also in this instances, unlike vertically transmitted bacteria, there is no evidence that they have lost genes (Suh et al., 2001). There are only few Saccharomycotina exceptions to the extracellular localization of yeasts: *Candida* spp. symbionts of long-horned beetles (Nardon and Grenier, 1989) and *Coccidiascus legeri* of *Drosophila*, both of the yeast symbionts sequestered within host vacuoles (Lushbaugh et al., 1976; Nardon and Grenier, 1989). The role of yeast symbionts in insect biology spans from the nutritional role to the protection from biotic stresses (Gibson and Hanter, 2010; Janson et al., 2008). Their localization, mainly extracellularly in the gut of their insect host, may reflect their roles in symbiosis. Known nutritional roles of fungi symbionts include sterol and vitamine synthesis, lipid and sugar providing and N recycling (Gibson and Hanter, 2010; Blackwell, 2017). Fungi produce sugars and glycerol in order to maintain their hypertonic state (Heritage et al., 1996), thus, in cost-free mutualisms, they could provide sugars and fats to insects (Douglas, 2008). The association of insects with deficient diet with fungi can provide numerous benefits to them: in fact, fungi can synthesize vitamins, especially B ones (Gusteleva,1975), and sterols to enrich insects' diet and, although fungi can not fix nitrogen, they are able to aid in its uptake from the insect host's diet and provide amino acids for their hosts (Pant et al., 1960). YLS are also involved in the enzymatic detoxification of plant allelochemicals, mycotoxins, insecticides and herbicides (Shen and Dowd, 1991) and in the production of enzymes required to insects' digestion (Brues and Glaser, 1921), but there are not strong evidences that support this role for true yeasts yet. Exception is the study of Ba and Phillips (1996) in which Saccharomycotina yeast *Candida guilliermondii* and *Debaromyces hansenii* isolated from the gut of the red fire ant (*Solenopsis invicta*) were able to degrade the toxin salicin. Notably, microbial symbionts can protect their insect hosts against pathogens and harmful microorganisms, directly or by inducing immune and tissue response in the arthropod (Flórez et al., 2015). In free-life, yeasts can antagonize other microorganisms competing with them for nutrients, changing the environmental pH by organic acid production and releasing killer toxins with a wide spectrum (Muccilli and Restuccia, 2015). These competitive characteristics are considered promising for the use of yeasts as agents of bioprotection in agriculture, sanity and food industry. Numerous killer toxins, also known as mycocins or mycotoxins, have been described and isolated, especially in high density yeast populations and in stressful and high competitive environments (Muccilli and Restuccia, 2015). Furthermore, yeasts can produce killer toxins *in vivo* in the body of the arthropod as was demonstrated for

the yeast *Wickerhamomyces anomalus*, that can produce mycocins in the midgut and gonads of mosquitoes after its reintroduction within the mosquito by the diet (Cappelli et al., 2014).

1.9. The yeast *Wickerhamomyces anomalus*.

Wickerhamomyces anomalus is a yeast belonging to the phylum Ascomycota, subphylum Saccharomycotina, class Saccharomycetes, order Saccharomycetales, family Saccharomycetaceae. Its physiology, metabolism and ability to adapt make it a highly versatile and ubiquitous yeast. It has, indeed, been isolated from different habitats: plants, flowers, soil, fruits, dairy products, insect tissues, human feces, contaminated oil, tree resins, wastewater and marine environments (Kurtzman et al., 1998). It has also been isolated from fermented beverages, food and feed (Masoud et al., 2004; Sujaya et al., 2004). *W. anomalus* is highly tolerant of environmental stresses and has adapted to a wide range of growth conditions: temperature range of 3° C-37° C (its optimum is 26° C-30° C), pH 2-12, high osmotic pressure, anaerobic conditions and low water activity (Fredlund et al., 2002; Walker, 2011). At genetic level it has been poorly characterized; it is known that *W. anomalus* strains present a number of chromosomes varying between nine and 12, with a range of dimensions between 850 and 3500 Kb (Naumov et al., 2001). *W. anomalus* is renowned for its biotechnological potential (Walker, 2011). This yeast is already exploited for the production of therapeutic molecules, for biofuel production and as an agent of biocontrol and environmental bioremediation (Passoth et al., 2011). *W. anomalus* possesses an antimicrobial activity towards microorganisms of different phylogenetic groups such as viruses, bacteria, fungi and yeasts (Walker, 2011). Very important in the antimicrobial activity is the role played by its killer proteins, called WaKTs, and by toxic volatile compounds that this yeast can produce (Passoth et al., 2011; Grzegorczyk et al., 2017), but the activity is also carried out through the subtraction of oxygen to others microorganisms (Passoth et al., 2011). WaKTs secreted by the yeast bind to specific receptors on the microbial wall and act as β-1-3-glucanase. They have a fundamental role in the inhibition of susceptible strains of *Candida albicans* and *Saccharomyces cervisiae* that has been demonstrated *in vitro* and *in vivo* (Cappelli et al., 2014). Toxin production confers a significant advantage in the competition with other yeast strains for nutrients available in the environment (Starmer et al., 1987). WaKTs have also been used as a matrix to generate antibodies capable of killing bacteria, yeasts, filamentous fungi and even inactivated viruses (Magliani et al., 2002; Polonelli et al., 2011). Notably, the presence of this yeast in the gut and in reproductive organs of some mosquito vectors of diseases of medical interest, including *Anopheles stephensi*, vector of malaria, *Aedes aegypti* and *Aedes albopictus*, has been reported (Ricci et al., 2011). A study

conducted by Cappelli and collaborators (2014) demonstrated that the strain F17.12 of *W. anomalus*, isolated from the mosquito *An. stephensi*, is able to produce killer toxins both *in vitro* and *in vivo* and owns antimicrobial activity against certain strains of yeasts and bacteria. These discoveries have led to the conclusion that the association of this yeast with mosquito vectors could represent a specific symbiosis (Ricci et al., 2011; Cappelli et al., 2014). Moreover, a recent encouraging study highlights a strong anti-plasmodial effect of the WaKT produced by the strain F17.12 of *W. anomalus* (Valzano et al., 2016).

1.10. The yeast *Meyerozyma guilliermondii*.

Meyerozyma guilliermondii is a complex of yeast species belonging to the phylum Ascomycota, subphylum Saccharomycotina, class Saccharomycetes, order Saccharomycetales, family Debaryomycetaceae. *M. guilliermondii* species belongs to the Saccharomycotina CTG clade, characterized by the translation of CTG as serine instead of leucine. *M. guilliermondii* is widely distributed in the natural environment; it has been isolated from vegetables, soil, tree exudates, food and seawater (Papon et al., 2013). Moreover, it's a saprophyte of human skin and part of human mucosal microflora (Savini et al., 2011). *M. guilliermondii* could be considered a "generalist yeast" being associated with arthropods of different taxa (Gibson and Hanter, 2010). In fact, it has been recovered from long-horned beetles (Nardon and Grenier, 1989), scarab beetles (Vishniac and Johnson, 1990), metallic wood-boring beetles (Phaff and Starmer, 1987), fire ants (Ba et al., 1995), adrenid bees, a mushroom-feeding fly (Zacchi and Vaughn-Martini, 2002) and several genera of mosquitoes (Bozic et al., 2017). *M. guilliermondii* has been the object of several studies in the last 40 years due to its biotechnological interest, biocontrol potential and emerging pathogen status (Papon et al., 2013). It is employed in riboflavin production (Tanner Jr et al., 1945), bioconversion of xylose into xylitol (Zou et al., 2010) and is a feasible source of enzymes (Gong et al., 2007), biofuel (Wang et al. 2012) and aromas (Wah et al. 2013). This yeast has been used as a biocontrol agent in agriculture and food industry (Wisniewski et al., 1991; Hashem and Abo-Elyousr, 2011) thanks to his antimicrobial activity against bacteria (Zhao et al., 2010), fungi (Coda et al., 2013), and even nematodes (Hashem and Abo-Elyousr, 2011) and protozoa (Dantán-González et al., 2015). The antagonistic activity of *M. guilliermondii* is due to the combination of multiple actions such as the attachment to the hyphae of moulds (Arras et al., 1999), the competition for nutrients (Guetsky et al., 2002), the extracellular release of the cell wall-degrading enzyme β-1,3-glucanase (Zhang et al., 2011) and the induction of disease resistance in host plants (Zhao et al., 2008). *M. guilliermondii* is considered an uncommon emerging pathogen with the propensity to develop multidrug

resistance (Savini et al., 2011). Furthermore, this yeast is responsible for 1–11.7 % of all candidemia (Savini et al., 2011). Nevertheless, *M. guilliermondii* remains an "*experimentally tractable organism*" and is now extensively used as a model yeast (Papon et al., 2013).

1.11. References.

- Alekseev AN. (2006). The effects of global climatic changes on bloodsucking ectoparasites and pathogens they transmit. Vestn Ross Akad Med Nauk. (3), 21-25.
- Alma A, Daffonchio D, Gonella E, Raddadi N. (2010). Microbial symbionts of *Auchenorrhyncha* transmitting phytoplasmas: a resource for symbiotic control of phytoplasmoses. In: Phytoplasmas: Genomes, Plant Hosts and Vectors, Weintraub P. & Jones P. Ed., CAB International, Wallingford, UK, pp. 272-292.
- Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M; WHO Leishmaniasis Control Team. (2012). Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 7(5), e35671.
- Amato VS, Tuon FF, Bacha HA, Neto VA, Nicodemo AC. (2008). Mucosal leishmaniasis. Current scenario and prospects for treatment. Acta Trop. 105(1), 1-9.
- Aronson N, Herwaldt BL, Libman M, Pearson R, Lopez-Velez R, Weina P, Carvalho EM, Ephros M, Jeronimo S, Magill A. (2016). Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). Clin Infect Dis. 63(12), 1539-1557.
- Ba AS and Phillips Jr SA. (1996). Yeast biota of the red imported fire ant. Mycol Res. 100(6), 740-746.
- Ba AS, Guo DA, Norton RA, Phillips SA, Nes WD. (1995). Developmental differences in the sterol composition of *Solenopsis invicta*. Arch Insect Biochem. 29, 1-9.
- Bandi C, Anderson TJC, Genchi C, Blaxter ML. (1998). Phylogeny of *Wolbachia* in filarial nematodes. Proc R Soc Lond B Biol Sci. 265, 2407-2413.

- Bandi C, McCall JW, Genchi C, Corona S, Venco L, Sacchi L. (1999). Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. Int J Parasitol. 29, 357-364.
- Bates PA and Rogers ME. (2004). New insights into the developmental biology and transmission mechanisms of *Leishmania*. Curr Mol Med. 4, 601-609.
- Bazzocchi C, Mortarino M, Grandi G, Kramer LH, Genchi C, Bandi C, Genchi M, Sacchi L, McCall JW. (2008). Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity against *Dirofilaria immitis* in experimentally infected dogs. Int J Parasitol. 38, 1401-1410.
- Beard CB, Cordon-Rosales C, Durvasula RV. (2002). Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. Annu Rev Entomol. 47, 123-141.
- Beard CB, Durvasula RV, Richards FF. (1998). Bacterial symbiosis in arthropods and the control of disease transmission. Emerg Infect Dis. 4, 581-591.
- Beier JC, Keating J, Githure JI, Macdonald MB, Impoinvil DE, Novak RJ. (2008). Integrated vector management for malaria control. Malar J. 7 Suppl 1, S4.
- Bennis I, Belaid L, De Brouwere V, Filali H, Sahibi H, Boelaert M. (2017). "The mosquitoes that destroy your face". Social impact of Cutaneous Leishmaniasis in South-eastern Morocco, A qualitative study. PLoS One. 12(12), e0189906.
- Bern C, Maguire JH, Alvar J. (2008). Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Negl Trop Dis. 2 (10), e313.
- Bettini S, Gramiccia M, Gradoni L, Atzeni MC. (1986). Leishmaniasis in Sardinia: II. Natural infection of *Phlebotomus perniciosus* Newstead, 1911, by *Leishmania infantum* Nicolle, 1908, in the province of Cagliari. Trans R Soc Trop Med Hyg. 80, 458–459.
- Blackwell M. (2017). Made for Each Other: Ascomycete Yeasts and Insects. Microbiol Spectr. 5(3).
- Bongiorno G, Paparcone R, Foglia Manzillo V, Oliva G, Cuisinier AM, Gradoni L. (2013). Vaccination with LiESP/QA-21 (CaniLeish®) reduces the intensity of infection in *Phlebotomus perniciosus* fed on *Leishmania infantum* infected dogs — a preliminary xenodiagnosis study. Vet. Parasitol. 197 (3-4), 691-695.

- Borja-Cabrera GP, Correia Pontes NN, da Silva VO, de Souza EP, San-tos WR, Gomesa EM, Luzb KG, Palatnik M, Palatnik-de-Sousa CB. (2002). Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante RN). Vaccine. 20, 3277–3284.
- Bozic J, Capone A, Pediconi D, Mensah P, Cappelli A, Valzano M, Mancini MV, Scuppa P, Martin E, Epis S, Rossi P, Favia G, Ricci I. (2017). Mosquitoes can harbour yeasts of clinical significance and contribute to their environmental dissemination. Environ Microbiol Rep. 9(5), 642-648.
- Brues CT and Glaser RW. (1921). A symbiotic fungus occurring in the fat-body of *Pulvinaria innumerabilis* Rath. Biol Bull. 40, 299-U10.
- Bukhari T, Takken W, Koenraadt CJM. (2011). Development of *Metarhizium anisopliae* and *Beauveria bassiana* formulations for control of malaria mosquito larvae. Parasit Vectors. 4, 23.
- Busani L, Mughini Gras L, Romi R, Boccolini D, Severini F, Bongiorno G, Khoury C, Bianchi R, Gradoni L, Capelli G. (2012). Zanzare, flebotomi e zecche: atlante bibliografico delle specie di interesse sanitario in Italia (1985-1989). Rapporti ISTISAN 12/22.
- Cappelli A, Ulissi U, Valzano M, Damiani C, Epis S, Gabrielli MG, Conti S, Polonelli L, Bandi C, Favia G, Ricci I. (2014). A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*. PLoS One. 9(5).
- Coda R, Rizzello CG, Di Cagno R, Trani A, Cardinali G, Gobbetti M. (2013). Antifungal activity of *Meyerozyma guilliermondii*: identification of active compounds synthesized during dough fermentation and their effect on long-term storage of wheat bread. Food microbiol. 33(2), 243-251.
- Dantán-González E, Quiroz-Castañeda RE, Cobaxin-Cárdenas M, Valle-Hernández J, Gama-Martínez Y, Tinoco-Valencia JR, Serrano-Carreón L, Ortiz-Hernández L. (2015). Impact of *Meyerozyma guilliermondii* isolated from chickens against *Eimeria* sp. protozoan, an in vitro analysis. BMC Vet Res. 11, 278.
- Douglas AE. (2008). Conflict, cheats and the persistence of symbioses. New Phytol. 177, 849-858.
- Durvasula RV, Gumbs A, Panackal A, Kruglov O, Aksoy S, Merrifield RB, Richards FF, Beard CB. (1997). Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc Natl Acad Sci USA. 94, 3274-3278.

- Fang W, Vega-Rodriguez J, Ghosh AK, Jacobs-Lorena M, Kang A, St Leger RJ. (2011). Development of transgenic fungi that kill human malaria parasites in mosquitoes. Science. 331, 1074-1077.
- Faulde MK, Heyl G, Amirih ML. (2006). Zoonotic Cutaneous Leishmaniasis, Afghanistan. Emerg Infect Dis. 12(10), 1623-1624.
- Fernandes AP, Costa MM, Coelho EA, Michalick MS, de Freitas E, Melo MN, Luiz Tafuri W, Resende Dde M, Hermont V, Abrantes Cde F, Gazzinelli RT. (2008). Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. Vaccine. 26(46), 5888-5895.
- Flórez LV, Biedermann PH, Engl T, Kaltenpoth M. (2015). Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. Nat Prod Rep. 32(7), 904-936.
- Fredlund E, Druvefors U, Boysen ME, Lingsten KJ, Schnürer J. (2002). Physiological characteristics of the biocontrol yeast *Pichia anomala* J121. FEMS Yeast Res. 2(3), 395-402.
- Ghorbani M and Farhoudi R. (2017). Leishmaniasis in humans: drug or vaccine therapy? Drug Des Devel Ther. 12, 25-40.
- Gibson CM and Hunter MS. (2010). Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects. Ecol Lett. 13(2), 223-234.
- Gong F, Sheng J, Chi Z, Li J. (2007). Inulinase production by a marine yeast P*ichia guilliermondii* and inulin hydrolysis by the crude inulinase. J Ind Microbiol Biotechnol. 34(3), 179-185.
- Gossage SM, Rogers ME, Bates PA. (2003). Two separate growth phases during the development of *Leishmania* in sand flies: implications for understanding the life cycle. Int J Parasitol. 33, 1027-1034.
- Gould EA, Higgs S. (2009). Impact of climate change and other factors on emerging arbovirus diseases. Tran Roy Soc Trop Med Hyg. 103,109-121.
- Gradoni L and Gramiccia M. (2004). Phylum Sarcomastigophora: Classe Zoomastigophorea. In: De Carneri I., Parassitologia generale e umana, Genchi C & Pozio E. Ed, Casa Editrice Ambrosiana, Rozzano (MI), Italy, pp. 158-171.

- Gradoni L, Soteriadou K, Louzir H, Dakkak A, Toz SO, Jaffe C, Dedet JP, Campino L, Cañavate C, Dujardin JC. (2008). Drug regimens for visceral leishmaniasis in Mediterranean countries. Trop Med Int Health. 13(10), 1272-1276.
- Gradoni L. (2015). Canine Leishmania vaccines: still a long way to go. Vet Parasitol. 208(1-2), 94-100.
- Grimaldi G Jr, Teva A, Dos-Santos CB, Santos FN, Pinto ID, Fux B, Leite GR, Falqueto A. (2017). Field trial of efficacy of the Leish-tec® vaccine against canine leishmaniasis caused by *Leishmania infantum* in an endemic area with high transmission rates. PLoS One. 12(9), e0185438.
- Grzegorczyk M, Żarowska B, Restuccia C, Cirvilleri G. (2017). Postharvest biocontrol ability of killer yeasts against *Monilinia fructigena* and *Monilinia fructicola* on stone fruit. Food Microbiol. 61, 93-101.
- Guetsky R, Shtienberg D, Elad Y, Fischer E, Dinoor A. (2002). Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology. 92, 976-985.
- Gusteleva LA. (1975). Biosynthesis of vitamins of B group by yeasts symbiotic on xylophagous insects. Microbiol. 44, 36-38.
- Gyapong JO, Kumaraswami V, Biswas G, Ottesen EA. (2005). Treatment strategies underpinning the Global Programme to Eliminate Lymphatic Filariasis. Expert Opin Pharmacother. 6, 179-200.
- Hashem M, Abo-Elyousr KA. (2011). Management of the root-knot nematode *Meloidogyne incognita* on tomato with combinations of different biocontrol organisms. Crop Protection. 30(3), 285-292.
- Heritage J, Evans EGV, Killington RA. (1996). Introductory Microbiology. Cambridge University Press, New York, USA.
- Herwaldt BL. (1999). Leishmaniasis. The lancet. 354(9185), 1191–1199.
- Hotez PJ. (2018). The rise of neglected tropical diseases in the "new Texas". PLoS Negl Trop Dis. 12(1), e0005581.
- Hughes JL, Koga R, Xue P, Fukatsu T, Rasgon JL. (2011). *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. PLoS Pathog. 7, 1-8.

- Hurwitz I, Fieck A, Read A, Hillesland H, Klein N, Kang A, Durvasula R. (2011). Paratransgenic control of vector borne diseases. Int J Biol Sci. 7(9), 1334-1344.
- Jain K, Jain NK. (2015). Vaccines for visceral leishmaniasis: A review. J Immunol Methods. 422, 1-12.
- Janson EM, Stireman JO, Singer MS, Abbot P. (2008). Phytophagous insect–microbe mutualisms and adaptive evolutionary diversification. Evolution. 62(5), 997-1012.
- Kambris Z, Cook PE, Phuc HK, Sinkins SP. (2009). Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science. 326, 134-136.
- Kamhawi S. (2006). Phlebotomine sand flies and *Leishmania* parasites: friends or foes? Trends Parasitol. 22(9), 439-445.
- Kbaier-Hachemi H, Guerbouj S, Turki-Mannoubi L, Kaabi B, Guizani I. (2012). *In vitro* growth kinetics, differentiation and morphological characterisation of Tunisian *Leishmania infantum* parasites. Trans R Soc Trop Med Hyg. 106(1), 20-25.
- Killick-Kendrick R. (1999) The biology and control of Phlebotomine sandflies. Clin Dermatol. 17, 279-289.
- Kurtzman CP. (1998). The Yeasts, A Taxonomic Study, 4th ed. Elsevier Science BV, Amsterdam, the Netherlands, pp. 273-352.
- Lewis DJ. (1982). A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). Bull Br Mus Nat Hist Entomol. 45, 121-209.
- Liu D, Uzonna JE. (2012). The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. Front Cell Infect Microbiol. 2, 83.
- Lucius R, Loos-Frank B, Lane RP, Poulin R, Roberts C, Grencis RK. (2017). The Biology of Parasites. John Wiley & Sons. New York, USA.
- Lushbaugh WB, Rowton ED, McGhee RB. (1976). Redescription of *Coccidiascus legeri* Chatton, 1913 (Nematosporaceae: Hemiascomycetidae), an intracellular parasitic yeastlike fungus from intestinal epithelium of *Drosophila melanogaster*. J Invertebr Path. 28, 93-107.
- Magliani W, Conti S, Cassone A, De Bernardis F, Polonelli L. (2002) New immunotherapeutic strategies to control vaginal candidiasis. Trends Mol Med. 8, 121-126.

- Maroli M, Cavallini C, Khoury C, Miceli N, Manilla G. (1991). Entomological survey of sandflies (Diptera, Psychodidae) in the province of L'Aquila (Abruzzo). Parassitologia. 33(2-3), 127-131.
- Maroli M, Mizzon V, Siragusa C, D'Oorazi A, Gradoni L. (2001). Evidence for an impact on the incidence of canine leishmaniasis by the mass use of deltamethrin-impregnated dog collars in southern Italy. Med Vet Entomol. 15(4), 358-363.
- Maroli M, Pozio E, Gradoni L, Gramiccia M, Bettini S. (1980). Osservazioni sui vettori della leishmaniosi in provincia di Grosseto. Parassitologia, 22, 331-332.
- Maroli M. (1983). Laboratory colonization of *Phlebotomus perfiliewi* (Parrot). Trans R Soc Trop Med Hyg. 77(6), 876-877.
- Maroli M. (1986). Biology of a colony of *Phlebotomus perniciosus* (Diptera, Psychodidae). Ann Ist Super Sanità. 22 (1), 63-67.
- Martin V, Vouldoukis I, Moreno J, McGahie D, Gueguen S, Cuisinier AM. (2014). The protective immune response produced in dogs after primary vaccination with the LiESP/QA-21 vaccine (CaniLeish®) remains effective against an experimental challenge one year later. Vet Res. 45, 69.
- Mascari TM, Hanafi HA, Jackson RE, Ouahabi S, Ameur B, Faraj C, Obenauer PJ, Diclaro JW 2nd, Foil LD. (2013). Ecological and control techniques for sand flies (Diptera: Psychodidae) associated with rodent reservoirs of leishmaniasis. PLoS Negl Trop Dis. 7(9), e2434.
- Masoud W, Cesar LB, Jespersen L, Jakobsen M. (2004). Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. Yeast. 21(7), 549-556.
- Mazloumi-Gavgani AS, Hodjati MH, Mohite H, Davies CR. (2002). Effect of insecticide-impregnated dog collars on incidence of zoonotic visceral leishmaniasis in Iranian children: a matched-cluster randomised trial. The Lancet, 360, 374-379.
- McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF, O'Neill S. (2009). Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Science. 323, 141-144.

- Moreno J, Vouldoukis I, Schreiber P, Martin V, McGahie D, Gueguen S, Cuisinier AM. (2014). Primary vaccination with the LiESP/QA-21 vaccine (CaniLeish) produces a cell-mediated immune response which is still present 1 year later. Vet Immunol Immunopathol. 158 (3-4), 199-207.
- Muccilli S, Restuccia C. (2015). Bioprotective Role of Yeasts. Microorganisms. 3(4), 588-611.
- Mutinga MJ, Kihara SM, Lohding A, Mutero CM, Ngatia TA, Karanu F. (1989). Leishmaniasis in Kenya: description of leishmaniasis of a domestic goat from Transmara, Narok District, Kenya. Trop Med Parasitol. 40(2), 91-96.
- Nardon P, Grenier AM. (1989). Endosymbiosis in Coleoptera: biological, biochemical, and genetic aspects. In: Insect Endocytobiosis: Morphology, Physiology, Genetics, Evolution, Schwemmler W. & Gassner, G. eds. CRC Press, Boca Raton, USA. pp. 175-216.
- Naumov G, Naumova ES, Schnürer J. (2001). Genetic characterization of the nonconventional yeast *Hansenula anomala*. Res Microbiol. 152(6), 551.
- Otranto D, Dantas-Torres F, de Caprariis D, Di Paola G, TaralloVD, Latrofa MS, Lia RP, Annoscia G, Breitshwerdt EB, Cantacessi C, Capelli G, Stanneck D. (2013). Prevention of canine leishmaniosis in a hyper-endemic area using a combination of 10% imidacloprid/4.5% flumethrin. PLoS ONE. 8, e56374.
- Pant NC, Gupta P, Nayar JK. (1960). Physiology of intracellular symbiotes of *Stegobium paniceum* L with special reference to amino acid requirements of the host. Experientia. 16, 311-312.
- Papon N, Savini V, Lanoue A, Simkin AJ, Crèche J, Giglioli-Guivarc'h N, Clastre M, Courdavault V, Sibirny AA. (2013). *Candida guilliermondii*: biotechnological applications, perspectives for biological control, emerging clinical importance and recent advances in genetics. Curr Genet. 59(3), 73-90.
- Passoth V, Olstorpe M, Schnürer J. (2011). Past, present and future research directions with *Pichia anomala*. Antonie van Leeuwenhoek. 99, 121-125.
- Phaff HJ, Starmer WT. (1987). Yeasts associated with plants, insects and soil. In: The Yeasts. Rose A.H. & Harrison J.S. eds. Academic, New York, USA. Pp. 123-180.

- Polonelli L, Magliani W, Ciociola T, Giovati L, Conti S. (2011). From *Pichia anomala* killer toxin through killer antibodies to killer peptides for a comprehensive anti-infective strategy. Antonie Van Leeuwenhoek. 99, 35-41.
- Quinnell RJ, Courtenay O. (2009). Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 136(14), 1915-1934.
- Ricci I, Damiani C, Scuppa P, Mosca M, Crotti E, Rossi P, Rizzi A, Capone A, Gonella E, Ballarini P, Chouaia B, Sagnon N, Esposito F,Alma A,Mandrioli M, Sacchi L, Bandi C, Daffonchio D, Favia G. (2011). The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*. Environ Microbiol. 13(4), 911-21.
- Ricci I, Mosca M, Valzano M, Damiani C, Scuppa P, Rossi P, Crotti E, Cappelli A, Ulissi U, Capone A, Esposito F, Alma A, Mandrioli M, Sacchi L, Bandi C, Daffonchio D, Favia G. (2011b). Different mosquito species host *Wickerhamomyces anomalus* (*Pichia anomala*): perspectives on vector-borne diseases symbiotic control. Antonie Van Leeuwenhoe. 99(1), 43-50.
- Ricci I, Valzano M, Ulissi U, Epis S, Cappelli A, Favia G. (2012). Symbiotic control of mosquito borne disease. Pathog Glob Health. 106(7), 380-385.
- Romi R, Khoury C, Bianchi R, Severini F (Ed.). Artropodi di interesse sanitario. Roma: Istituto Superiore di Sanità; 2012. (Rapporti ISTISAN 12/41).
- Romi R. (2010). Arthropod-borne diseases in Italy: from a neglected matter to an emerging health problem. Ann Ist Super Sanita. 46(4), 436-443.
- Rossi E, Bongiorno G, Ciolli E, Di Muccio T, Scalone A, Gramiccia M, Gradoni L, Maroli M. (2008). Seasonal phenology, host-blood feeding preferences and natural *Leishmania infection* of *Phlebotomus perniciosus* (Diptera, Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome province, Italy. Acta Trop. 105, 158-165.
- Savini V, Catavitello C, Onofrillo D, Masciarelli G, Astolfi D, Balbinot A, Febbo F, D'Amario C, D'Antonio D. (2011). What do we know about *Candida guilliermondii*? A voyage throughout past and current literature about this emerging yeast. Mycoses. 54, 434-441.
- Schlein Y, Jacobson RL, Messer G. (1992). *Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. Proc Natl Acad Sci USA. 89, 9944-9948.

- Shen SK, Dowd PF. (1992). Detoxifying enzymes and insect symbionts. J Chem Educ. 69, 796-799.
- Starmer WT, Ganter PF, Aberdeen V. (1987). The ecological role of killer yeasts in natural communities of yeasts. Can J Microbiol. 33, 783-796.
- Suh SO, McHugh JV, Pollock DD Blackwell M. (2005). The beetle gut: a hyperdiverse source of novel yeasts. Mycol Res. 109, 261-265.
- Suh SO, Noda H, Blackwell M. (2001). Insect symbiosis: derivation of yeast-like endosymbionts within an entomopathogenic filamentous lineage. Mol Biol Evol. 18, 995-1000.
- Sujaya IN, Antara NS, Sone T, Tamura Y, Aryanta WR, Yokota A, Asano K, Tomita F. (2004). Identification and characterization of yeasts in brem, a traditional Balinese rice wine. World J Microbiol Biotechnol. 20, 143-150.
- Takken W, Knols BGJ (2007) (Ed.) Emerging pests and vector borne diseases in Europe. Wageningen Academic Publishers, Wageningen, the Netherlands.
- Tanner Jr F, Vojnovich C, Van Lanen JM. (1945). Riboflavin production by *Candida* species. Science (Washington), 101, 180-161.
- Taylor MJ, Makunde WH, McGarry HF, Turner JD, Mand S, Hoerauf A. (2005). Macrofilaricidal activity after doxycycline treatment *Wuchereria bancrofti*: a doble blind, randimised placebo-controlled trial. The Lancet. 365, 2116-2121.
- Valzano M, Cecarini V, Cappelli A, Capone A, Bozic J, Cuccioloni M, Epis S, Petrelli D, Angeletti M, Eleuteri AM, Favia G, Ricci I. (2016). A yeast strain associated to *Anopheles* mosquitoes produces a toxin able to kill malaria parasites. Malar J. 15, 21.
- Vega FE, Dowd PF. (2005). The role of yeasts as insect endosymbionts. In: Insect-Fungal Assocations: Ecology and Evolution, Vega F.E. & Dowd P.F. Ed. Oxford University Press, Oxford, UK, pp. 211-243.
- Vishniac HS Johnson DT. (1990). Development of a yeast flora in the adult green June beetle (*Cotinis nitida*, Scarabaeidae). Mycologia. 82, 471-479.
- Vora N. (2008). Impact of anthropogenic alteration on vector borne diseases. Medscape Med. 10(10), 238-224.
- Wah TT, Walaisri S, Assavanig A, Niamsiri N, Lertsiri S. (2013). Co-culturing of *Pichia guilliermondii* enhanced volatile flavor compound formation by *Zygosaccharomyces rouxii* in the model system of Thai soy sauce fermentation. Int J Food Microbiol. 160, 282-289

- Walker GM. (2011). *Pichia anomala*: cell physiology and biotechnology relative to other yeasts. Antonie Van Leeuwenhoek. 99(1), 25-34.
- Wang GY, Chi Z, Song B, Wang ZP, Chi ZM. (2012). High level lipid production by a novel inulinase-producing yeast *Pichia guilliermondii* Pcla22. Bioresour Technol. 124, 77-82.
- Wanji S, Tendongfor N, Nji T, Esum M, Che JN, Nkwescheu A, Alassa F, Kamnang G, Enyong PA, Taylor MJ, Hoerauf A, Taylor DW. (2009). Community-directed delivery of doxycycline for the treatment of onchocerciasis in areas of co-endemicity with loiasis in Cameroon. Parasit Vectors. 2, 39.
- WHO (2004). Global strategic framework for integrated vector management. World Health Organization 10, 1-12. WHO/CDS/CPE/PVC/2004
- WHO (2010). Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010.
- WHO (2015). Investing to overcome the global impact of neglected tropical diseases: third WHO report on neglected diseases 2015.
- WHO (2017). Global leishmaniasis update, 2006–2015: a turning point in leishmaniasis surveillance. Weekly epidemiological record 38 (92), 557–565.
- WHO (2017b). Vector control in: Seventieth World Health Assembly update, 30 May 2017.GENEVA.
- Wisniewski M, Biles C, Droby S, McLaughlin R, Wilson C, Chalutz E. (1991). Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*.1. Characterization of attachment to *Botrytis cinerea*. Physiol Mol Plant Pathol. 39, 245-258.
- Zacchi I, Vaughn-Martini A. (2002). Yeasts associated with insects in agricultural areas of Perugia, Italy. Annu Rev Microbiol. 52, 237-244.
- Zhang DP, Spadaro D, Valente S, Garibaldi A, Gullino ML. (2011). Cloning, characterization and expression of an exo-1,3-beta-glucanase gene from the antagonistic yeast, *Pichia guilliermondii* strain M8 against grey mold on apples. Biol Control. 59, 284-293.
- Zhao J, Mou Y, Shan T, Li Y, Zhou L, Wang M, Wang J. (2010). Antimicrobial metabolites from the endophytic fungus *Pichia guilliermondii* isolated from *Paris polyphylla* var. *yunnanensis*. Molecules. 15(11), 7961-7970.

- Zhao Y, Tu K, Shao XF, Jing W, Su ZP. (2008). Effects of the yeast *Pichia guilliermondii* against *Rhizopus nigricans* on tomato fruit. Postharvest Biol Technol. 49, 113-120.
- Zou YZ, Qi K, Chen X, Miao X L, Zhong JJ. (2010). Favorable effect of very low initial k L a value on xylitol production from xylose by a self-isolated strain of *Pichia guilliermondii*. J Biosci Bioeng. 109(2), 149-152.

2. PUBLISHED PAPERS

2.1. Article 1

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

environmental microbiology

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

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Running title: yeast community of *Phlebotomus perniciosus*

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Summary

The knowledge of the fungal mycobiota of arthropods, including the vectors of human and animal diseases, is still limited. Here, we investigated 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. The dominant species was *Meyerozyma guilliermondii,* known for its biotechnological applications. We focused the attention on this yeast and we investigated its prevalence in adults, pupae and larvae of reared sand flies (overall prevalence: 57.5%) and of field-collected individuals (overall prevalence: 9%). Using whole-mount FISH and microscopic examination, we 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 to predict 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.

Introduction

In recent decades, the role of microbes (fungi and bacteria) as insect symbionts has drawn attention (Gibson and Hunter, 2010). One of the most promising fields of investigation is the interaction between yeasts and insects, due to the diversity and metabolic capacity of yeasts and their presence in almost every environment (e.g. Blackwell, 2017). However, the current knowledge of yeasts occurring in the gut, and thereby belonging to the intestinal microbiota of arthropod vectors, of medical and veterinary importance, remains still limited. Some publications report the presence of the yeasts in the gut of mosquitoes (Diptera: Culicidae). Gusmão *et al*. (2007 and 2010) identified yeasts of the genera *Pichia* and *Candida* in the *diverticulum* of the mosquito *Aedes aegypti*. Ricci *et al*. (2011a and b) observed the presence of *Wickerhamomyces anomalus* (also known as *Pichia anomala*) in the midgut of adult mosquitoes, of both sexes, belonging to different species (*Anopheles stephensi*, *Anopheles*

gambiae, *Aedes albopictus* and *Ae. aegypti*); male gonads were also shown to be positive to yeast presence, as well as larvae and pupae. Recently, *W. anomalus* has also been isolated and phylogenetically characterized in laboratory-reared adults and larvae of *Phlebotomus perniciosus* (Diptera: Psychodidae), the main phlebotomine vector of human and canine leishmaniasis in the Mediterranean area (Martin *et al.*, 2016). Previous surveys on the fungal microflora in sand flies, using routine microbiological methods, identified fungal strains associated with different species of these vectors, collected in North-Western Iran (Akhoundi *et al.*, 2012). In addition, the yeast-like fungus *Pseudozyma* sp. has been isolated from wild-collected *Lutzomyia longipalpis*, a major *Leishmania* vector in central/south America (Sant'Anna *et al.,* 2014); this study also presents an interesting investigation on the interaction between *Leishmania* and component of the microbiota, in laboratory reared *Lu. longipalpis.*

Larvae of sand flies feed on the decomposing organic materials and can acquire a part of their microbiota (including bacteria and fungi), whereas as adults they likely acquire microorganisms through their daily feeding on natural sugar solutions, especially nectars, lymph and water from plants. Even though yeasts do not appear to be as frequent/abundant as prokaryotes in the intestinal microbiota of sand flies, they can account for an important part of the microbiota biomass, as they have a cell volume 30- to 100-fold higher than bacteria (Gatesoupe, 2007). Intestinal yeasts are likely to interact with the other components of the microbiota, including pathogens, and are thus likely to influence the insect biology and its vector competence. Therefore, the development of new strategies for the control of sand fly-borne diseases should also consider the mycobiota of these insects.

The aim of our study was to investigate the diversity of the fungal microbiota that colonizes laboratory-reared and wild-collected *P. perniciosus* individuals, at different developmental stages, with the goal to identify yeast species, with the potential to be exploited for future development and applications in the control of leishmaniasis and other vector-borne diseases. *P. perniciosus* was selected as model of investigation because of its medical and veterinary importance, being the main vector, in the western Mediterranean area, of the protozoan parasite *Leishmania infantum* (Trypanosomatida: Trypanosomatidae), the causative agent of canine and human leishmaniases (Killick-Kendrick, 1990; Alten *et al*, 2016).

Results

Yeast isolation and characterization

Yeasts from adults and L4 larvae of laboratory reared sand flies *P. perniciosus* were isolated and identified at the species level by 26S rRNA gene amplification, sequencing and comparison with sequences in NCBI database. As reported in tables 1 and 2, we obtained a total of 112 yeast isolates derived from whole adults, from digestive apparatus of adults and from L4 larvae; table 1 shows that we were not able to obtain yeast isolation from all the processed samples.

Table 1. Proportion between positive specimens of *P. perniciosus* out of all tested samples

	Adult males		Adult females		L4 larvae
	Whole body n=10	Digestive system n=10; pool n=1	Whole body n=10	Digestive system n=10; pool n=1	Digestive system n=10
Specimens positive for yeast isolation	2	3; pool:1	4	6; pool:1	6

Table 2. Identification of yeast isolates associated to *P. perniciosus* specimens.

Yeast species	Adult males			Adult females			L4 larvae
	Whole body	Digestive system	Digestive system pool	Whole body	Digestive system	Digestive system pool	Digestive system
Meyerozyma guilliermondii	4	3	2	26	41	2	8
Rhodotorula mucilaginosa	0	0	0	7	0	0	0
Trichosporon sp.	0	0	0	0	0	0	4
Wickerhamomyces anomalus	2	0	0	10	0	0	3
Total yeast isolates	6	3	2	43	41	2	15

The molecular identification of all the isolated yeasts is reported in table 2. The dominant yeast species was *Meyerozyma guilliermondii*, isolated from both sexes of whole adults and from the digestive apparatus of adults and L4 larvae. The yeast *Wickerhamomyces anomalus*, derived

from both whole adults and from the digestive system of the L4 larvae, was also frequently isolated. Finally, the Basidiomycota yeasts *Trichosporon* sp. and *Rhodotorula mucilaginosa* were also isolated from the digestive apparatus of larvae and from whole females, respectively. A total of eight species-specific 26S rRNA region sequences were submitted to GenBank (table 3). The analysis performed on the 26S rRNA sequences showed a variable sequence identity ranging from 99% to 100% (E-value: 0) with *Meyerozyma guilliermondii* KU729151 (strain ATCC 6260).

Table 3. 26S rRNA sequence analysis and homology with GenBank sequences

Yeast species	GenBank acc. no.	26S D1/D2 sequence comparison	
	D1/D2 26S	Ident (%)	Species (GenBank acc. no.)
Meyerozyma guilliermondii	LT905498	99%	KY463383
Meyerozyma guilliermondii	LT905499	100%	KY463383
Rhodotorula mucilaginosa	LT905496	99%	LC176999
Rhodotorula mucilaginosa	LT905497	100%	KY296083
Trichosporon sp.	LT905494	98%	AF444740
Trichosporon sp.	LT905495	99%	AF444740
Wickerhamomyces anomalus	LN871207	100%	LT594899
Wickerhamomyces anomalus	LN871208	100%	KY296073

Amplicon-based analysis of fungal community

After removal of chimeras and low-quality base call, a total of 37,154 amplicons of the fungal ITS rRNA were obtained by larvae and adults of the sand fly. Applying a similarity threshold of 97%, these amplicons were clustered into a total of 295 operational taxonomic units (OTUs). In particular 103 OTUs were amplified from adult females, 114 OTUs from adult males and 142 OTUs from larvae. The taxonomic assignment is synthesized in fig. 1 and in table S1-S3. The fungal community of *P. perniciosus* is dominated by taxa belonging to the phylum Ascomycota (average 96.6% ± 3.1%). Analyzing the samples according with the developmental stages, Ascomycota predominates in both adults (98.1% ± 2.4% of the total retrieved OTUs) and larvae (93.5%). Zygomycota represents the second taxon in term of abundance in larvae (6.2%) and is roughly null in adults (0.04% in females, 0% in males); meanwhile Basidiomycota are more present in adult females (3.5%) than in adult males (0.3%) and larvae (0.2%). The most represented class in the analyzed sand flies is Saccharomycetes (49.4% in

adults, in detail 53.7% in adult males and 45.2% in adult females, 33.4% in larvae). Sordariomycetes (40.9% in adults, in particular 45.5% in adult males and 36.2% in adult females, 29% in larvae) and Eurotiomycetes (0.3% in adults, in particular 0.2% in adult males and 0.3% in adult females, 27.5% in larvae) are also represented. A total of 14 genera were retrieved in the analyzed samples. Only four genera, namely *Candida*, *Engyodontium*, *Meyerozyma* and *Simplicillium*, are shared between the analyzed sand fly groups, being identified in both adult males, adult females and larvae. The great majority of amplicons in larvae belongs to the genera *Candida* (32.7%), *Aspergillus* (27.4%) and *Simplicillium* (24.7%). By contrast, the majority of amplicons obtained from adults (both males and females) were identified as *Meyerozyma* (44% in adult females, 53.6% in adult males) and as *Simplicillium* (34% in adult females, 45.4% in adult males). Nevertheless, the genera *Candida* and *Aspergillus* are poorly represented in adults (0.2% ± 0.1% and 0.1% ± 0.1% respectively) and the genus *Meyerozyma* is low in larvae (0.6%). The obtained 295 OTUs are not equally distributed between the analyzed sand fly groups; in fact 37% of OTUs are exclusive of larvae (see Fig. S1).

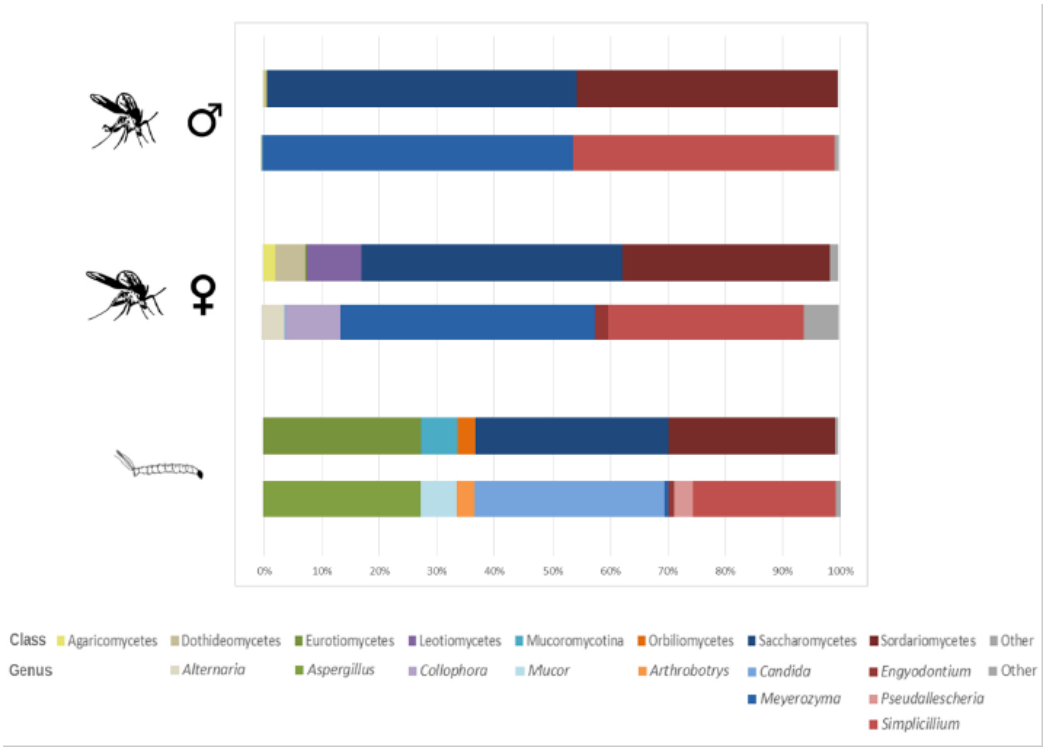


Figure 1. Fungal diversity associated with *P. perniciosus* analyzed by ITS rRNA amplicon-based sequencing. Only taxa represented more than 2% were considered. At the left of histograms are indicated sample stage/sex. Relative abundance of class and genera for each sample stage/sex are shown by histograms.

The OTU core of the population, consisting of the OTUs that are in common between larvae, adult males and adult females, is composed of 10 taxonomic units, therefore representing 3.4% of the *P. perniciosus* panmycobiota. The OTUs in the core were assigned to *Engyodontium*, *Meira*, *Meyerozyma* and *Simplicillium.* Among them, one OTU in the core represents the 63% of all the reads obtained from adults and it is assigned to the genus *Meyerozyma,* confirming the results obtained using culture dependent methods. Another noteworthy OTU in the core is assigned to *Simplicillium;* this OTU represents 25% of all the reads from larvae and 8.8% of all the reads obtained from adults.

Prevalence of the yeast *M. guilliermondii* in sand fly populations

In order to evaluate the prevalence of *M. guilliermondii* (the dominant yeast species), 80 reared specimens (including males, females, L4 larvae and pupae) and 100 field collected sand flies, were screened with species-specific primers. Due to the difficulty to collect larvae and pupae of wild sand flies, the molecular screening of field collected insects have been performed only on adults. Results are summarized in table 4.

Table 4. Results of the molecular screening for the detection of *M. guilliermondii* DNA from *P. perniciosus* specimens.

Location of capture/origin	Sex or sample type	Number of *M. guilliermondii* positive samples/ n. of analyzed samples
Frascati (RM)	Male	1/21
N 41,838810-EO 12,700712	Female	2/37
Roma	Male	1/8
N 41,936649-EO 12,367065	Female	0/2
SP 52 Vieste-Peschici (FG)	Male	4/21
N 41,901623-EO 16,138898	Female	1/11
Insectary of ISS (Rome, Italy)	Male	13/20
	Female	8/20
	L4 larvae	18/20
	Pupae	7/20

The prevalence of *M. guilliermondii,* evaluated by PCR, in the reared population was 65% (13/20) in males, 40% (8/20) in females, 90% (18/20) in larvae and 35% (7/20) in pupae, with an overall prevalence of 57.5% (95% CI: 46.7-68). By contrast, the overall prevalence of the yeast in field collected populations was 9% (95% CI: 3.4-14,6) that is statistically lower than

the prevalence in Istituto Superiore di Sanità (ISS) reared one ($\chi 2 = 49.268$, $P < 0.00001$). The analyzed wild individuals showed a prevalence of 12% (6/50) in males and 6% (3/50) in females. The prevalences in different sites of collection were not equal, although there is no statistically significant difference ($\chi 2$, P =0.250): in Frascati (RM) the prevalence was 5% (3/58), in Roma it was 10% (1/10) and in Vieste-Peschici (FG) the prevalence was 16% (5/32).

Microscopic analysis of fungi in sand flies and whole mount fluorescent *in situ* hybridization (FISH)

In order to confirm the presence of yeasts in digestive tract of sand flies, microscopic analyses were performed on males and females. Giemsa stained yeast cells and budding yeast cells were observed in 5/10 slide preparations made from digestive tracts and from the Malpighian tubules of *P. perniciosus* (2/5 from males, 3/5 from females). The fungi exhibited typical staining properties (as basophilic) and structure, as reported in fig. S2.

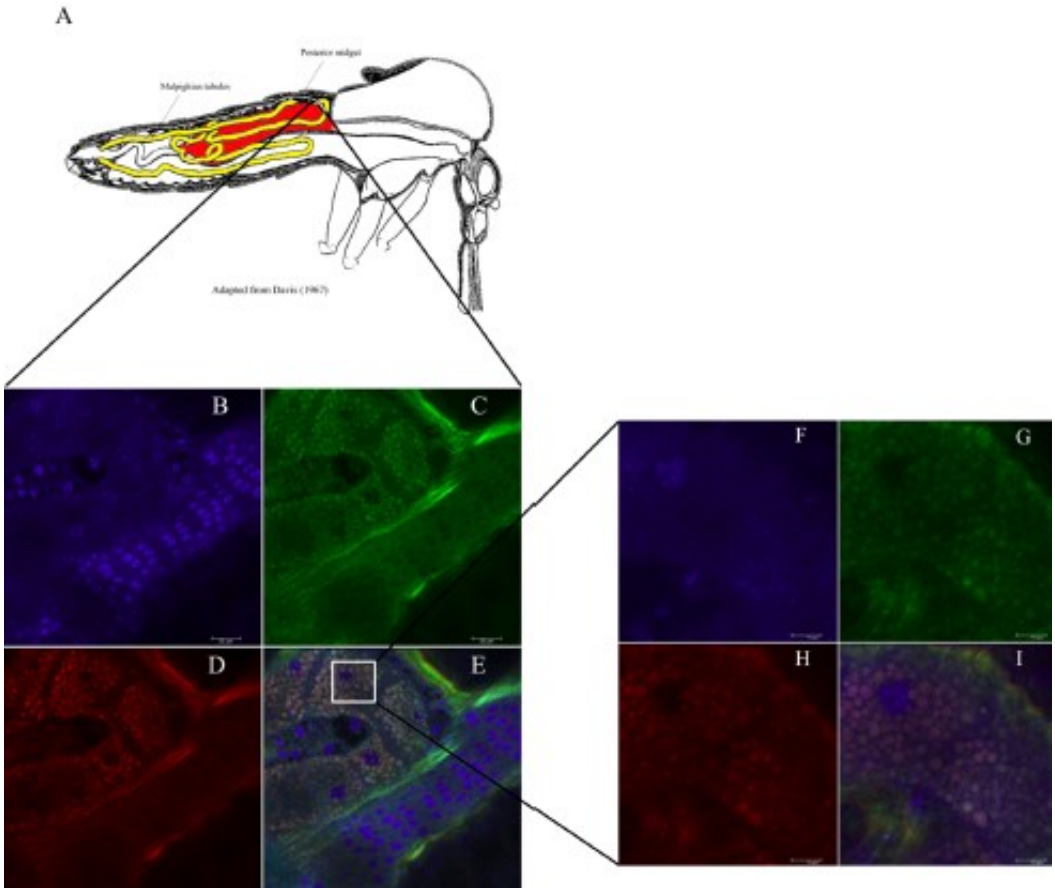


Figure 2. Localization of *M. guilliermondii* complex in a *P. perniciosus* female. (a) Schematic view of digestive apparatus of sand fly. (b) Cellular nuclei stained for cell viability with DAPI. (c) Staining with the universal yeast probe PF2 labeled with CY3. (d) Staining with FITC labeled probe, specific for *M.*

guilliermondii complex MGU26S410. (e) Overlap between the two probes. (f-i) Higher magnification of the Malpighian tubule using the same probes described in figures b to e.

The presence of *M. guilliermondii* in adult sand flies and L4 larvae was further investigated with whole mount FISH. The specificity of the probe MGU26S410, designed for *M. guilliermondii* complex, was evaluated against the yeast strains of *R. mucilaginosa, W. anomalus*, *Trichosporon* sp. and *M. guilliermondii* isolated from sand flies in this work. The probe resulted highly specific for *M. guilliermondii* (Fig. S3). The probe MGU26S410 for *M. guilliermondii* complex localized yeasts in the midgut of males (Fig. S4), females (Fig. 2) and larvae (Fig. S5) and in the distal part of the Malpighian tubules of females (Fig. 2), where yeast cells were in active division. However, *M. guilliermondii* was not detected in all the analyzed specimens, confirming what observed with PCR screening or yeast isolation (Fig. S6). In addition, the signal obtained using the PF2 probe (the universal yeast probe) in Malpighian tubules of females is comparable in intensity and localization to the signal obtained with the specific probe for *M. guilliermondii* complex, indicating the highest concentration of yeast of *M. guilliermondii* complex in this compartment. The probe non-MGU26S410 and the pre-treatment with RNase A (Fig. S7), utilized as negative controls, did not generate detectable fluorescence signals in the analyzed samples.

Phylogenomic analysis of a strain of *M. guilliermondii* isolated from *P. perniciosus*

After sequencing with an Illumina HiSeq 2500, 12,869,872 150bp paired-end reads were obtained. The reads were checked and resulted of good quality. The obtained assembly consists of 108 contigs and has an overall length of 10,666,172 base pairs (and 5,476 genes), comparable to the 10,609,954 base pairs (and 5,396 genes) of the only genome already published for *M. guilliermondii* (strain ATCC 6260) (table 5). In addition, the number of not shared genes is 21 for *M. guilliermondii* Pp, and four not shared genes for the *M. guilliermondii* ATCC 6260 (table 5). We retrieved 5,531 putative genes with an average length of 1,521.35 base pairs, composing most of the genome by totaling 8,414,591 nucleotides. Out of the total of putative genes, 82.73% were annotated. Starting from a comprehensive dataset of fungal genomes (table S4), OrthoMCL retrieved 190 shared orthologous clusters with a single copy in each genome. Each cluster of orthologs was multialigned with ClustalO and the resulting alignment was cleaned with Gblock. All cleaned alignments were concatenated to obtain the final input for the phylogenetic analysis, consisting of a total of 42,467 aminoacids. A maximum likelihood

analysis performed with RaxML using 100 bootstrap pseudoreplicates resulted in the phylogenetic tree showed in fig. 3a. *M. guilliermondii* Pp is strongly supported to be a sister clade to *M. guilliermondii* ATCC 6260 with *Clavispora lusitaniae* as their closest relative. The phylogenetic picture is consistent with other phylogeny studies conducted on yeasts, with the fission yeast *Schizosaccharomyces pombe* as the outgroup and a clear separation between Saccharomycotina and Pezizomycotina species (Kurtzman, 2014; Spatafora *et al.*, 2006).

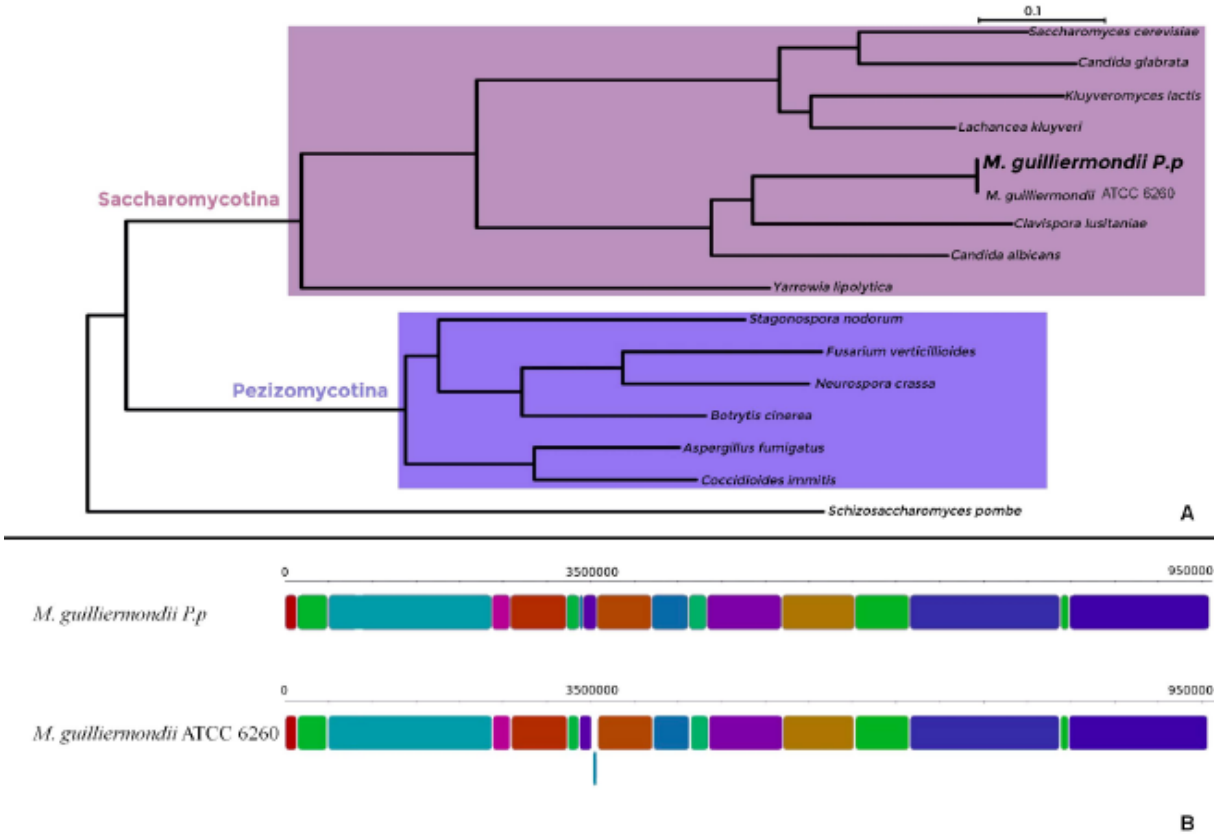


Figure 3. Phylogenomic analysis of a strain of *M. guilliermondii* isolated from *P. perniciosus.* (a) Phylogenetic yeast tree; Saccharomycotina species highlighted in lilac, Pezizomycotina species highlighted in blue lavender. (b) Genomic alignment obtained with Mauve; colors highlight regions sharing sequence homology between the two genomes

Table 5. Statistics of the obtained assembly compared with the ATCC 6260 strain genome.

Assembly	Total length	Number of contigs	Contig average length	N50	Number of genes	Number of non shared genes
Abyss-pe	10,666,172 bp	108	98,760.85 bp	232,015	5,476	21
ATCC 6260	10,609,954 bp	9	1,178,883.78 bp	1,701,016	5,396	4

Accepted

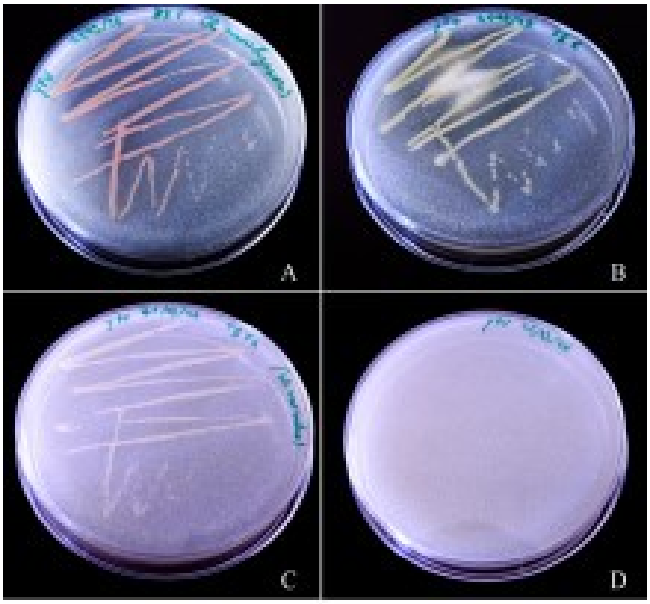
In order to perform the comparative genomics, species-specific and strain-specific clusters of orthologous genes were retrieved for *M. guilliermondii* Pp, *M. guilliermondii* ATCC 6260, and *C. lusitaniae* (Fig. 3). The biggest subset by far is composed of clusters shared between the three yeast species (4,242 clusters), whereas few clusters are strain specific. There is evidence for a substantial subset of *Meyerozyma* specific subset composed of 793 clusters. A visualization of the GO terms assigned to the three genomes presents a homogeneous landscape, implying an overall high similarity of general gene functions. The genomic alignment (Fig. 3b) between *M. guilliermondii* Pp and *M. guilliermondii* ATCC 6260 shows that synteny is mainly conserved between the two, with the exception of an inversed translocation of about 70 kb located roughly at 3,365 kb into the *M. guilliermondii* Pp genome.

Bioinformatic prediction of uric acid degradation pathway and uricolytic activity of *M. guilliermondii*

All components of the uric acid degradation pathway, except for Allantoine racemase, were found in the set of predicted proteins of *M. guilliermondii* Pp. Each enzyme had a reciprocal blast hit with a percentage of identity higher than 56%. Specifically, Mguil2443_1 was identified as Urate oxidase (percentage of identity 64%), Mguil178_1 as HIU Hydrolase (63%), Mguil553_1 as OHCU decarboxylase (56%), Mguil3309_1 as Allantoinase (66%), Mguil2347_1 as Allantoicase (67%), Mguil178_1 as Ureidoglycolate hydrolase (63%). Uric acid degradation pathway of *M. guilliermondii* Pp is shown on fig. 4c.

After the prediction of uric acid degradation pathway, and following the particular localization of the yeast in Malpighian tubules, we evaluated the yeast capability of degrading uric acid; the uricolytic activity assays were performed on two strains for each isolated yeast species. All tested *R. mucilaginosa, W. anomalus*, *Trichosporon* sp. and *M. guilliermondii* yeast isolates were able to grow on modified YPU containing 4 g/l of uric acid.

However, only *M. guilliermondii* and *R. mucilaginosa* strains were able to degrade uric acid showing a clear halo (suggestive of uric acid utilization) surrounding the yeast (Fig. 4a and 4b). The halos obtained from *M. guilliermondii* strains were comparable with those produced by *R. mucilaginosa* strains, the yeast was able to grow and consume the uric acid as sole nitrogen source (Vera-Ponce de Leon *et al*., 2016).



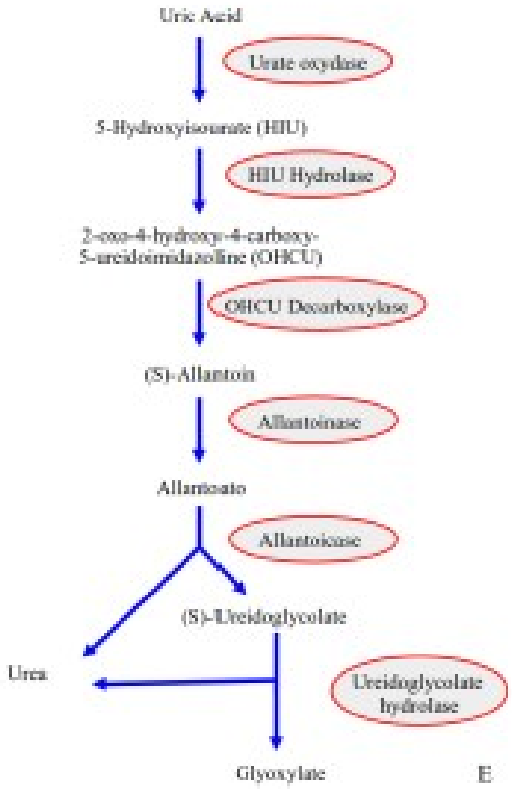


Figure 4. Uric acid degradation of *M. guilliermondii*. (a) Fungal uricolytic activity of the yeasts *R. mucilaginosa*, (b) *M. guilliermondii*, (c) *W. anomalus* and (d) the control plate only with modified YPU medium. (e) Uric acid degradation predicted pathway of *M. guilliermondii* Pp isolated from *P. perniciosus*.

Discussion

In this work, we investigated the mycobiota associated with the sand fly *P. perniciosus,* the main vector of canine and zoonotic visceral leishmaniasis. Culture-dependent analyses and ITS rRNA amplicon-based analysis show that the dominant yeast species is *M. guilliermondii*. This ascomycota yeast could be considered a "generalist yeast" that can form relationships with insects belonging to a variety insect taxa; in fact, it has been recovered from long-horned beetles (Nardon and Grenier, 1989), scarab beetles (Vishniac and Johnson, 1990), bark beetles (Rivera *et al.*, 2009), leaf beetle and crambid snout moths (Molnar *et al.*, 2008), fire ants (Ba *et al.*, 1995), adrenid bees and a mushroom-feeding fly (Zacchi and Vaughn-Martini, 2002), fishfly, dobsonfly and owlfly (Nguyen *et al.*, 2007), and several genera of mosquitoes (Gusmão *et al.*, 2010; Bozic *et al.,* 2017). Interestingly, it has never been recovered from sand flies. The prevalences recorded for *M. guilliermondii* in *P. perniciosus* indicate that this yeast is well distributed in the insectary population, in both sexes and in all analyzed life stages (with an overall prevalence of 57.5%). In wild-collected individuals, the yeast is less prevalent (overall: 9%) and less homogeneously distributed, with variability related also with the geographical location (Lazio region 7.5%; Puglia region: 16%). As for the diffusion of the yeast in the two sexes, we observed a higher prevalence in males, in both reared (65% in males, 40% in females) and field-collected individuals (12% in males and 6% in females).

The higher prevalence in lab-reared flies compared to wild collected ones could derive from different factors. First, it is reasonable to assume that laboratory rearing provides conditions that favors the horizontal transmission of the yeast among the insects, with cofeeding likely representing one of the main routes for transmission. Other ways for the horizontal transmission of the microbiota, that are known to occur in insects, are: trophallaxis (e.g. in termites; Nalepa *et al.,* 2001); venereal transfer (e.g. in mosquitoes; Damiani *et al.,* 2008), which includes the transmission of ejaculated components of the microbiota from males to females (e.g. in aphids; Moran and Dunbar, 2006). Whether similar phenomena occur in sand flies is however unknown. Assuming that horizontal transmission is responsible for the higher prevalence of *M. guilliermondii* in the reared flies, we would conclude that this yeast is at least neutral toward the insect host, otherwise we would have observed some mortality or fitness reduction; indeed, we have evidence that *M. guilliermondii* is present in the examined colony since 2013 (unpublished observations). Second, we could also suggest that *M. guilliermondii* plays a beneficial role in laboratory condition. Indeed, yeasts are known to provide insects with several advantages, which include the provision of nutrients, the detoxification of harmful substances

and the protection from biotic stresses (Gibson and Hunter, 2010; Janson *et al.,* 2008*).* It is reported that yeast cells can be a source of B vitamins, proteins and amino acids, that can be assimilated by the insects. We can thus hypothesize that, under laboratory conditions, *M. guilliermondii* contributes to the diet of reared sand flies with nutrients which are on the other side easily available in nature. Third, a founder-effect explanation could be proposed: the sand fly lab colony had been established from positive insects. Anyway, the maintenance of *M. guilliermondii* at a high prevalence, over a period of 30 years, would imply a neutral or beneficial effect of the yeast toward the insect. As for the higher prevalence of *M. guilliermondii* in males than in females, we could perhaps suggest that this difference derives from the different diet (males do not take a blood meal). The diet is indeed known to affect the composition of the microbiota, as shown in mosquitoes, in relation with the blood meal (Wang *et al.,* 2011).

Culture-independent ITS amplicon analysis of the fungal community in larvae and adults highlighted a core of 10 OTUs assigned to four genera of fungi (Fig. S1). Two genera that are worth to be considered are the already discussed yeast *Meyerozyma*, the predominant genus in adults, and the filamentous fungus *Simplicillium*. We could hypothesize that the genera that are in common between life stages and adults, represented in the core, can be transtadially transmitted. This hypothesis is also supported by the localization of the yeast *M. guilliermondii* in sand flies, i.e. in the gut of adults and larvae, and also in the Malpighian tubules of females (Fig. 2). A previous, and so far unique, report of the presence yeasts in this excretory organ in dipteran was published in 1984 (Schlein *et al.*, 1985), where unidentified yeast-like cells (about 2-3 micrometers), without filaments or hyphae, were observed in the gut and in the Malpighian tubules of laboratory reared *P. papatasi* and *P. tobbi*.

The fact that *M. guilliermondii* can be housed in the Malpighian tubules of larvae and of adults opens the possibility that the yeast persistence could also be due to escape from the metamorphosis using the Malpighian tubules as a refuge. It is reported that in Diptera the larval Malpighian tubules remain intact during metamorphosis (Singh *et al.,* 2009) and tubular fluid including the microbes could then drain from Malpighian tubules towards the midgut.

In mosquitoes, the main role of Malpighian tubules after a blood meal switches from diuresis to detoxification and purine catabolism, and uric acid (an end product of purine catabolism) is accumulated in such structures within 24 h after a blood meal (Esquivel *et al*., 2016). The physiology of Malpighian tubules in sand flies could be similar to that in mosquitoes, being both hematophagous nematoceran Diptera. The degradation of purines to uric acid is conserved

in all organisms, but the resulting uric acid can either be excreted or further degraded and, in particular, Diptera excrete allantoin as the end product. Most fungi possess all the necessary nitrogen catabolic enzymes to completely degrade uric acid; the yeast *Saccharomyces cerevisiae*, where the pathway begins at the step of allantoin degradation, appears as an exception. Ammonia, the final product of uric acid degradation, is readily assimilated as a nitrogen source utilized by most bacteria and fungi (Lee *et al*., 2013). In cockroaches, in which uric acid is accumulated in the fat body in periods of diet rich in proteins and decreases in times of shortness (Cochran *et al*., 1979), the stored nitrogen can then be recycled thanks to the activity of his primary endosymbiont *Blattabacterium cuenoti* (Valovage and Brooks, 1979). In particular, Patiño-Navarrete *et al*. (2014) demonstrated that in the cockroach *Blattella germanica* the nitrogen recycling pathway is chimeric, with participation of enzymes from the host and the bacterial symbiont *B. cuenoti.*

Here, considering the detection of *M. guilliermondii* in Malpighian tubules of females of *P. perniciosus*, we can speculate that the yeast can play a role in uric acid degradation at the end of purine catabolism, in particular after the blood meal, explaining this unusual localization only in female individuals. The analysis of the genome of *M. gulliermondii* Pp. from *P. perniciosus*, supported this evidence: after the bioinformatic prediction of uric acid degradation pathway we demonstrated that all of the components of the uric acid degradation pathway, up to ammonia (except for Allantoine racemase), were found in the set of predicted proteins of *M. guilliermondii* Pp. The uricolytic activity of *M. guilliermondii* yeast strains isolated from sand flies in this work was also investigated determining the degradation halo in YPU medium (containing uric acid); we observed a clear halo surrounding yeast colonies,which is suggestive for uric acid degradation. We can thus propose that this yeast, associated with female *P. perniciosus* sand flies, could contribute to the degradation of urates, facilitating the removal of excesses of nitrogenous wastes in the Malpighian tubules after the blood meal. Ammonia could indeed be easily be drained towards the gut and, within the gut, ammonia could then be recycled by the resident microbial community, or eliminated with the fecal material.

Judging from genomic analysis, the genome of *M. guilliermondii* isolated from *P. perniciosus* is very similar to that of the available environmental strain (ATCC 6260) of the same species. A phylogenomic analysis performed on 190 genes shared between 15 yeast species placed the two *Meyerozyma* genomes as sister clades. Comparative genomics provides results coherent with those of phylogenomics, showing that, in fact, the two genomes have a comparable number of genes with overall similar general functions. Lastly, a genomic alignment points out that

synteny is conserved, meaning that, apart from a minor translocation, blocks of similarity are present in the same order in the two genomes.

Finally, as reported in Papon *et al*. (2013), the yeast *M. guilliermondii* can display a large range of applications in various areas of fundamental and applied scientific research. Some *M. guilliermondii* strains have been shown to be useful as biological control agents due to their ability to compete for nutrients and living space with pathogens. The presence of the yeast *M. guilliermondii* in phlebotominae sand flies could also play a protective role against other microorganisms, including transmitted pathogens (e.g. *Leishmania* spp. or viruses). Indeed, as in the case of other yeast species (e.g. *Wickerhamomyces anomalus*, symbiont of arthropod vectors, including sand flies), *M. guilliermondii* likely compete for nutrients or could be a source of enzymes, or could protect the host insect against infectious agents (Wang *et al*., 2008; Cappelli *et al*., 2014). As already demonstrated for bacteria, yeasts can also benefit insects by promoting the development of other mutualistic and beneficial organisms in the community, while decreasing the presence of insect pathogens (Zindel *et al*., 2011).

In conclusion, although further studies will be required, the investigation of the mycobiota of *P. perniciosus* may have a great impact in a better understanding of some aspects of phlebotominae biology and towards the development of strategies aimed to reduce the vectorial capacity and/or inhibiting pathogen transmission using engineered yeast.

Experimental Procedures

Samples analyzed in this study

Laboratory-reared sand flies

A laboratory colony of *Phlebotomus perniciosus*, originated from samples collected in Madrid area (Spain) and established at the Institute of Health Carlos III of Madrid in 1983, was employed. The colony has been reared since June 2012 at the Unit of Vector-Borne Diseases of Istituto Superiore di Sanità, Rome, and at time of this study the sand flies were at the 27th generation. Adults were routinely maintained in thin mesh cages in thermostated cabinets at standard conditions of temperature, humidity and photoperiod (28 ± 1ºC; RU 95-100%; light:dark period 7:17 hrs) and daily provided of 30% sugar solution (Maroli *et al*., 1987). Larvae were grown in plastered jars at same standard conditions, supplemented by sterilized

larval food following the mass-rearing technique (Modi and Tesh, 1983), with strict regulation of food quantity and moisture.

Wild-caught sand flies

Sample collection was carried out in central and south Italy between 2012 and 2015, in the months of July and August, in three rural sites using five CDC miniature light traps equipped with a fine net cage for each site and placed near animal shelters. Site 1 was an agricultural area of Frascati commune (Rome province, central Italy) (N 41,838810-EO 12,700712) where available blood sources were horses, sheep and dogs. Site 2 consisted of a horse stable placed in Rome outskirts (N 41,936649-EO 12,367065). Site 3 was a farm hosting goats, sheep and dogs, and placed along a main road (SP 52) connecting two towns (Vieste and Peschici, Foggia province, south Italy) (N 343 41,901623-EO 16,138898). The sites were selected considering a variety of parameters consistent with *P. perniciosus* presence, and represented by different environmental conditions and biotic factors that may affect the sand fly microbiome.

All field collected specimens were morphologically identified to species level according to Theodor (1958) and Léger *et al*. (1983), by cutting the last abdominal segments in both sexes and the head in females, with subsequent clarification in chloro lactophenol and permanent mounting. The rest of the insect body was stored in absolute ethanol to preserve DNAs of both the host and the harbored microorganisms.

Yeast isolation

Laboratory reared sand flies were surface sterilized with ethanol and washed twice with sterile physiological solution (NaCl 0.9%) supplemented with detergent, in order to eliminate external contaminants and their hair. Ten whole females and 10 whole males were homogenized in 200 µl of sterile NaCl 0.9% and diluted serially up to 10^{-3}. 100 µl of each dilution were plated on YM agar medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l dextrose, 20 g/l agar, pH 6.2) and 100 µl on PDA medium (Sigma Aldrich, St. Luis, USA), both supplemented with chloramphenicol 100 mg/l to avoid bacterial growth. Fifteen females, 15 males and 10 L4 larvae, after surface cleaning, were aseptically subjected to dissection under a stereomicroscope (Leica M50, Leica Microsystems, Wetzlar, Germany) with sterile needles in order to separate the digestive system. Ten digestive systems from females, 10 digestive systems from males and all the digestive systems excised from larvae were homogenized in 100 µl NaCl 0.9% and plated individually directly on YM and on PDA agar medium. The digestive system of five females

and the digestive system of five males were pooled for sex and homogenized in 200 µl NaCl 0.9% (each pool), diluted up to 10^{-2} and both the dilutions were plated on YM and on PDA medium. Plates were incubated at 28°C for 48-72 hours (Varotto Boccazzi *et al.,* 2017).

Characterization of yeast isolates

Growing yeast colonies from whole adult sand flies, digestive systems of adults and larvae were selected and collected based on colony morphology (Kurtzman and Fell, 2000), re-plated twice and the pure cultures of yeast strains, grown in liquid potato dextrose broth (PDB) medium (Sigma Aldrich, St. Luis, USA), were stored in 20% glycerol at − 80 °C. DNAs from yeast isolates were extracted using the enzyme lyticase (Sigma Aldrich, St. Luis, USA) and DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the supplementary protocol for purification of total DNA from yeasts. Total extracted DNAs were quantified by spectrophotometry (Nanodrop 2000, Thermo Scientific, Wilmington, USA). In order to perform the characterization of the isolated strains, a PCR protocol for the amplification of 26S rRNA gene was carried out using primers NL1 (5′- GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Ferreira *et al*., 2010) in a total volume of 25 µl containing: 1X Green GoTaq Reaction Buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1.25 U GoTaq G2 (Promega, Madison, USA), 7-10 ng of template DNA following the thermal protocol reported in Ferreira *et al*. (2010). All amplicons, obtained from 26S rRNA gene amplification, were sequenced and the obtained sequences were corrected and subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) in order to compare our sequences with the available sequences of fungi in GenBank (nucleotide collection nr/nt; http://www.ncbi.nlm.nih.gov/genbank/). Two sequences for each yeast species isolated in this study were deposited in GenBank database.

Microscopic analysis of fungal infection in sand flies

Ten adult reared sand flies (five males and five females) were macerated in 10% KOH for 24 h at room temperature, dehydrated with steps in H_2O, 70% EtOH, 100% EtOH, finally xylol, following the protocol reported in Schlein and collegues (1985). Digestive systems and Malpighian tubules from male and female sand flies were placed on microscopic slides in a drop of 1X PBS, smashed and smeared. The smears were fixed with methanol, stained with Giemsa and examined with a light microscope (Axioskop 2, Zeiss, Oberkochen, Germany).

ITS rRNA amplicon-based analysis of the fungal community of sand flies

ITS rRNA amplicon-based next-generation sequencing was carried out from pooled DNA of five reared specimens (in total, two pool of males, two pool of females and two pool of L4 larva) using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). DNAs were extracted as reported below. After PCRs, amplicons were purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) and then sequenced using Roche FLX 454 titanium. PCR reactions and amplicons sequencing were performed commercially by MR DNA (Shallowater, TX, USA). QIIME (Caporaso *et al*., 2010) was used to remove pyrosequencing adaptors and low quality base calls (< 30 Phred score), perform the amplicon size selection (retained sequences between 200 and 400 bp) and purge the chimeric sequences. ITS2 gene sequences obtained by 454 pyrosequencing assays were deposited in European Nucleotide Archive with accession number PRJEB22022 . Highquality 454 sequence reads were clustered into operational taxonomic units (OTUs) using UCLUST (Edgar, 2010) with a 97% sequence-identity threshold. The most abundant sequence of each OTU was selected as representative and used to build the overall OTU table. These OTUs were taxonomically assigned by RDP Classifier (Wang *et al.*, 2007) using, as reference, the Warcup ITS fungal database (Deshpande *et al.*, 2016).

M. guilliermondii-specific primers design and PCR screening on wild-caught and laboratory reared sand flies

Before DNA extraction, 40 adults (20 males and 20 females), 20 pupae and 20 L4 larvae of *P. perniciosus,* reared in the insectary of ISS (Roma, Italy), and 100 field collected adults of *P. perniciosus* (50 males and 50 females), as reported in table 4, were carefully washed in sterile 1X PBS and dried. Samples were homogenized using sterile pestles in 200 µl of lysis buffer (1 M sorbitol, 0.1 M sodium EDTA and 200 units of lyticase (Sigma Aldrich, St. Luis, USA)) and incubated for 45 min at 30°C. Then, the extraction was carried out using the commercial kit DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the protocol for purification of total DNA from animal tissues. DNAs were eluted in 25 µl of AE buffer. Each DNA was quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA). To investigate the prevalence of the yeast *M. guilliermondii*, a specific primer set mguITS_F108 (5'-CTTTGGTTTGGCCTAGAG-3') and mguITS_R481 (5'-ATAAACCTAATACATTGAGAGG-3'), that amplifies a fragment of 373 bp, was designed on ITS rRNA region. The amplification was carried out in a total volume of 25 µl containing: 1X Green GoTaq Reaction Buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1.25 U GoTaq G2

(Promega, USA) 7-10 ng of template DNA with the following thermal protocol: initial denaturation at 94°C for 7 min, followed by 35 cycles of 94°C for 45 sec, 56°C for 45 sec, 72°C for 45 sec and a final extension step of 72°C for 10 min. The PCR protocol was validated on three isolates of *M. guilliermondii* identified in this work by 26S rRNA gene sequencing. The *M. guilliermondii*-specific PCR was used to screen the prevalence of *M. guilliermondii* on the previously extracted DNAs from whole insects (wild-caught and laboratory-reared sand flies). The obtained amplicons were visualized on agarose gel. Only for negative samples, 1 µl of the amplification products was subjected to a second round of the same PCR.

Fluorescence *in situ* hybridization (FISH): probe design and evaluation of the specificity

A specific 26S rRNA oligonucleotide probe was designed to target yeasts of the complex *M. guilliermondii* associated with sand flies (MGU26S410_5'-FITC: 5'-GGCTCACAAAATATCGAGTCTG-3'). The MGU26S410 probe was designed on the basis of an alignment with sequences of a selection of yeast strains commonly isolated in arthropods derived from the NCBI Nucleotide database. The oligonucleotide sequence was further checked for its suitability with the software probeCheck (http://www. microbial-ecology.net/probecheck) (Loy *et al.*, 2008). In addition, the probe PF2_5'-CY3: 5'-CTCTGGCTTCACCCTATTC-3' (Kempf *et al.*, 2000) was used as universal fungal probe and the probe non-MGU26S410, which is the complementary (antisense) sequence to the probe MGU26S410, was tested as a negative control for non-specific binding. As supplementary negative controls, males, females and larvae of sand flies were treated with RNase A (USB, Thermo Fisher Scientific, Paisley, United Kingdom), prior to incubation with probes. All FISH probes were purchased from Eurofins Genomics (Ebersberg, Germany). The specificity of the probe MGU26S410 was tested against the yeast strains of *R. mucilaginosa, W. anomalus*, *Trichosporon* sp. and *M. guilliermondii*, isolated from sand flies in this work, as previously described (Xufre *et al.*, 2006). Briefly, yeast cells were grown in PDB medium and harvested at the exponential growth phase, washed ones with 1X PBS and fixed for 4h with 4% paraformaldehyde at 4°C. Cells were hybridized in hybridization buffer (0.9 M sodium chloride, 0.01% w/v sodium dodecyl sulphate, 20 mM Tris–HCl) with 1.5 ng of each probe per microlitre, at 46°C for 2 h. After hybridization, cells were pelleted and washed with prewarmed hybridization buffer without probe for 30 min at 46 °C. The suspension was mixed with 200 µl 1× PBS and spotted onto microscope slides that were dried at 37°C and mounted with buffered glycerol 4% propyl gallate (80% glycerol, 4% n-propyl gallate, 0,02M Tris). The slides were examined using a laser-scanning confocal microscope SP8 (Leica, Wetzlar, Germany).

Whole mount fluorescence *in situ* hybridization (FISH)

Whole mount *in situ* hybridization was performed on reared *P. perniciosus*. Males, females and L4 larvae of *P. perniciosus* were cut near the last abdominal segment area utilizing a microclipper for dissection, fixed overnight in 4% parafolmaldehyde at 4 °C and preserved in ethanol/PBS 1:1 at - 20°C until processing. The samples were decolourized in 6% H_2O_2 for 30 minutes at room temperature and treated with 1.5 ng of the probe specific for *M. guilliermondii* complex MGU26S410_5'-FITC and of the universal yeast probe PF2_5'-CY3 (Kempf *et al.*, 2000) per microlitre in hybridization buffer (Xufre *et al.*, 2006) overnight at 42°C. After incubation, sand flies were washed with hybridization buffer without probe for 20 minutes at room temperature. Finally, 75 ng/ml of 4′,6-diamidino-2-phenylindole (DAPI) were added for nuclei detection and incubated for 15 min at RT. The treated samples were mounted on slides with buffered glycerol 4% propyl gallate. The same protocol was applied for negative controls. The slides were observed using a laser-scanning confocal microscope SP8 (Leica, Wetzlar, Germany).

Phylogenomic analysis of a strain of *M. guilliermondii* isolated from *P. perniciosus*

DNA extraction and genome sequencing

We selected a yeast strain, isolated from the whole body of a reared male specimen of *P. perniciosus*, previously identified as *M. guilliermondii* by sequencing the 26S rRNA region, for the genome sequencing. The yeast strain, here referred to as *M. guilliermondii* Pp, was grown on YM agar medium for 30 hours, as previously described. Total DNA was sequenced by an external company (Mr. DNA, Shallowater, USA) in one run of 2x150 paired-end reads on a HiSeq-2500 platform (Illumina). The assembled contigs are available on the European Nucleotide Archive under accession number GCA_900174495.1.

Reads assembly

After an assessment of the quality of the reads using FastQC (Andrews, 2010), the paired-end reads were assembled using the abyss-pe (Simpson *et al.*, 2009) *de novo*-assembler for short reads, setting all parameters at default values. The resulting contigs were filtered by length, retaining only contigs with a length greater than 1,000 nucleotides.

ORF calling

We performed yeast specific ORF calling using the software GeneMark (Besemer and Borodovsky, 2005) with an optional parameter for fungal sequences. The resulting putative genes were translated using transeq (Rice *et al.*, 2000; Goujon *et al.*, 2010). Putative genes were annotated using BLASTP (Camacho *et al.*, 2009) with a cut-off evalue of 0.000001 against a custom SwissProt database with the taxonomic filter "Diakarya" (Boeckmann *et al.*, 2003).

Phylogenetic study

Following a research on current literature, we identified a subset of yeast species representative of the Ascomycota phylum with a published genome sequence. We gathered 15 species (table S2), comprising the only published *M. guilliermondii* genome to date (strain ATCC 6260). Orthologous genes were retrieved using the OrthoMCL software (Li *et al.*, 2003). We selected from the resulting clusters of orthologs only genes present in a single copy in all species. A multiple sequence alignment was performed on each cluster of sequences using the ClustalO software (Sievers and Higgins, 2014). Alignments were cleaned with Gblock (Castresana, 2000), concatenated and used for a maximum likelihood phylogenetic analysis using raxml (Stamatakis, 2014) using 100 bootstrap.

Annotation and Comparative genomics

The genome of *M. guilliermondii* isolated from *P. perniciosus* (*M. guilliermondii* Pp) was compared with environmental *M. guilliermondii* ATCC 6260 and with *C. lusitaniae*, its closest relative on the phylogenetic tree. Species-specific and strain-specific cluster of orthologous genes were identified with OrthoMCL. Cluster of orthologs were manually curated to remove artifacts caused by repetitive elements. GO terms (Harris *et al.*, 2004) and putative domains were assigned to all three genomes with InterProScan5 (Jones *et al.*, 2014). The functional characterization of specific genes and genes shared between the three species was visualized using WEGO (Ye *et al.*, 2006). The genome of *M. guilliermondii* Pp was aligned to *M. guilliermondii* ATCC 6260 using progressiveMauve (Darling *et al.*, 2010) to visualize large-scale evolutionary events such as rearrangements and inversions and to check the synteny of the two genomes.

Fungal uricolytic activity

The uricolytic activity of two strains of *R. mucilaginosa, W. anomalus*, *Trichosporon* sp. and *M. guilliermondii* isolated from sand flies in this work was tested on modified YPU medium (10 g/l yeast extract, 10 g/l peptone, 4 g/l uric acid (UA), 20 g/l agar). Yeast strains were

maintained on YM agar until plating a single colony of each strain on YPU. After incubation at 26°C for 48 h, the degradation halo, suggesting uric acid utilization (Vera-Ponce de León *et al.*, 2016), was evaluated. The assay was performed three times for each tested isolate.

Bioinformatic analyses for prediction of uric acid degradation pathway of *M. guilliermondii*

Protein sequences of characterized Ascomycota uric acid catabolic enzymes were downloaded from Uniprot (UniProt Consortium, 2011). These proteins were queried against *M. guilliermondii* Pp predicted proteins using reciprocal BLASTP (Camacho *et al.*, 2009) searches.

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References

Akhoundi, M., Bakhtiari, R., Guillard, T., Baghaei, A., Tolouei, R., Sereno, D., *et al.* (2012) Diversity of the bacterial and fungal microflora from the midgut and cuticle of phlebotomine sand flies collected in North-Western Iran. PLOS ONE 7(11): e50259.

Alten, B., Maia, C., Afonso, M.O., Campino, L., Jiménez, M., González, E., *et al.* (2016) Seasonal Dynamics of Phlebotomine Sand Fly Species Proven Vectors of Mediterranean Leishmaniasis Caused by *Leishmania infantum.* PLOS Neglected Tropical Diseases 10(2): e0004458.

Andrews, S. (2010) FastQC: A quality control tool for high throughput sequence data. URL http://www.bioinformatics.bbsrc.ac.uk/projects/*fastqc*.

Ba, A.S., Guo, D.A., Norton, R.A., Phillips, S.A., and Nes, W.D. (1995) Developmental differences in the sterol composition of *Solenopsis invicta*. Arch Insect Biochem 29: 1-9.

Besemer, J., and Borodovsky, M. (2005) GeneMark: web software for gene finding in prokaryotes, eukaryotes and viruses. Nucleic Acids Res 33(suppl 2): W451-W454.

Blackwell, M. (2017) Made for Each Other: Ascomycete Yeasts and Insects. Microbiol Spectr 5(3).

Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M.C., Estreicher, A., Gasteiger, E., *et al.* (2003) The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003.

Nucleic Acids Res 31(1): 365-370. Bozic, J., Capone, A., Pediconi, D., Mensah, P., Cappelli, A., Valzano, M., *et al.* (2017) Mosquitoes can harbour yeasts of clinical significance and contribute to their environmental dissemination. Environ Microbiol Rep doi: 10.1111/1758-2229.12569.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. (2009) BLAST+: architecture and applications. BMC Bioinformatics 10: 421.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al.* (2010) QIIME allows analysis of high–throughput community sequencing data. Nat Methods 7: 335-336.

Cappelli, A., Ulissi, U., Valzano, M., Damiani, C., Epis, S., Gabrielli, M.G., *et al.* (2014) A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*. PLoS One. 9(5): e95988.

Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17(4): 540-552.

Cochran, D.G., Mullins, D.E., and Mullins, K.J. (1979) Cytological changes in the fat body of the American cockroach, *Periplaneta americana*, in relation to dietary nitrogen levels. Ann Entomol Soc Am 72: 197-205.

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., *et al*. (2008) Paternal transmission of symbiotic bacteria in malaria vectors. Curr Biol 18(23):R1087-1088.

Darling, A.E., Mau, B., and Perna, N.T. (2010) ProgressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PloS one 5(6): e11147.

Deshpande, V., Wang, Q., Greenfield, P., Charleston, M., Porras-Alfaro, A., Kuske, C.R., *et al.* (2016) Fungal identification using a Bayesian classifier and the Warcup training set of internal transcribed spacer sequences. Mycologia 108(1): 1-5.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19): 2460-2461.

Esquivel, C.J., Cassone, B.J., and Piermarini P.M. (2016) A de novo transcriptome of the Malpighian tubules in non-blood-fed and blood-fed Asian tiger mosquitoes *Aedes albopictus*: insights into diuresis, detoxification, and blood meal processing. PeerJ 4: e1784.

Ferreira, N., Belloch, C., Querol, A., Manzanares, P., Vallez, S., and Santos, A. (2010) Yeast microflora isolated from brazilian cassava roots: taxonomical classification based on molecular identification. Curr Microbiol 60: 287-293.

Gatesoupe, F.J. (2007) Live yeasts in the gut: natural occurrence, dietary introduction, and their effects on fish health and development. Aquaculture 267: 20-30.

Gibson, C.M., and Hunter, M.S. (2010) Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects. Ecol Lett 13(2): 223-234.

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., and Lopez, R. (2010) A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res 38 Suppl: W695-W699

Gusmão D.S., Santos, A.V., Marini, D.C., Bacci, M.Jr., Berbert-Molina, M.A., and Lemos, F.J. (2010) Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti* (Diptera: Culicidae) (L.) and dynamics of bacterial colonization in the midgut. Acta Trop 115(3): 275-281.

Gusmão, D.S., Santos, A.V., Marini, D.C., Russo Ede, S., Peixoto, A.M., Bacci, M.Jr., *et al.* (2007) First isolation of microorganisms from the gut diverticulum of *Aedes aegypti* (Diptera: Culicidae): new perspectives for an insect-bacteria association. Mem Inst Oswaldo Cruz 102(8): 919-924.

Harris, M.A., Clark, J., Ireland, A., Lomax, J., Ashburner, M., Foulger, R., *et al.* (2004) The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res 32(suppl 1): D258-D261.

Janson, E.M., Stireman, J.O., Singer, M.S., and Abbot, P. (2008) Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. Evolution 62(5): 997-1012.

Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., *et al.* (2014) InterProScan 5: genome-scale protein function classification. Bioinformatics 30(9): 1236-1240.

Kempf, V.A.J., Trebesius, K., and Autenrieth, I.B. (2000) Fluorescent in situ hybridization allows rapid identification of microorganisms in blood cultures. J Clin Microbiol 38: 830-838.

Killick-Kendrick, R. (1990) Phlebotomine vectors of the leishmaniases: a review. Med Vet Entomol 4(1): 1-24.

Kurtzman, C.P. (2014) Use of gene sequence analyses and genome comparisons for yeast systematics. Int J Syst Evol Microbiol 64.2: 325-332.

Kurtzman, C.P., and Fell, J.W. (2000) The yeasts; a taxonomic study, 4th edn. Amsterdam, Netherlands: Elsevier Science.

Lee, I.R., Yang, L., Sebetso, G., Allen, R., Doan, T.H., Blundell, R., *et al.* (2013) Characterization of the complete uric acid degradation pathway in the fungal pathogen *Cryptococcus neoformans*. PLoS One 8(5): e64292.

Léger, N., Pesson, B., Madulo-Leblond, G., and Abonnenc, E. (1983) Sur la différenciation des femelles du sous-genre Larroussius Nitzulescu, 1931 (Diptera-Phlebotomidae) de la ragion méditerranéenne. Ann Parasitol Hum Comp 58: 611–623.

Li, L., Stoeckert, C.J., and Roos, D.S. (2003) OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res 13(9): 2178-2189.

Loy, A., Arnold, R., Tischler, P., Rattei, T., Wagner, M., and Horn, M. (2008) ProbeCheck – a central resource for evaluating oligonucleotide probe coverage and specificity. Environ Microbiol 10: 2894-2898.

Maroli, M., Fiorentino, S., and Guandalini, E. (1987) Biology of a laboratory colony of *Phlebotomus perniciosus* (Diptera: Psychodidae). J Med Entomol 24(5): 547-551.

Martin, E., Bongiorno, G., Giovati, L., Montagna, M., Crotti, E., Damiani, C., *et al.* (2016) Isolation of a *Wickerhamomyces anomalus* yeast strain from the sandfly *Phlebotomus perniciosus*, displaying the killer phenotype. Med Vet Entomo 30(1):101-106.

Modi, G.B., and Tesh, R.B. (1983) A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. J Med Entomol 20(5): 568-569.

Molnar, O., Wuczkowski, M., and Prillinger, H. (2008) Yeast biodiversity in the guts of several pests on maize; comparison of three methods: classical isolation, cloning and DGGE. Mycol Prog 7(2): 111-123.

Moran, N.A., and Dunbar, H.E. (2006) Sexual acquisition of beneficial symbionts in aphids. Proc Natl Acad Sci USA 103(34): 12803-12806.

Nalepa, C.A., Bignell, D.E. and Bandi, C. (2001) Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. Insectes Sociaux 48(3): 194-201.

Nardon, P., and Grenier, A.M. (1989) Endosymbiosis in Coleoptera: biological, biochemical, and genetic aspects. In Insect Endocytobiosis: Morphology, Physiology, Genetics, Evolution. Schwemmler, W., and Gassner, G. (eds). Boca Raton: CRC Press, pp. 175-216.

Nguyen, N.H., Suh, S.O., and Blackwell, M. (2007) Five novel *Candida* species in insectassociated yeast clades isolated from *Neuroptera* and other insects. Mycologia 99(6): 842-858.

Papon, N., Savini, V., Lanoue, A., Simkin, A.J., Crèche, J., Giglioli-Guivarc'h, N., *et al.* (2013) *Candida guilliermondii*: biotechnological applications, perspectives for biological control, emerging clinical importance and recent advances in genetics. Curr Genet 59(3): 73-90.

Patiño-Navarrete, R., Piulachs, M.D., Belles, X., Moya, A., Latorre, A., and Peretó, J. (2014) The cockroach *Blattella germanica* obtains nitrogen from uric acid through a metabolic pathway shared with its bacterial endosymbiont. Biol Lett 10(7): 20140407.

Ricci, I., Damiani, C., Scuppa, P., Mosca, M., Crotti, E., Rossi, P., *et al.* (2011a) The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*. Environ Microbiol 13(4): 911-921.

Ricci, I., Mosca, M., Valzano, M., Damiani, C., Scuppa, P., Rossi, P., *et al.* (2011b) Different mosquito species host *Wickerhamomyces anomalus* (*Pichia anomala*): perspectives on vectorborne diseases symbiotic control. Antonie Van Leeuwenhoek 99(1): 43-50.

Rice, P., Longden, I., and Bleasby, A. (2000) EMBOSS: The European Molecular Biology Open Software Suite. Trends Genet 16(6): 276-277

Rivera, F.N., Gonzalez, E., Gomez, Z., Lopez, N., Hernandez-Rodriguez, C., Berkov, A., and Zuniga, G. (2009) Gut-associated yeast in bark beetles of the genus *Dendroctonus Erichson* (Coleoptera: Curculionidae: Scolytinae). Biol J Linn Soc 98(2): 325-342.

Sant'Anna, M.R., Diaz-Albiter, H., Aguiar-Martins, K., Al Salem, W.S., Cavalcante, R.R., Dillon, V.M., *et al.* (2014) Colonisation resistance in the sand fly gut: *Leishmania* protects *Lutzomyia longipalpis* from bacterial infection. Parasit Vectors 23(7): 329.

Schlein, Y., Polacheck, I., and Yuval, B. (1985) Mycoses, bacterial infections and antibacterial activity in sand flies (Psychodidae) and their possible role in the transmission of leishmaniasis. Parasitology 90: 57-66.

Sievers, F., and Higgins, D.G. (2014) Clustal Omega, accurate alignment of very large numbers of sequences. Methods Mol Biol 1079: 105-116.

Simpson, J.T., Wong, K., Jackman, S.D., Schein, J.E., Jones, S.J., and Birol, I. (2009) ABySS: a parallel assembler for short read sequence data. Genome Res 19(6): 1117-1123.

Singh, S.R., and Hou, S.X. (2009) Multipotent stem cells in the Malpighian tubules of adult *Drosophila melanogaster*. J Exp Biol 212: 413-423.

Spatafora, J.W., Sung, G.H., Johnson, D., Hesse, C., O'Rourke, B., Serdani, M., *et al*. (2006) A five-gene phylogeny of Pezizomycotina. Mycologia 98(6):1018-1028.

Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312-1313.

Theodor, O. (1958) Psychodidae–Phlebotomine. In Die Fliegen der Palaearktischen Region, 9c. Lindner, E. (ed). Stuttgart: E. Schweizerbart'sche Verlagsbuchhandlung, pp.1–55.

UniProt Consortium (2011) Reorganizing the protein space at the Universal Protein Resource (UniProt). Nucleic Acids Res 40: D71-D75.

Valovage, W.D., and Brooks, M.A. (1979) Uric acid quantities in the fat body of normal and aposymbiotic German cockroaches, *Blattella germanica*. Ann Entomol Soc Am 72: 687-689.

Varotto Boccazzi, I., Ottoboni, M., Martin, E., Comandatore, F., Vallone, L., Spranghers, T., Eeckhout, M., Mereghetti, V., Pinotti, L., Epis, S. (2017) A survey of the mycobiota associated with larvae of the black soldier fly (*Hermetia illucens*) reared for feed production. PLOS ONE: 12(8):e0182533.

Vera-Ponce de León, A., Sanchez-Flores, A., Rosenblueth, M., and Martínez-Romero, E. (2016) Fungal community associated with *Dactylopius* (Hemiptera: Coccoidea: Dactylopiidae) and its role in uric acid metabolism. Front Microbiol 7: 954.

Vishniac, H.S., and Johnson, D.T. (1990) Development of a yeast flora in the adult green June beetle (*Cotinis nitida*, Scarabaeidae). Mycologia 82: 471-479.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol 73(16): 5261-5267.

Wang, Y., Gilbreath, T.M. III, Kukutla, P., Yan, G., and Xu, J. (2011) Dynamic Gut Microbiome across Life History of the Malaria Mosquito *Anopheles gambiae* in Kenya. PLOS ONE 6(9): e24767.

Wang, L., Yue, L., Chi, Z., and Wang, X. (2008) Marine killer yeasts active against a yeast strain pathogenic to crab *Portunus trituberculatus*. Dis Aquat Organ 80(3): 211-218.

White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A guide to Methods and Applications. Innis, M.A., Gelfand, D.H., Sninsky, J.J and White, T.J. (eds). San Diego, USA: Academic Press, pp. 315-322.

Xufre, A., Albergaria, H., Inácio J., Spencer-Martins, I., and Gírio, F. (2006) Application of fluorescence in situ hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. Int J Food Microbiol 108: 376-384.

Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., *et al.* (2006) WEGO: a web tool for plotting GO annotations. Nucleic Acids Res 34(suppl 2): W293-W297.

Zacchi, I., and Vaughn-Martini, A. (2002) Yeasts associated with insects in agricultural areas of Perugia, Italy. Annu Rev Microbiol 52: 237-244.

Zindel, R., Gottlieb, Y., and Aebi, A. (2011) Arthropod symbioses: a neglected parameter in pest- and disease-control programmes. J Appl Ecol 48: 864-872.

2.2. Article 2

The genomes of four *Meyerozyma caribbica* isolates and novel insights into the *Meyerozyma guilliermondii* species complex

GENOME REPORT

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The genomes of four *Meyerozyma caribbica* isolates and novel insights into the *Meyerozyma guilliermondii* species complex

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DATA REFERENCE NUMBERS

Reads are available in the European Nucleotide Archives (https://www.ebi.ac.uk/ena) under the accession numbers ERX2126952-5. The assembled contigs of each genome are available at ENA under accession numbers GCA_900231965, GCA_900231995, GCA_900232055, and GCA_900232065.

RUNNING TITLE Genomes of four *Meyerozyma caribbica*

KEYWORDS *Meyerozyma caribbica*, Genome Report, *Drosophila suzukii*, *Culex quinquefasciatus*,

Anopheles stephensi, *Aedes aegypti*

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ABSTRACT

Yeasts of the *Meyerozyma guilliermondii* species complex are widespread in nature and can be isolated from a variety of sources, from the environment to arthropods to hospital patients. To date, the species complex comprises the thoroughly studied and versatile *M. guilliermondii*, and the hard to distinguish *M. caribbica*, and *Candida carpophila*. Here we report the whole-genome sequencing and *de novo* assembly of four *M. caribbica* isolates, identified with the most recent molecular techniques, derived from four Diptera species. The four novel assemblies present reduced fragmentation and comparable metrics (genome size, gene content) to the available genomes belonging to the species complex. We performed a phylogenomic analysis comprising all known members of the species complex, to investigate evolutionary relationships within this clade. Our results show a compact phylogenetic structure for the complex and indicate the presence of a sizable core set of genes. Furthermore, *M. caribbica*, despite a broad literature on the difficulties of discerning it from *M. guilliermondii*, seems to be more closely related to *C. carpophila*. Finally, we believe that there is evidence for considering these four genomes the first published for the species *M. caribbica*. Raw reads and assembled contigs have been made public to further the studies on these organisms.

INTRODUCTION

Meyerozyma caribbica (anamorph *Candida fermentati*) and *Meyerozyma guilliermondii* (anamorph *Candida guilliermondii*) are two closely related yeast species belonging to the *M. guilliermondii* species complex [Bai et al. 2000; Vaughan-Martini et al. 2005]. *M. guilliermondii* has been object of several studies, with a broad bibliography describing its multiple interesting properties and applications [Papon et al. 2013], and is extensively used in biotechnology in a variety of tasks. *M. guilliermondii* is employed in riboflavin production [Tanner Jr et al. 1945], bioconversion of xylose into xylitol [Zhou et al. 2010], and is a promising source of enzymes [Gong et al. 2007] and biofuel [Wang et al. 2012]. Moreover, it is considered a killer yeast, having a broad range anti-microbial activity against bacteria [Zhao et al. 2010], fungi [Coda et al. 2013], and even protozoa [Dantan- Gonzalez et al. 2015]. This has led to its use as a biocontrol agent in agriculture and food industry [Wisniewski et al. 1991;

Hashem et al. 2011]. Another killer yeast species, *Wickerhamomyces anomalus*, has been suggested as possible candidate for integrated vector control [Ricci et al. 2011; Martin et al, 2016]; interestingly, yeasts of the *M. guilliermondii* clade possess a similar antimicrobial activity and can be found in insect hosts as well, opening the possibility of envisioning similar approaches. Although *C. guilliermondii,* the anamorph of *M. guilliermondii*, is considered safe and classified as a biosafety level 1 organism, it has been described as an occasional opportunistic pathogen in immunocompromised patients [Pflaller et al. 2006]. It is estimated to be the sixth most frequent nosocomial yeast [Pflaller et al. 2006], causing more than 11% of all episodes of systemic candidiasis [Girmenia et al. 2006].

The *Meyerozyma* species complex belongs to the Saccharomycotina CTG clade, a group of yeasts which has been thoroughly studied in the last 40 years, however its fine phylogenetic structure remains unclear. Taxonomy has been traditionally ruled by phenotypic (e.g. morphologic and metabolic) features, making it a challenging task in yeasts due to the paucity of discriminative morphological characters. More recently, molecular features (e.g. Single Nucleotide Polimorphysms in a single gene/sets of genes) have been widely adopted by taxonomists in general, and this shift clarified a number of phylogenetic relationships in all Kingdoms of life, including Fungi [Kurtzman, Cletus P. 1994]. Yeast taxonomy however remains a complicated matter, with multiple synonyms for each species and an exception to the "one species one name" rule concerning yeasts in different sexual stages (teleomorph/anamorph) [Taylor 2011].

Specifically, *M. guilliemondii*, formerly known as *Pichia guilliermondii*, has recently been placed into its own genus [Kurtzman 2010] and it is thought to form a species complex with close relatives *M. caribbica* and *Candida carpophila* (no known teleomorph) [Vaughan-Martini et al. 2005]. Furthermore, given its emerging pathogen status, it is important to be able to correctly identify yeasts belonging to this species complex, particularly *M. guilliermondii* and *M. caribbica*, the latter of which is less frequent and does not seem to present antibiotic resistance [Pfaller et al. 2006]. Since a morphological identification within the complex is impossible, several molecular protocols using microsatellites, internal transcribed spacer (ITS) polymorphisms, and ITS restriction fragment length polymorphism (RFLP) fingerprinting, have been developed [Romi et al. 2014, Merseguel et al. 2015 , Wrent et al. 2016].

We isolated four yeast strains from the gut of four different diptera species, namely *Drosophila suzukii*, *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti*. First, we carefully

identified them at the species level with RFLP fingerprinting of the ribosomal ITS, one of the most effective molecular protocols for the task [Romi et al. 2014]. We then performed whole genome sequencing and we *de novo* assembled the reads. The resulting genomes were employed for phylogenomic and comparative genomic analyses.

The objective of this study was twofold, first to examine the genomes of arthropodassociated yeasts, second to draw a comprehensive phylogenetic picture of the *M. guilliermondii* species complex exploiting whole genome data. To do so, we integrated our dataset with the reference genomes of the three members of the species complex and one close relative, *Clavispora lusitaniae*, completing it with an already published genome of *Phlebotomus*-associated *M. guilliermondii* [Martin et al. submitted].

Here we describe these four novel genomes in the context of the *M. guilliermondii* species complex, make them available to the public, and discuss the phylogenetic implications of our results.

METHODS

Yeast isolation and characterization

All arthropod samples employed in this study derive from insect colonies maintained at the University of Camerino and at the University of Torino. Diptera were maintained in cages at standard conditions of temperature, humidity and photoperiod, as previously reported (Ricci et al, 2011, Vacchini et al, 2017). The yeast strains derived from the three mosquito species (isolates Clone C2, Clone 8, and Clone 1, respectively from *C. quinquefasciatus*, *An. stephensi,* and *Ae. aegypti*), were isolated following a published protocol (Bozic et al. 2017). Briefly, homogenised mosquitoes guts were pre-inoculated in YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and suspended in saline solution before plating on selective media 6.5% Sabouraud (Sabouraud powder prepared by the manufacturer, BDR Sabouraud Dextrose Agar) with Rifampicin 40µg/ml. Isolate AF2.6.P.231 was obtained from an adult individual of *D. suzukii*. After surface sterilization by washing once with ethanol and twice with deionized water, serial dilutions of the insect homogenate were plated on Potato Dextrose Agar (PDA). Once growth was visible, a colony was purified on solid PDA for three times and then conserved at -80°C. For identification, DNA was extracted from the isolate using boiling lysis (Marasco et al., 2012).

Genomic DNA was extracted from individual yeast samples grown in YPD medium, using JetFlex Genomic DNA Purification Kits (Genomed, Lohne, Germany). Quantity and quality of the recovered DNAs were checked by spectrophotometer and stored at − 20 °C. After incubation for 48h at 28°C, yeast colonies were subjected to PCR to amplify a polymorphic fragment of the 18S rRNA gene using oligos yeast-F1 and yeast-R1 (Ricci et al., 2011) or a region comprising the 5.8 rRNA gene and the two sideward regions, ITS1 and ITS2 using primers ITS1F and ITS4 (Manter and Vivanco, 2007). Subsequently the amplification products were sequenced and BLAST was used to characterize the isolated yeasts at the genus level.

Restriction Fragment Length Polymorphism (RFLP) was performed on all isolates to specifically discriminate between *M. guilliermondii* and *M. caribbica*, as previously described (Romi et al., 2014). Briefly PCR amplification using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TC CTCCGCTTATTGATATGC-3′) was carried out to amplify a polymorphic ITS fragment. The PCR product (4 μL) was digested with 5 U of TaqI (Promega, Madison, USA) in a 10 μL reaction volume at 65°C for 2 hours as per manufacturer's instructions. The restriction patterns were analyzed by electrophoresis of the 10 μL reaction volume on 2.0% (w/v) agarose gel.

Sequencing, assembly and annotation

Total DNA was sequenced by an external company (Mr. DNA, Shallowater, USA) in one run of 2x150 paired-end reads on a HiSeq-2500 platform (Illumina). Reads are available in the European Nucleotide Archives (https://www.ebi.ac.uk/ena) under the accession numbers ERX2126952-5.

Quality of the raw reads was assessed for each sample using FastQC [Simon 2010]. De novo assembly was performed using SPAdes version 3.8.2 [Bankevich et al. 2012] employing different k-mer lengths (21, 33, 55, 77, 99, 111), setting the --cov-cutoff parameter to auto, and using the --careful option. The assembled contigs of each genome are available at ENA under accession numbers GCA_900231965, GCA_900231995, GCA_900232055, GCA_900232065 respectively for *C. quinquefasciatus*, *An. stephensi, D. suzukii*, and *Ae. aegypti*. All genomes analyzed in this study, newly sequenced and reference ones, were annotated with the following procedure. First, gene calling was performed using GeneMark-ES Suite version 4.32 [Ter-Hovhannisyan et al. 2008] with the parameter --min_contig set to 10000 and using the --fungus option. Then, Clusters of Orthologous Groups (COGs) [Tatusov et al. 2000] were assigned to the obtained translated genes by the COGnitor software [Tatusov et al. 2000] .

Genomic analysis

The genomes of members of the *M. guilliermondii* species complex and of *C. lusitaniae* were retrieved from NCBI with the following GeneBank assembly accessions: *M. guilliermondii* (GCA_000149425.1 and GCA_900174495.1), *M. caribbica* (GCA_000755205.1), *C. carpophila* (GCA_001599235.1), *C. lusitaniae* (GCA_000003835.1).

Orthogroups were inferred with Orthofinder 1.1.4 [Emms et al. 2015] from the predicted sets of proteins. Single Copy Orthogroups (SCO) were then selected, defined as orthogroups with exactly one protein in all samples, with a custom Python script. Each SCO was aligned using MUSCLE 3.8.31[Edgar et al. 2004] and consecutively tested each Multiple Sequence Alignment (MSA) for recombination using the software PhiPack [Bruen et al. 2005]. A SCO group was considered not having signs of recombination if it passed all three tests run by PhiPack. Then, non-recombinant SCO MSAs were polished with Gblocks 0.91b [Castresana 2000] and concatenated with a custom Python script. Finally, the concatenated alignment was used as input for RAxML version8.2.8 [Stamatakis 2014] under the PROTCAT approximation, using the LG substitution matrix and 100 bootstrap replicates. A comparative genomic approach was designed, integrating the functional and phylogenetic data obtained. Phylogenetic clades were analyzed for COG content using inhouse Python scripts.

RESULTS AND DISCUSSION

Yeast isolation and characterization

Yeasts were isolated from four insect species and characterized at the species complex level using PCR and Sanger sequencing. 18S fragments were sequenced for the three yeasts isolated from mosquitoes, were identical and presented 99% sequence identity with an 18S gene belonging to *M. guilliermondii*, GenBank accession KX258468.1. For what concerns the yeast isolated from *D. suzukii*, a fragment of the ITS was sequenced, showing a 100% sequence identity with sequence KU216711.1 of *M. guillermondi*. These results allowed to identify the isolates at the genus level, however, in order to discriminate between *M. guilliermondii* and *M. caribbica*, a specific RFLP protocol [Romi et al. 2014] was performed, which clearly showed that all novel isolates exhibit the restriction fragment pattern typical of *M. caribbica*. The same RFLP protocol was performed on the yeast derived from *P. perniciosus* (characterized in Martin et al submitted) confirming its identification as *M. guilliermondii*.

Assembly and annotation

Raw reads were high quality for each of the samples analyzed. The four novel draft genomes present a reduced amount of fragmentation (from 43 to 144 contigs longer than 1000bp) and are sized coherently compared to the reference genomes of the *Meyerozyma* genus (table 1). It has to be noted that, while the two reference genomes were sequenced with a combination of long and short reads, we only employed a paired-end short reads library thus obtaining a larger amount of contigs. Nevertheless, GeneMark-ES, a selftraining gene calling algorithm, predicted a comparable amount of genes (from 5111 to 5774) for all genomes analyzed (table 1), including the reference genomes. Additionally, COGnitor assigned a similar number of COGs (from 3288 to 3598) to a similar number of unique genes (from 3029 to 3312) for all sets of predicted proteins (table 1).

Species	Isolate	Genome size – bp	Contigs #	Genes #	Genes with COG
M. guilliermondii	ATCC6260	10609954	9	5401	3312
M. caribbica	MG20W	10609282	9	5390	3305
C. carpophila	JCM9396	10242926	10	5296	3219
C.lusitaniae	ATCC42720	12114892	9	5111	3029
M. guilliermondii	*P. perniciosus*	10642597	31	5487	3362
M. caribbica	*D. melanogaster* *	10387257	43	5367	3242
M. caribbica	*Cx. quinquefasciatus* *	10553449	51	5453	3301
M. caribbica	*An. stephensi* *	11040470	144	5774	3481
M. caribbica	*Ae. aegypti* *	10347015	48	5359	3237

Table 1. Assembly and annotation statistics of the four novel genomes (marked with an asterisk) and of the published genomes used for comparative analysis; contigs shorter than 1kbp were discarded.

Genomic analysis

All analyzed genomes show high similarity for what concerns the inferred orthogroups. Almost the totality of proteins (98.7%) was assigned to an orthogroup; moreover, out of a total 5371 orthogroups, 5142 had at least one protein in each genome of the *M. guilliermondii* species complex (all genomes analyzed except the outgroup *C. lusitaniae*), while 4050 had all species represented, indicating the presence of a strong core set of genes.

We retrieved 3408 SCOs which were then aligned with MUSCLE and tested for recombination with PhiPack. We retained 2147 non recombining SCOs and processed their MSAs with Gblocks. The polished MSAs were concatenated obtaining a 870,212 bp long alignment. We

used this final MSA as input for RAxML, obtaining a phylogenetic tree with 100% bootstrap support for all branches (figure 1). Four clear clades can be seen in the tree: 1) *C. lusitaniae* as the outermost single species clade; 2) the reference genomes of *M. guilliermondii* and *M. caribbica* clustering together with the *M. guilliermondii* genome isolated from *P. perniciosus* in a clade with reduced branch lengths; 3) *C. carpophila* as a single species clade; 4) all novel *M. caribbica* genomes clustering together in a clade with reduced branch lengths, closer to the *C. carpophila* clade than to the reference *M. guilliermondii*/*M. caribbica* clade.

This is the first phylogenetic study attempting to describe the *M. guilliermondii* species complex employing Whole Genome Sequencing data. The genome of *M. guilliermondii* has already been included in a phylogenomic analysis in a study which, among other things, clarifies its position inside the CTG clade [Butler et al. 2009]. In this work we expand the genomic dataset of the *M. guilliermondii* species complex, including genomes of *M. caribbica* and *C. carpophila*. Literature on these two species is scarce compared to *M. guilliermondii* and past phylogenetic inferences were based on single or few genes and did not include all members of the species complex, leaving doubts about fine characterization [Kurtzman et al. 2010, 2013]. Considering these facts, it would have been presumptuous to make assumptions on the phylogenetic structure of the complex.

Our phylogenetic tree has one glaring issue: the position of the reference genome of *M. caribbica* (figure 1). At first sight, its closeness to the genome of *M. guilliermondii* would not be suspicious since they share the genus name and they are notoriously difficult to differentiate, even with traditional molecular markers. The aforementioned difficulty to discern the two species, in our opinion, is the key to solve this phylogenetic enigma, considering two facts: 1) the reference genome of *M. caribbica* is sister group to the genome of *M. guilliermondii* isolated from *P. perniciosus*; 2) our genomes of *M. caribbica* isolated from arthropods form a distinct clade, closer to *C. carpophila*. If we accept the identification as *M. caribbica* for the isolate previously sequenced [Kim et al. 2015], it would seem that two distinct *M. caribbica* exist: one is phylogenetically indistinguishable from *M. guilliermondii* while the other forms a distinct clade, sister group to *C. carpophila*. We think that the most likely and parsimonious explanation is however that the reference genome of *M. caribbica* has been misidentified and actually belongs to *M. guilliermondii*.

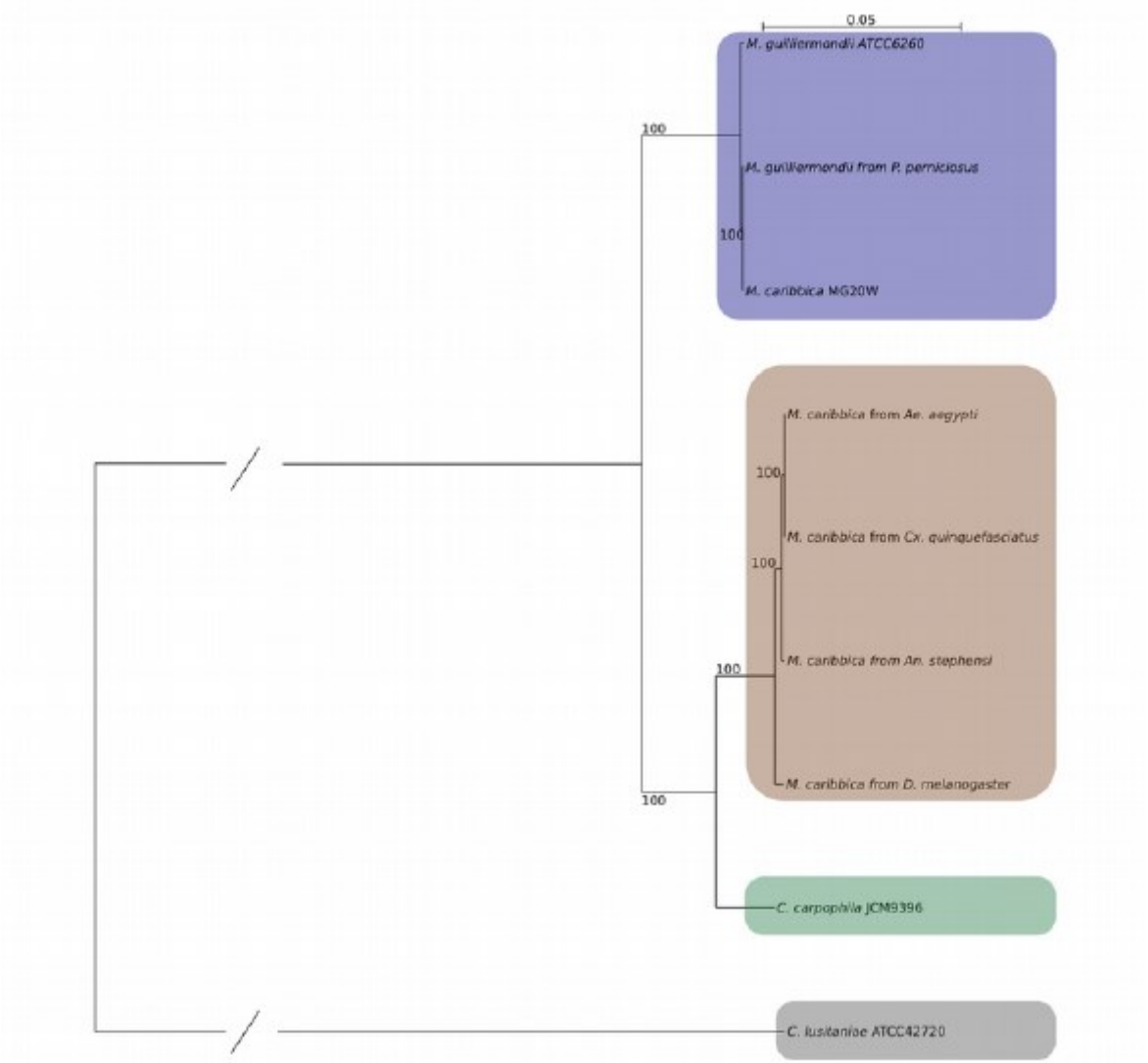

Figure 1. Maximum likelihood tree. Resulting clades are highlighted: *M. guilliermondii* (blue), *M. caribbica* (orange), *C. carpophila* (green), *C. lusitaniae* (grey).

As a follow-up to the phylogeny, the COG content of the four described clades was compared (figure 2). In total, 1093 COGs are shared between the four clades, constituting the vast majority of COGs assigned to each clade. This result confirms the evolutionary similarity of all analyzed genomes and a probable corresponding functional closeness. The outgroup, *C. lusitaniae*, holds the most unique COGs at 145 followed by the *M. caribbica* clade (128); the *M. guilliermondii* clade has a comparable number of unique COGs (107) while *C. carpophila* presents the least with 57. We think that this results confirm the phylogenetic distance between the *M. caribbica* clade and *M. guilliermondii* clade observed in the phylogenetic tree.

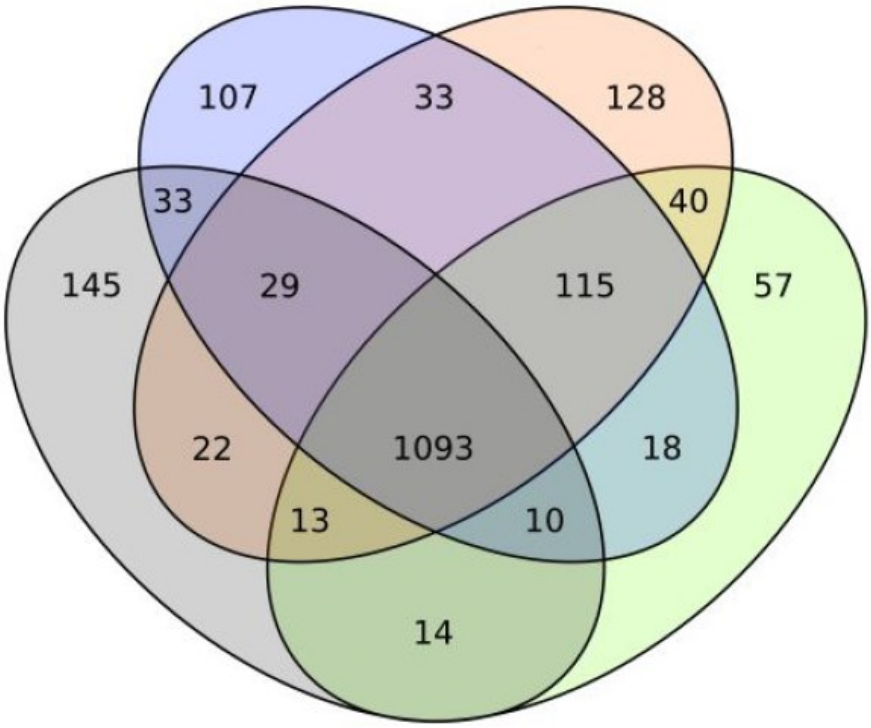

Figure 2. Venn diagram representing COG content in the clades resulting from the phylogenomic analysis: *M. guilliermondii* (blue), *M. caribbica* (orange), *C. carpophila* (green), *C. lusitaniae* (grey).

CONCLUSIONS

Yeasts of the *M. guilliermondii* species complex are relevant for multiple reasons. They are widely used in industry due to their useful properties, have recently emerged as nosocomial pathogens and due to their proprieties they can be envisioned as potential tools for the control of arthropod-borne diseases. Our study provides useful genomic resources and a detailed phylogenomic analysis of this species complex, providing novel insights into the evolutionary history of these yeasts. Additionally, we provide to the public four novel genomes belonging to *M. caribbica*, arguably the first of this species.

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LITERATURE CITED

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.

Bai, F. Y., Liang, H. Y., & Jia, J. H. (2000). Taxonomic relationships among the taxa in the *Candida guilliermondii* complex, as revealed by comparative electrophoretic karyotyping. International journal of systematic and evolutionary Microbiology, 50(1), 417-422.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of computational biology, 19(5), 455-477.

Bozic, J., Capone, A., Pediconi, D., Mensah, P., Cappelli, A., et al. (2017). Mosquitoes can harbour yeasts of clinical significance and contribute to their environmental dissemination. Environmental microbiology reports.

Bruen, T., & Bruen, T. (2005). PhiPack: PHI test and other tests of recombination. McGill University, Montreal, Quebec.

Butler, G., Rasmussen, M. D., Lin, M. F., Santos, M. A., Sakthikumar, S., et al. (2009).

Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature, 459(7247), 657-662.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular biology and evolution, 17(4), 540-552.

Coda, R., Rizzello, C. G., Di Cagno, R., Trani, A., Cardinali, G., et al. (2013). Antifungal activity of *Meyerozyma guilliermondii*: identification of active compounds synthesized during dough fermentation and their effect on long-term storage of wheat bread. Food microbiology, 33(2), 243-251.

Dantan-Gonzalez, E., Quiroz-Castaneda, R. E., Cobaxin-Cardenas, M., Valle-Hernandez, J., Gama-Martinez, Y., et al. (2015). Impact of *Meyerozyma guilliermondii* isolated from chickens against *Eimeria sp*. protozoan, an in vitro analysis. BMC veterinary research, 11(1), 278.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research, 32(5), 1792-1797.

Emms, D. M., & Kelly, S. (2015). OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome biology, 16(1), 157.

Ferreira, N., Belloch, C., Querol, A., Manzanares, P., Vallez, S., et al. (2010). Yeast microflora isolated from brazilian cassava roots: taxonomical classification based on molecular identification. Current microbiology, 60(4), 287-293.

Girmenia, C., Pizzarelli, G., Cristini, F., Barchiesi, F., Spreghini, E., et al. (2006). *Candida guilliermondii* fungemia in patients with hematologic malignancies. Journal of clinical microbiology, 44(7), 2458-2464.

Gong, F., Sheng, J., Chi, Z., & Li, J. (2007). Inulinase production by a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the crude inulinase. Journal of industrial microbiology & biotechnology, 34(3), 179-185.

Hashem, M., & Abo-Elyousr, K. A. (2011). Management of the root-knot nematode *Meloidogyne incognita* on tomato with combinations of different biocontrol organisms. Crop Protection, 30(3), 285-292.

Kim, J. S., Baek, J. H., Park, N. H., & Kim, C. (2015). Complete genome sequence of halophilic yeast *Meyerozyma caribbica* MG20W isolated from rhizosphere soil. Genome announcements, 3(2), e00127-15.

Kurtzman, C. P. (1994). Molecular taxonomy of the yeasts. Yeast, 10(13), 1727-1740.

Kurtzman, C. P., & Suzuki, M. (2010). Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*. Mycoscience, 51(1), 2-14.

Kurtzman, C. P., & Robnett, C. J. (2013). Relationships among genera of the *Saccharomycotina* (*Ascomycota*) from multigene phylogenetic analysis of type species. FEMS yeast research, 13(1), 23-33.

Manter, D. K., & Vivanco, J. M. (2007). Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. Journal of Microbiological Methods, 71(1), 7-14.

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., et al. (2012). A drought resistance-promoting microbiome is selected by root system under desert farming. PLoS One, 7(10), e48479.

Martin, E., Bongiorno, G., Giovati, L., Montagna, M., Crotti, E., et al. (2016). Isolation of a *Wickerhamomyces anomalus* yeast strain from the sandfly *Phlebotomus perniciosus*, displaying the killer phenotype. Medical and veterinary entomology, 30(1), 101-106.

Martin, E., Varotto Boccazzi, I., De Marco, L., Bongiorno, G., Montagna, M., et al. The mycobiota of the sand fly *Phlebotomus perniciosus*: involvement of yeast 2 symbionts in uric acid metabolism. Submitted.

Merseguel, K. B., Nishikaku, A. S., Rodrigues, A. M., Padovan, A. C., e Ferreira, R. C., et al. (2015). Genetic diversity of medically important and emerging *Candida* species causing invasive infection. BMC infectious diseases, 15(1), 57.

Papon, N., Savini, V., Lanoue, A., Simkin, A. J., Creche, J., et al. (2013). *Candida guilliermondii*: biotechnological applications, perspectives for biological control, emerging clinical importance and recent advances in genetics. Current genetics, 59(3), 73-90.

Pfaller, M. A., Diekema, D. J., Mendez, M., Kibbler, C., Erzsebet, P., et al. (2006). *Candida guilliermondii*, an opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. Journal of clinical microbiology, 44(10), 3551-3556.

Ricci, I., Mosca, M., Valzano, M., Damiani, C., Scuppa, P., et al. (2011). Different mosquito species host *Wickerhamomyces anomalus* (*Pichia anomala*): perspectives on vector-borne diseases symbiotic control. Antonie Van Leeuwenhoek, 99(1), 43-50.

Romi, W., Keisam, S., Ahmed, G., & Jeyaram, K. (2014). Reliable differentiation of *Meyerozyma guilliermondii* from *Meyerozyma caribbica* by internal transcribed spacer restriction fingerprinting. BMC microbiology, 14(1), 52.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics, 30(9), 1312-1313.

Tanner Jr, F., Vojnovich, C., & Van Lanen, J. M. (1945). Riboflavin production by *Candida* species. Science (Washington), 101, 180-161.

Tatusov, R. L., Galperin, M. Y., Natale, D. A., & Koonin, E. V. (2000). The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic acids research, 28(1), 33-36.

Taylor, J. W. (2011). One fungus= one name: DNA and fungal nomenclature twenty years after PCR. IMA fungus, 2(2), 113-120.

Ter-Hovhannisyan, V., Lomsadze, A., Chernoff, Y. O., & Borodovsky, M. (2008). Gene prediction in novel fungal genomes using an ab initio algorithm with unsupervised training. Genome research, 18(12), 1979-1990.

Vacchini, V., Gonella, E., Crotti, E., Prosdocimi, E. M., Mazzetto, F., et al. (2017). Bacterial diversity shift determined by different diets in the gut of the spotted wing fly *Drosophila suzuki*i is primarily reflected on acetic acid bacteria. Environmental microbiology reports, 9(2), 91-103.

Vaughan-Martini, A., Kurtzman, C. P., Meyer, S. A., & O'Neill, E. B. (2005). Two new species in the *Pichia guilliermondii* clade: *Pichia caribbica sp. nov*., the ascosporic state of *Candida fermentati*, and *Candida carpophila comb. nov*. FEMS yeast research, 5(4-5), 463-469.

Wang, G. Y., Chi, Z., Song, B., Wang, Z. P., & Chi, Z. M. (2012). High level lipid production by a novel inulinase-producing yeast *Pichia guilliermondii* Pcla22. Bioresource technology, 124, 77-82.

Wisniewski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C., et al. (1991). Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I. Characterization of attachment to *Botrytis cinerea*. Physiological and Molecular Plant Pathology, 39(4), 245- 258.

Wrent, P., Rivas, E. M., Peinado, J. M., & de Siloniz, M. I. (2016). Development of an affordable typing method for *Meyerozyma guilliermondii* using microsatellite markers. International journal of food microbiology, 217, 1-6.

Zhao, J., Mou, Y., Shan, T., Li, Y., Zhou, L., et al. (2010). Antimicrobial metabolites from the endophytic fungus *Pichia guilliermondii* isolated from *Paris polyphylla* var. yunnanensis. Molecules, 15(11), 7961-7970.

Zou, Y. Z., Qi, K., Chen, X., Miao, X. L., & Zhong, J. J. (2010). Favorable effect of very low initial k L a value on xylitol production from xylose by a self-isolated strain of *Pichia guilliermondii*. Journal of bioscience and bioengineering, 109(2), 149-152.

2.3. Article 3

Isolation of a *Wickerhamomyces anomalus* yeast strain from the sandfly *Phlebotomus perniciosus*, displaying the killer phenotype

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SHORT COMMUNICATION

Isolation of a *Wickerhamomyces anomalus* yeast strain from the sandfly *Phlebotomus perniciosus*, displaying the killer phenotype

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Abstract.

The yeast *Wickerhamomyces anomalus* has been studied for its wide biotechnological potential, mainly for applications in the food industry. Different strains of *W. anomalus* have been isolated from diverse habitats and recently from insects, including mosquitoes of medical importance. This paper reports the isolation and phylogenetic characterization of *W. anomalus* from laboratory-reared adults and larvae of *Phlebotomus perniciosus* (Diptera: Psychodidae), a main phlebotomine vector of human and canine leishmaniasis. Of 65 yeast strains isolated from *P. perniciosus*, 15 strains were identified as *W. anomalus*; one of these was tested for the killer phenotype and demonstrated inhibitory activity against four yeast sensitive strains, as reported for mosquito-isolated strains. The association between *P. perniciosus* and *W. anomalus* deserves further investigation in order to explore the possibility that this yeast may exert inhibitory/killing activity against *Leishmania* spp.

Key words. *Leishmania*, biocontrol, phlebotomine sandflies, yeast community.

Wickerhamomyces anomalus (also known as *Pichia anomala* or *Hansenula anomala*) is a Saccharomycetes yeast of great interest for its biotechnological applications. Like other yeast species, strains of *W. anomalus* are known to produce killer toxins (*Wa*KT) with antimicrobial activity against fungi, including other yeasts, and bacteria (Passoth *et al.*, 2011). Given these characteristics, strains of *W. anomalus* have been exploited in food production and preservation, environmental bioremediation, and the production of therapeutic molecules and biofuel (Walker, 2011). Although some cases of human infection caused by *W. anomalus* have been reported (Kalkanci *et al.*, 2010; Oliveira *et al.*, 2014), this yeast can only be considered as an opportunistic pathogen (Epis *et al.*, 2015) and is classed at biosafety level 1 by the European Food Safety Authority (De Hoog, 1996).

Wickerhamomyces anomalus has been isolated from different substrates and habitats, including fruits, plants, dairy and baked food products, cereal silos, oil-contaminated soil and marine environments (Walker, 2011). Recently, *W. anomalus* was also isolated from arthropods, in particular from *Drosophila* sp. (Diptera: Drosophilidae) (Zacchi & Vaughan-Martini, 2002), *Doubledaya bucculenta* beetles (Coleoptera: Erotylidae) (Toki *et al.*, 2012) and from different mosquito species (Ricci *et al.*, 2011a). Interestingly, one of the strains isolated from the malaria mosquito vector *Anopheles stephensi* (Diptera: Culicidae) has been shown to produce a killer toxin active against other yeast species (Cappelli *et al.*, 2014). This result may suggest a potential involvement of *W. anomalus* in protection against pathogens in mosquitoes, as previously reported for the crab *Portunus trituberculatus* (Decapoda: Portunidae) (Wang *et al.*, 2008).

The present paper reports the isolation and characterization of strains of *W. anomalus* from laboratory-reared *Phlebotomus perniciosus*, the main phlebotomine vector in the western Mediterranean area of the protozoan parasite *Leishmania infantum* (Trypanosomatida: Trypanosomatidae), the causative agent of canine and human visceral leishmaniasis (Killick-Kendrick, 1990).

A laboratory colony of *P. perniciosus*, originating from samples collected in the Madrid area (Spain) and established at the Institute of Health Carlos III of Madrid in 1983, was employed. The colony has been maintained since June 2012 at the Unit of Vector-Borne Diseases of Istituto Superiore di Sanità, Rome, and at the time of this study was in its 27th generation. Adults were routinely maintained in thin mesh cages in thermostat-controlled cabinets at

standard conditions of temperature (28±1 ∘C) and relative humidity (95–100%), under a photoperiod of LD 7 : 17 h and were provided with 30% sugar solution daily (Maroli *et al.*, 1987). Larvae were grown in plaster jars under the same standard conditions and were supplemented with larval food according to the mass-rearing technique of Modi & Tesh (1983), with strict regulation of food quantity and moisture.

A total of seven females, seven males and seven fourth-stage larvae were preserved alive until microbiological analysis. In order to isolate yeasts associated with *P. perniciosus*, two different media, universally used for the isolation and cultivation of yeasts, were employed: (a) yeast malt (YM) agar medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, 20 g/L agar, pH 6.2), and (b) potato dextrose agar (PDA) medium (Sigma-Aldrich Corp., St Louis, MO, U.S.A.), both supplemented with chloramphenicol 100 mg/L to avoid bacterial growth. Adult sandflies were surface-sterilized in 100% ethanol andwashed twice in 1× phosphate-buffered saline (PBS) supplemented with detergent. Whole bodies were individually homogenized with a sterile pestle by grinding each body in 200 μL of sterile 0.9% NaCl water solution. A sample of 100 μL of each homogenate was spread directly on to a YM agar plate supplemented with chloramphenicol and the remaining 100 μL of homogenate were spread on a PDA plate supplemented with the same antibiotic. The plates were incubated for 48–72 h at 30 ∘C. The L4 larvae were dissected in a drop of sterile 1× PBS using sterile needles under a stereomicroscope (Leica M50; Leica Microsystems GmbH, Wetzlar, Germany). The midguts of larvae were individually homogenized with a sterile micropestle by grinding them in 100 μL of sterile 0.9% NaCl solution, spread directly on PDA (50 μL) and YM (50 μL) plates supplemented with chloramphenicol and incubated for 48–72 h at 30 ∘C. Growing yeast colonies were then selected and collected based on colony morphology (Kurtzman & Fell, 2000), re-plated twice in order to purify them, and pure cultures of yeast strains [grown in liquid potato dextrose broth (PDB) medium; Sigma-Aldrich Corp.] were stored in 20% glycerol (Sigma-Aldrich Corp.) at −80 ∘C.

DNA from each yeast colony was extracted using the enzyme lyticase (Sigma-Aldrich Corp.) and the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) following the supplementary protocol for purification of total DNA from yeasts. *Wickerhamomyces anomalus*-specific polymerase chain reaction (PCR), targeted on the *β*-tubulin gene, was performed on all DNAs from selected yeast colonies using the specific primers SpWanom-170F (5′-TTATCCATCCACCAATTG-3′) and SpWanom-374R (5′-GGAACTAAGTTCACAGCTA-3′) (Huang *et al.*, 2012) in a total volume of 25 μL containing

1× Green GoTaq Reaction Buffer, 0.2mm dNTPs, 0.5 μm of each primer, 1.25U GoTaq G2 (Promega Corp., Madison, WI, U.S.A.) and 7–10 ng of template DNA, following the thermal protocol reported in Huang *et al.* (2012), except that the annealing temperature was decreased to 52 ∘C. Subsequently, in order to characterize the isolated strains, PCRs for the amplification of the 18S–26S rRNA internal transcribed spacer (ITS) and the 26S rRNA gene were carried out, as suggested in Schoch *et al.* (2012), for samples that tested positive in the previous *β*-tubulin-targeted PCR. This PCR amplification of the ITS fragment was carried out using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Manter & Vivanco, 2007), whereas PCR amplification of the D1/D2 domain of the 26S rRNA gene was conducted using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Ferreira *et al.*, 2010). All amplicons obtained from the ITS and 26S rRNA gene were sequenced.

Before DNA extraction, 17 adults and 10 L4 larvae of *P. perniciosus*, previously stored in 100% ethanol, were carefully washed in sterile 1× PBS. DNA extraction was performed in an extractor hood using a commercial kit, following the protocol previously described for yeasts. DNAs were eluted in 25 μL of AE buffer. A *W. anomalus*-specific PCR was carried out on DNA from whole insects using the previously described *β*-tubulin-targeted PCR (Huang *et al.*, 2012).

In order to perform molecular identification, the sequences obtained from 18S–26S rRNA ITS and 26S rRNA gene were subjected to blast analysis (http://www.ncbi.nlm.nih.gov/blast) and compared with sequences available in GenBank (nucleotide collection nr/nt; http://www.ncbi.nlm.nih.gov/genbank/). In addition, the clustering pattern among the sequences amplified in the present study and previous data was determined through phylogenetic analyses. Homologous sequences of both markers were retrieved from GenBank. Sequences from previous studies were included (Toki *et al.*, 2012, 2013) and two sets of data were produced, referring to, respectively, 18S–26S rRNA ITS and 26S rRNA gene sequences.

The two datasets of sequences were aligned using mafft Version 6.0 (http://mafft.cbrc.jp/alignment/server/) (Katoh *et al.*, 2005) implementing a G-INS-i search strategy (Katoh & Toh, 2008). The appropriate model of nucleotide substitution was estimated with jModelTest 2 (Darriba *et al.*, 2012) using Akaike's information criterion (AIC) to select the best-fit model. The best model of nucleotide evolution comprised a general time reversible (GTR) model (Lanave *et al.*, 1984) with gamma distribution for both sets of data. Maximum

likelihood phylogenetic inference and the hierarchical cluster analysis adopting the unweighted pair group method with arithmetic mean (UPGMA) were performed on the aligned datasets using, respectively, PhyML Version 3.0 (Guindon *et al.*, 2010) and mega 6 (Tamura *et al.*, 2013). Branch support was estimated using the approximate likelihood ratio test (aLRT) approach (Anisimova & Gascuel, 2006) in maximum likelihood analyses and by bootstrap analysis (1000 replicates) in UPGMA.

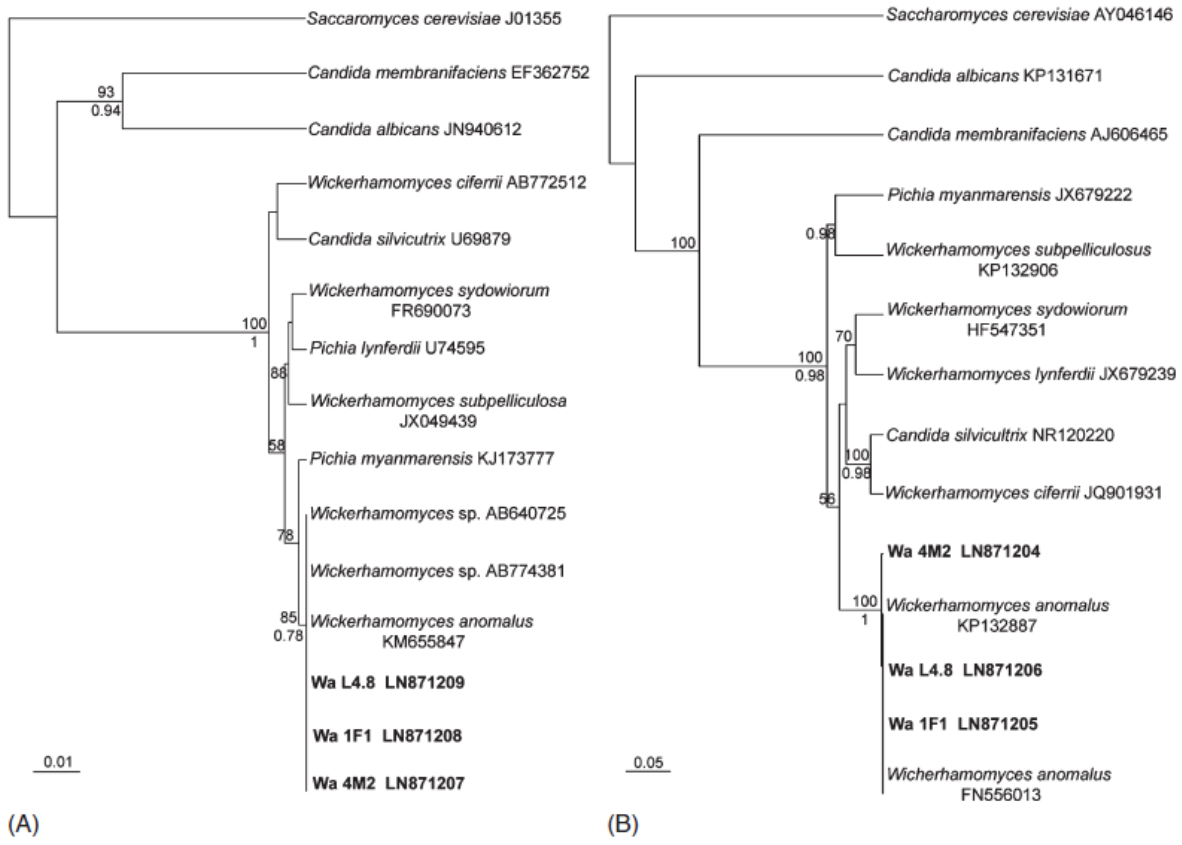

Fig. 1. UPGMA dendrograms inferred from (A) 26S rRNA and (B) 18S–26S rRNA ITS gene sequence datasets. Support values, respectively, the approximate likelihood ratio values for the maximum likelihood tree (A) and UPGMA bootstrap percentages (B) are reported on the branches. Support values below 0.5 and 50% are not reported. The dendrograms obtained are rooted on the outgroup, represented by *Saccaromyces cerevisiae*. Isolates obtained in this study are reported in bold. The scale bar indicates the distance in substitutions per site.

An assay to test for the killer phenotype was performed on one of the 15 isolates of *W. anomalus* recovered from *P. perniciosus* (isolate *Wa*_1F1) using the killer-sensitive reference strains

NEQAS 8706 (*Candida glabrata*), NEQAS 6208 (*Candida lusitaniae*), NCYC 1006 (*Saccharomyces cerevisiae*) and *Wa*UM3 [*W. anomalus* (a strain not producing toxins)] (Polonelli *et al.*, 1997). The killer strain *Wa*ATCC 96603 was used as a *Wa*KT-producing positive control (Guyard *et al.*, 2001). The killing assays were performed following a previously described method with minor modifications (Polonelli *et al.*, 1983). Briefly, each reference strain was resuspended in water to a final concentration of 0.5 McFarland and plated (100 μL) on methylene blue agar (MBA: 2% peptone, 2% glucose, 1% yeast extract, 2% agar, 0.003% methylene blue, buffered at pH 4.5 with 0.1 m citric acid and 0.2M Na2HPO4, and autoclaved at 121 ∘C for 15 min). Strains *Wa*_1F1 and *Wa*ATCC 96603 reactivated in Sabouraud dextrose agar were spotted (5mm diameter) on to the surface of previously prepared MBA, and the plate was incubated at 25 ∘C for 48 h. The killer-positive strain was characterized by the presence of a clear surrounding zone.

To date, most scientific research on the microbial community that colonizes haematophagous insects has been performed in mosquitoes (Dillon & Dillon, 2004). Very little is known about sandfly fungi microflora and their possible impact on the biology and reproduction of sandflies and sandfly–pathogen interaction (Zacchi & Vaughan-Martini, 2002; Sant'Anna *et al.*, 2014).

In particular, among insect vectors, the yeast *W. anomalus* has been described only in mosquitoes of public health importance; in these arthropod species, the yeast was observed in the midgut as well as in the gonads (Ricci *et al.*, 2011b). The present paper reports the first isolation o*f W. anomalus* from the sandfl*y P. perniciosus* and its antimicrobial activity against susceptible yeast strains.

Culture-based screening for yeasts was effected using 21 *P. perniciosus* sandfly individuals (14 whole adults and seven midguts of larvae) and led to the recovery of 65 yeast isolates from 12 flies (four females, two males, six larvae). Two media were used in order to increase the likelihood of obtaining yeast colonies; more yeast colonies were obtained from the YM agar medium than from the PDA medium, from which moulds were also retrieved. Specific PCR, for the amplification of the β-tubulin gene, identified 15 of the 65 isolates as *W. anomalus*. These *W. anomalus* isolates derived from both whole adults (four isolates from two males and four isolates from three females) and from the midguts of the L4 larvae (seven isolates from three larvae). In summary, *W. anomalus* isolates were obtained from a total of eight *P. perniciosus* individuals. The 15 isolates identified as *W. anomalus* were further characterized by amplification and sequencing of the 18S–26S rRNA ITS and the D1/D2 domain of the 26S

rRNA gene in order to confirm identification and to perform a phylogenetic analysis. Concerning the 26S rRNA gene, all sequences were identical at nucleotide level and showed 100% identity (E-value: 0) with *W. anomalus*, as revealed by the blast search.

With respect to the ITS marker, sequences of the 15 isolates from larvae, females and most males were identical (represented by *Wa*_L4.8 and *Wa*_1F1) (Fig. 1); one of the sequences from male isolates (*Wa*_4M2) showed one nucleotide deletion and one nucleotide substitution in comparison with the above sequences. blast analysis performed on ITS sequences confirmed the previous results, showing 100% and 99% identity, respectively, (E-value: 0) with *W. anomalus*. The 26S rRNA and ITS sequences of *W. anomalus*, obtained from isolates from one male (*Wa*_4M2), one female (*Wa*_1F1) and one larva (*Wa*_L4.8), were deposited in the database [European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database, accession nos. LN871204–LN871209] and used for phylogenetic analysis. Phylogenetic analyses, performed on the two markers in order to elucidate the relationships of the new fungal isolates (*Wa*_4M2, *Wa*_1F1, *Wa*_L4.8) from the sandfly *P. perniciosus*, confirmed their clustering with *Wickerhamomyces* spp. and the identification as *W. anomalus* (Fig. 1). Based on the analyses performed on the 26S rRNA gene sequence dataset, sandfly isolates cluster with *W. anomalus* and with a *Wickerhamomyces* sp. isolated from a fungus-growing beetle [accession nos. AB640725 and AB774381 (Toki *et al.*, 2012, 2013)]. On the basis of the ITS marker, the new isolated strains cluster in a well-supported clade (node support of 0.99 aLRT and 100% of bootstrap) with two specimens of *W. anomalus*, one of which was isolated from the mosquito *An. stephensi* (Fig. 1) [accession no. FN556013 (Ricci *et al.*, 2011a)].

In addition, to further verify the presence of*W. anomalus* in the analysed population of *P. perniciosus*, a *W. anomalus*-specific PCR assay was carried out on DNA from whole insects; one of nine females, two of eight males and three of 10 L4 larvae tested positive for the presence of*W. anomalus*. As in other cases of arthropod–symbiont associations, this suggests that yeasts associated with sandflies provide the host with some benefit. Recently, Sant'Anna *et al.* (2014) reported that pre-feeding yeast or bacteria to the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae) (bacteria *Asaia* sp. and *Ochrobactrum intermedium*, and yeast *Pseudozyma* sp.) can prevent the establishment of *Leishmania mexicana* infection within the insect vector. As demonstrated in plant systems, the yeast *Pseudozyma* can secrete extracellular metabolites that inhibit the pathogen, but may also prime the plant immune system to induce a local and systemic immune response towards the pathogen. Insect associations with yeasts may also benefit insects by promoting the development of other mutually beneficial organisms in the community while

decreasing the presence of insect pathogens. Davis *et al.* (2011) reported that volatiles produced by the yeast *Ogatea pini* found in the western pine beetle inhibited the development of the entomopathogenic fungi and increased the development of mutualist fungi, demonstrating that yeasts can selectively shape the microbial community.

As has been reported for mosquitoes, *W. anomalus* is not present in 100% of *P. perniciosus* individuals. This supports the speculation that as the yeast is not fixed in the population, it is not required for host survival. Nevertheless, non-obligate symbionts can exert beneficial roles and can be acquired by ingestion or through interaction with co-specifics and the environment (Engel & Moran, 2013).

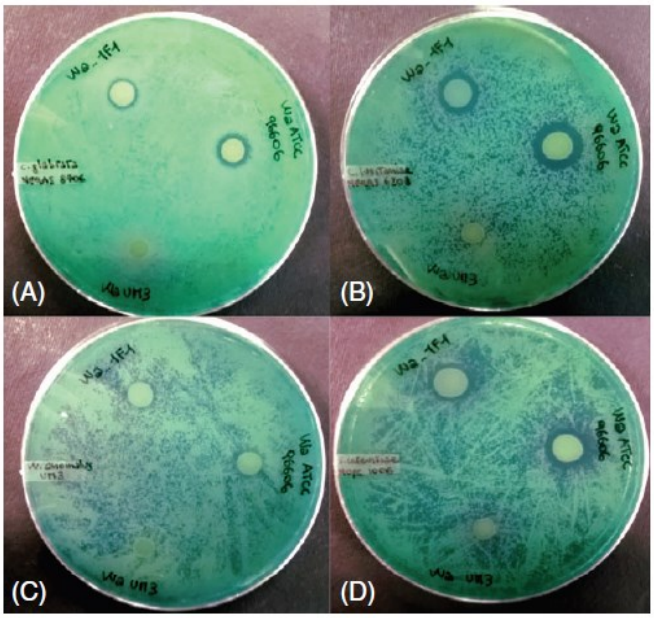

Fig. 2. Evidence of growth inhibition of the environmental strain *Wickeramomyces anomalus Wa*ATCC 96603 (right of the plates) and the strain *W. anomalus* Wa_1F1 isolated from *Plebotomus perniciosus* (left of the plates) determined by a transparent halo, in the plates with the sensitive strains: (A) *Candida glabrata* NEQAS 8706; (B) *Candida lusitaniae* NEQAS 6208; (C) *Wickeramomyces anomalus Wa*UM3, and (D) *Saccharomyces cerevisiae* NCYC 1006. No growth inhibition was determined by the *Wa*UM3 (*W. anomalus*, a strain not producing toxins) in the plates with the sensitive strains (bottom left of the plates).

Some*W. anomalus* strains have demonstrated antifungal activity and, more generally, have been shown to inhibit harmful microorganisms in a great variety of habitats (Polonelli, 2000; Cappelli *et al.*, 2014). To establish whether the strain *Wa*_1F1 isolated from *P. perniciosus* possesses antifungal activity, susceptible yeast strains were submitted to specific killer phenotype assays. Results are shown in Fig. 2 and present evidence of the growth inhibition of tested microorganisms, determined by a transparent halo, in plates with susceptible strains (*C. glabrata*NEQAS 8706, *C. lusitaniae* NEQAS 6208, *W. anomalusWa*UM3 and *S. cerevisiae* NCYC 1006). The transparent halo was similar to that generated by *Wa*ATCC 96606, the positive strain, known to produce the killer toxin (Guyard *et al.*, 2001). As previously reported

for the mosquito *An. stephensi* (Cappelli *et al.*, 2014), the present results show that *W. anomalus* from *P. perniciosus* inhibits the growth of selected fungi, and can thus be regarded as a killer yeast that is probably able to produce a killer toxin. Further studies should investigate whether killer toxin-producing strains of *W. anomalus* are commonly found in wild populations of *P. perniciosus* and if they possess anti-*Leishmania* activity.

It is important to note that Adler & Theodor (1929) have proposed that females of *Phlebotomus papatasi* infected by fungi were significantly more resistant to *Leishmania major* infection than uninfected females. Using the same study model, Schlein *et al.* (1985) reported a high prevalence of microbial infection in the digestive tract of laboratory-reared *P. papatasi* and hypothesized that these might have a negative effect on *Leishmania* transmission in endemic areas. In conclusion, the present discovery of *W. anomalus* yeasts displaying the killer phenotype in the sandfly *P. perniciosus*, the main Mediterranean vector of *L. infantum*, opens new avenues for research aimed at investigating the potential of this yeast as an agent for the biocontrol of leishmaniasis.

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References

Adler, S. & Theodor, O. (1929) Attempts to transmit *Leishmania tropica*: the transmission of *L. tropica* by *Phlebotomus sergenti*. *Annals of Tropical Medicine and Parasitology*, **23**, 1–18.

Anisimova, M. & Gascuel, O. (2006) Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Systematic Biology*, **55**, 539–552.

Cappelli, A., Ulissi, U., Valzano, M. *et al.* (2014) A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*. *PLoS ONE*, **9**, e95988.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, **9**, 772.

Davis, T.S., Hofstetter, R.W., Foster, J.T., Foote, N.E. & Keim, P. (2011) Interactions between the yeast *Ogataea pini* and filamentous fungi associated with the western pine beetle. *Microbial Ecology*, **61**, 626–634.

De Hoog, G.S. (1996) Risk assessment of fungi reported from humans and animals. *Mycoses*, **39**, 407–417.

Dillon, R.J. & Dillon, V.M. (2004) The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology*, **49**, 71–92.

Engel, P. & Moran, N.A. (2013) The gut microbiota of insects – diversity in structure and function. *FEMS Microbiology Reviews*, **37**, 699–735.

Epis, S., Capone, A., Martin, E. *et al.* (2015) A rapid qPCR method to investigate the circulation of the yeast *Wickerhamomyces anomalus* in humans. *New Microbiologica*, **38**, 577–581.

Ferreira, N., Belloch, C., Querol, A., Manzanares, P., Vallez, S. & Santos, A. (2010) Yeast microflora isolated from Brazilian cassava roots: taxonomical classification based on molecular identification. *Current Microbiology*, **60**, 287–293.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, **59**, 307–321.

Guyard, C., Evrard, P., Corbisier-Colson, A.M. *et al.* (2001) Immuno-crossreactivity of an anti-*Pichia anomala* killer toxin monoclonal antibody with a *Williopsis saturnus* var. *mrakii* killer toxin. *Medical Mycology*, **39**, 395–400.

Huang, C.H., Chang, M.T. & Huang, L. (2012) Species identification of *Wickerhamomyces anomalus* and related taxa using β-tubulin (β-tub) DNA barcode marker. *Yeast*, **29**, 531–535.

Kalkanci, A., Dizbay, M., Turan, O. *et al.* (2010) Nosocomial transmission of *Candida pelliculosa* fungemia in a pediatric intensive care unit and review of the literature. *Turkish Journal of Pediatrics*, **52**, 42–49.

Katoh, K. & Toh, H. (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinformatics*, **9**, 212.

Katoh, K., Kuma, K., Toh, H. & Miyata, T. (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research*, **33**, 511–518.

Killick-Kendrick, R. (1990) Phlebotomine vectors of the leishmaniases: a review. *Medical and Veterinary Entomology*, **4**, 1–24.

Kurtzman, C.P. & Fell, J.W. (2000) *The Yeasts, A Taxonomic Study*, 4th edn. Elsevier Science, Amsterdam. Lanave, C., Preparata, G., Saccone, C.&Serio, G. (1984) A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution*, **20**, 86–93.

Manter, D.K. & Vivanco, J.M. (2007) Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. *Journal of Microbiological Methods*, **71**, 7–14.

Maroli, M., Fiorentino, S. & Guandalini, E. (1987) Biology of a laboratory colony of *Phlebotomus perniciosus* (Diptera: Psychodidae). *Journal of Medical Entomology*, **24**, 547–551.

Modi, G.B. & Tesh, R.B. (1983) A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *Journal of Medical Entomology*, **20**, 568–569.

Oliveira, V.K., Ruiz Lda, S., Oliveira, N.A. *et al.* (2014) Fungemia caused by *Candida* species in a children's public hospital in the city of São Paulo, Brazil: study in the period 2007–2010. *Revista do Instituto de Medicina Tropical de São Paulo*, **56**, 301–305.

Passoth, V., Olstorpe, M. & Schnürer, J. (2011) Past, present and future research directions with *Pichia anomala*. *Antonie Van Leeuwenhoek*, **99**, 121–125.

Polonelli, L. (2000) Human, monoclonal and recombinant *Candida*cidal, pneumocysticidal and mycobactericidal antibodies. *Indian Journal of Chest Diseases and Allied Sciences*, **42**, 259–263.

Polonelli, L., Archibusacci, C., Sestito, M. & Morace, G. (1983) Killer

system: a simple method for differentiating *Candida albicans* strains. *Journal of Clinical Microbiology*, **17**, 774–780.

Polonelli, L., Séguy, N., Conti, S. *et al.* (1997) Monoclonal yeast killer toxin-like *Candida*cidal anti-idiotypic antibodies. *Clinical and Diagnostic Laboratory Immunology*, **4**, 142–146.

Ricci, I., Mosca, M., Valzano, M. *et al.* (2011a) Different mosquito species host *Wickerhamomyces anomalus* (*Pichia anomala*): perspectives on vector-borne diseases symbiotic control. *Antonie Van Leeuwenhoek*, **99**, 43–50.

Ricci, I., Damiani, C., Scuppa, P. *et al.* (2011b) The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*. *Environmental Microbiology*, **13**, 911–921.

Sant'Anna, M.R., Diaz-Albiter, H., Aguiar-Martins, K. *et al.* (2014) Colonization resistance in the sand fly gut: *Leishmania* protects *Lutzomyia longipalpis* from bacterial infection. *Parasites and Vectors*, **7**, 329.

Schlein, Y., Polacheck, I. & Yuva, B. (1985) Mycoses, bacterial infections and antibacterial activity in sand flies (Psychodidae) and their possible role in the transmission of leishmaniasis. *Parasitology*, **90**, 57–66.

Schoch, C.L., Seifert, K.A., Huhndorf, S. *et al.* (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 6241–6246.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013) MEGA6:molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, **30**, 2725–2729.

Toki, W., Tanahashi, M., Togashi, K. & Fukatsu, T. (2012) Fungal farming in a non-social beetle. *PLoS ONE*, **7**, e41893.

Toki, W., Takahashi, Y. & Togashi, K. (2013) Fungal garden making inside bamboos by a non-social fungus-growing beetle. *PLoS ONE*, **8**, e79515.

Walker, G.M. (2011) *Pichia anomala*: cell physiology and biotechnology relative to other yeasts. *Antonie Van Leeuwenhoek*, **99**, 25–34.

Wang, L., Yue, L., Chi, Z. &Wang, X. (2008) Marine killer yeasts active against a yeast strain pathogenic to crab *Portunus trituberculatus*. *Diseases of Aquatic Organisms*, **80**, 211–218.

Zacchi, L. & Vaughan-Martini, A. (2002) Yeasts associated with insects in agricultural areas of Perugia, Italy. *Annals of Microbiology*, **52**, 237–244.

2.4. Article 4

Candidacidal Activity of a Novel Killer Toxin from *Wickerhamomyces anomalus* against Fluconazole-Susceptible and -Resistant Strains

toxins

MDPI

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Article

Candidacidal Activity of a Novel Killer Toxin from *Wickerhamomyces anomalus* against Fluconazole-Susceptible and -Resistant Strains

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Abstract: The isolation and characterization from the sand fly *Phlebotomus perniciosus* of a *Wickerhamomyces anomalus* yeast strain (Wa1F1) displaying the killer phenotype was recently reported. In the present work, the killer toxin (KT) produced by Wa1F1 was purified and characterized, and its antimicrobial activity in vitro was investigated against fluconazole-susceptible and -resistant clinical isolates and laboratory strains of *Candida albicans* and *C. glabrata*displaying known mutations. Wa1F1-KT showed a differential killing ability against different mutant strains of the same species. The results may be useful for the design of therapeutic molecules based on Wa1F1-KT and the study of yeast resistance mechanisms.

Keywords: *Wickerhamomyces anomalus*; yeast killer toxin; *Candida albicans*; *Candida glabrata*; antifungal resistance

Key Contribution: A novel killer toxin produced by the recently isolated *Wickerhamomyces anomalus* strain 1F1 showed a differential killing ability against fluconazole-susceptible and -resistant *Candida* clinical isolates and laboratory strains displaying known mutations.

1. Introduction

Yeast strains belonging to diverse species produce and secrete proteins or glycoproteins, known as killer toxins (KTs), that are lethal to susceptible strains [1,2]. This property, which offers a competitive advantage to self-immune killer yeasts in their ecological niches, has found several applications in the biological control of plant pathogens and spoiling yeasts in the food and fermentation industries [3]. In the medical field, KTs have been used for the biotyping of pathogenic microorganisms, in epidemiological studies, and for the identification of novel cellular targets in microbial cells and the development of new antimicrobials [4–6]. Some KTs, such as K1 and K28 from *Saccharomyces cerevisiae*, have a narrow spectrum of activity, limited to susceptible strains of the same species, while other KTs show a wide killing spectrum [2]. In particular, some *Wickerhamomyces anomalus* (formerly *Pichia anomala*) KTs proved to be active against a wide range of microorganisms, including other yeast species, filamentous fungi, bacteria, and protozoan parasites [2,7,8]. Killer strains of *W. anomalus* have been isolated from different sources, including plants and food products [9–11], arthropods such as the crab *Portunus trituberculatus* [12], and mosquitoes of the species Anopheles stephensi [13,14]. Recently, the isolation and characterization of a *W. anomalus* strain displaying the killer phenotype was reported from specimens of the sand fly *Phlebotomus perniciosus* [15]. The ability to inhibit harmful microorganisms in a variety of habitats and the wide killing spectrum of the produced KTs have prompted the use of *W. anomalus* as a bio-control agent [7,16], since it could be classed as a low risk microorganism, rarely traced in human samples [17,18]. In addition, *W. anomalus* KTs activity against dermatophytes and pathogenic yeasts, especially *Candida* spp., led to the hypothesis that could be applied in medical mycology as alternative antifungal compounds [19–21]. *Candida* spp. are the most frequently isolated yeasts in clinical specimens. The frequency of invasive opportunistic fungal infections caused by species of this genus has significantly increased in recent years, particularly in immunosuppressed individuals and patients with indwelling medical devices [22]. Worldwide, the prevalent cause of invasive candidiasis remains *C. albicans*, although the epidemiology of Candidal infections has gradually shifted towards non-*albicans* species, such as *C. glabrata* and *C. krusei* [22–24]. Increasing concern is rising in view of growing reports of resistance to antifungal drugs, with particular reference to resistance to azoles in non-albicans *Candida* species [25,26].

In the aim of searching for new molecules, potentially effective against strains resistant to conventional antifungal drugs, we investigated the in vitro activity of a KT produced by the recently isolated *W. anomalus* strain 1F1 (*Wa*1F1-KT) [15] against both susceptible and azole-resistant clinical isolates as well as laboratory strains of *C. albicans* and *C. glabrata* displaying known mutations [27–31].

2. Results

2.1. Wa1F1- KT Production

The production of *Wa*1F1- KT by the strain *W. anomalus* 1F1 was analyzed over time. The activity of the concentrated culture supernatant obtained at different time periods was determined against the reference *C. lusitaniae* strain on solid medium and quantified by Arbitrary Units/mL (1 Arbitrary Unit (AU) is defined as the amount of KT producing an inhibition zone of 1 mm2). As shown in Figure 1, the results indicate that the activity of the *Wa*1F1- KT sample obtained after 24 h of incubation was low (559 AU/mL), although it increased after 48 h of incubation (1815 AU/mL), and reached a maximum after 72 h (2326 AU/mL). The killing activity decreased steeply thereafter.

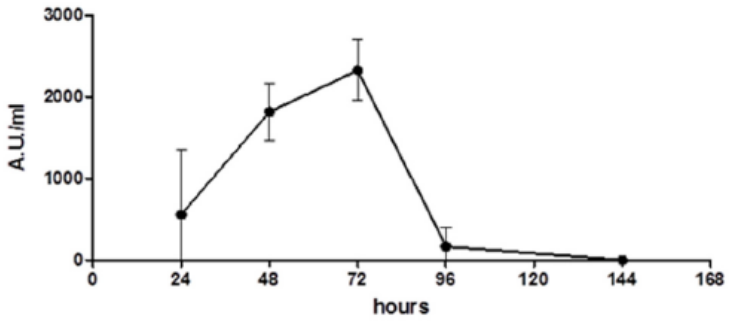

Figure 1. Time- course of *Wa*1F1- KT production by *Wickerhamomyces anomalus* 1F1. Overnight grown liquid seed cultures of the yeast were inoculated at 1% (*v*/*v*) into killer toxin (KT) production medium, then incubated at 20 °C under shaking (180 rpm). Samples were withdrawn at 24 h intervals, yeast cells were removed by centrifugation, and the filtered supernatants were concentrated 50- fold and assayed for their activity against the reference *Candida lusitaniae* strain grown on solid medium. Arbitrary Unit (AU): amount of KT which produces an inhibition zone of 1 mm^2.

2.2. Western Blot Analysis of Wa1F1- KT

Western blot analysis was carried out on crude extracts from 72- h cultures of the *W. anomalus*

strains 1F1 and ATCC 96603 (KT- producing, positive control strain). As a negative control, a crude extract from *W. anomalus* UM3 (KT- nonproducing strain) culture and 50- fold concentrated YPD medium were used. Bands were detected with mAbKT4, a monoclonal antibody directed against a KT produced by the reference ATCC 96603 strain (*Wa*96603- KT) and shown to cross- react with KTs produced by other *W. anomalus* strains and other killer yeasts [14,32,33]. The results showed that mAbKT4 reacts with high- molecular mass proteins in samples obtained from *W. anomalus* 1F1 and ATCC 96603, but not from controls (Figure 2). A single band of approximately 220 kDa was revealed in extracts from the reference strain, while a single band with a lower molecular mass (approximately 160–170 kDa) was detected in the extract from 1F1 strain, indicating the secretion of KTs with a common epitope, although with some structural differences.

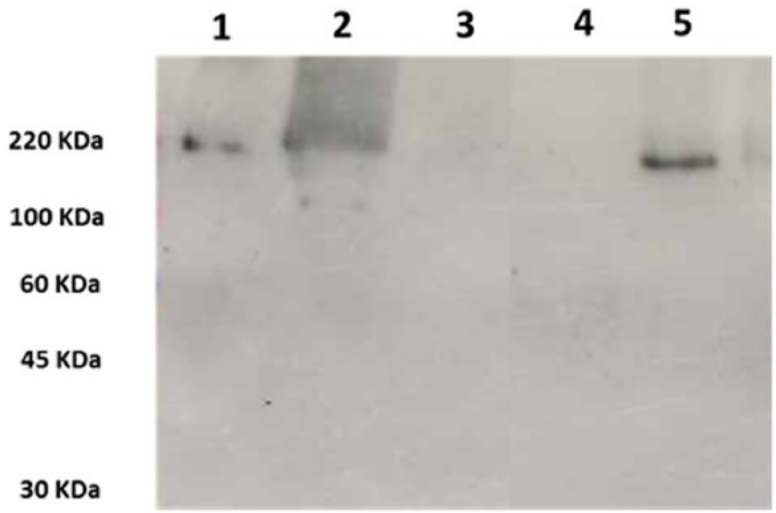

Figure 2. Western blot analysis using mAbKT4 to probe 50- fold concentrated crude extracts of (1) *Wickerhamomyces anomalus* ATCC 96603, 2 µL; (2) *W. anomalus* ATCC 96603, 5 µL; (3) *W. anomalus* UM3, 5 µL; (4) YPD medium, 5 µL; (5) *W. anomalus* 1F1, 5 µL. Molecular masses (kDa) are shown on the left.

2.3. Characterization of Wa1F1- KT Using Size Exclusion Chromatography

The concentrated extract from *W. anomalus* 1F1 culture was analyzed by size exclusion chromatography and eluted fractions were assayed for killing activity against the reference *C. lusitaniae* strain. In agreement with the immunoblot data, the results indicated the elution of active *Wa*1F1- KT in fractions 34–39 of the size exclusion chromatogram (Figure 3), corresponding to the highest molecular mass separation zone.

Strain	Features and Genotype	*Wa*1F1-KT Sensitivity (mm Growth Inhibition) [a]
C. albicans SC5314	Reference laboratory strain, FluS	0
C. albicans DSY347	FluS clinical strain [27]	0
C. albicans DSY289	FluR, DSY347 *ERG11*: S405F, Y132H; *TAC1*: A736V [27,29]	14
C. albicans DSY544	FluS clinical strain [30]	0
C. albicans DSY775	FluR, DSY544 *ERG11*: G464S, *TAC1*: G980W [30]	0
C. glabrata DSY562	FluS clinical strain [28]	13
C. glabrata DSY565	FluR clinical strain [28]	12
C. glabrata SFY93	FluR, DSY562 pdr1Δ [31]	12
C. glabrata SFY105	FluR, DSY562 pdr1Δ-T588A [31]	13
C. glabrata SFY115	FluR, DSY562 pdr1Δ-L280F [31]	13
C. glabrata SFY116	FluR, DSY562 pdr1Δ-P822L [31]	13
C. lusitaniae NEQAS6208	Reference laboratory strain, FluS	17

[a] Diameter (mm) of growth inhibition zone, mean values (±1 mm) from four independent experiments. FluS, Fluconazole-susceptible; FluR, Fluconazole-resistant.

Table 1. Sensitivity of laboratory strains and clinical isolates of *Candida* spp. susceptible or resistant to fluconazole against killer toxin Wa1F1-KT.

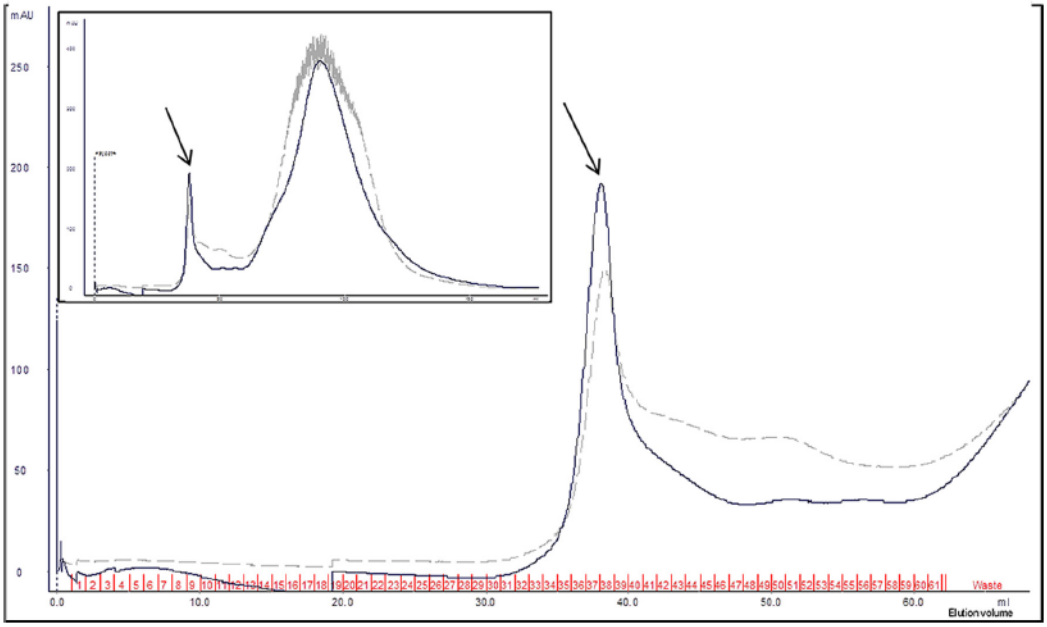

Figure 3. *Wickerhamomyces anomalus* 1F1 crude extract separation on HiPrep Sephacryl S- 200 column. Partial chromatographic traces, displaying the active fractions (34–39), as determined by killing assay on *Candida lusitaniae* NEQAS 6208. Continuous and dashed lines refer to absorbance values (milli Absorbance Units, mAU) at 280 nm (mainly Trp absorbance) and 215 nm (peptide bond absorbance), respectively. Inset: complete chromatogram traces. The arrows indicate the elution peak of active fractions.

2.4. Activity of Wa1F1- KT

*Wa*1F1- KT was tested by an agar diffusion assay against the reference *C. lusitaniae* NEQAS 6208 strain and clinical isolates and laboratory strains of *C. albicans*and *C. glabrata*susceptible or resistant to fluconazole. The results showed that, when tested at 25 °C, *Wa*1F1- KT was active towards both fluconazole- susceptible and - resistant strains of *C. glabrata*, with some quantitative differences (Table 1). *Wa*1F1- KT showed no effect against the reference *C. albicans*SC5314 strain and against two fluconazole- susceptible clinical isolates (DSY347 and DSY544) and the DSY544- derived fluconazoleresistant mutant strain (DSY775) of *C. albicans*. On the contrary, a good effect was detected against the DSY347- derived fluconazole- resistant mutant *C. albicans*DSY289 strain. No effect against any of the tested strains was detected when the assay was carried out at 30 and 37 °C.

2.5. Exo- β- 1,3- Glucanase Activity

To investigate if *Wa*1F1- KT could act on susceptible yeast strains by hydrolyzing major cell wall components, its ability to digest the soluble β- 1,3- glucan laminarin was assayed in comparison with *Wa*96603- KT, the KT produced by the *Williopsis saturnus* var. *mrakii* MUCL 41968 (*Wm*41968- KT), with known β- glucanase activity [34], and laminarinase. The results showed that all KTs could hydrolyze laminarin to an end product presenting similar relative mobility to the product of the laminarinase reaction (Figure 4).

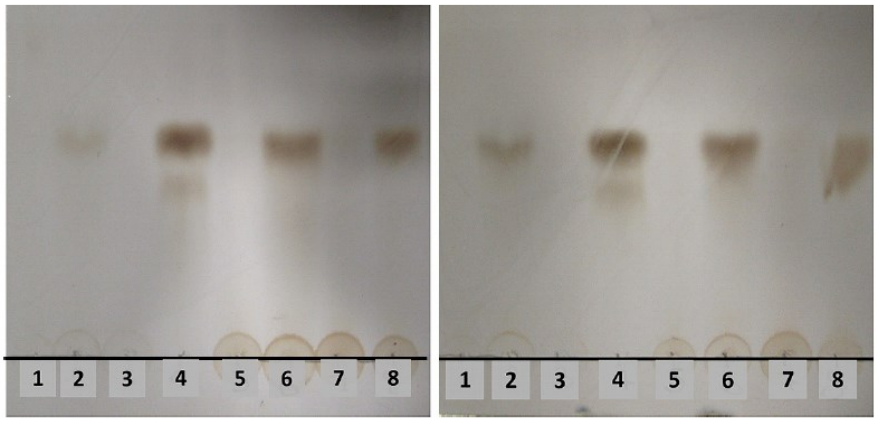

Figure 4. Thin- layer chromatogram of hydrolysis products of laminarin by crude killer toxins (KTs). Before loading, laminarin was incubated for 2 (left panel) or 4 (right panel) hours at 25 °C with: (1) heat- inactivated *Wa*1F1- KT; (2) *Wa*1F1- KT; (3) heat- inactivated laminarinase; (4) laminarinase; (5) heat- inactivated *Wa*96603- KT; (6) *Wa*96603- KT; (7) heat- inactivated *Wm*41968- KT; (**8**) *Wm*41968- KT.

3. Discussion

Mucosal and invasive candidiasis are the most common mycoses in humans [22–24,35,36]. Invasive candidiasis and candidemia, in particular, are an emerging health problem, especially in hospitalized and immunosuppressed individuals and patients with indwelling medical devices [22]. Additionally, the diffusion of antifungal drugs resistance in *Candida* spp., particularly non-*albicans*, makes it necessary to look for new treatments against these infections [37].

In the present study, with the aim of searching for alternative antifungal agents, a KT produced by the newly isolated *W. anomalus* 1F1 strain [15] was assayed in vitro against clinical isolates and laboratory strains of *C. albicans* and *C. glabrata* displaying known mutations and different susceptibility to fluconazole [27–31].

KTs produced by strains of *W. anomalus* have previously shown to be active against *Candida* spp. both *in vitro* and *in vivo* [20,21,38,39], although little is known on their activity against resistant strains. *W. anomalus* may produce different KTs with variable molecular mass (8–300 kDa), structural characteristics, pH and temperature optima, and antimicrobial activity range [7,40]. KTs from *W. anomalus* and other killer yeasts have been shown to exert a _-1,3-glucanase activity, and may cause damage to the cell wall of susceptible yeasts as a result of degradation of the main _-glucans cell wall components [20,34,41,42].

Our results showed that Wa1F1-KT shares at least one epitope with the KT produced by *W. anomalus* ATCC 96603, although the molecular mass of the two toxins is slightly different, indicating that they are related but not identical. Both Wa96603-KT and Wa1F1-KT degraded the soluble _-glucan laminarin in a manner similar to laminarinase, an endo-1,3(4)-_-glucanase, as did Wm41968-KT, whose glucanase activity had been already suggested [34].

The spectrum of activity of Wa96603-KT and Wa1F1-KT, however, appeared to be different, as the latter proved to be active against the *C. glabrata*isolates but did not affect the majority of C. *albicans* strains tested in this study, while Wa96603-KT was previously shown to kill different clinical C. *albicans* isolates [43,44]. Selective killing of non-*albicans* species by *W. anomalus* KTs with β-glucanase activity has previously been reported [20]. This differential spectrum may be explained with differences in the specificity of β-glucanase activity of KTs, which may selectively recognize different glycosidic linkages and glucan receptors on target yeast cells [45,46].

Although some KTs from *W. anomalus* display high stability at 37 °C and even higher temperatures [47], many KTs show lower thermostability [7]. We found that the optimal temperature for Wa1F1-KT activity was lower than the physiological value in the human body. Nevertheless, the absence of β-glucans on mammalian cells suggests its potential application against fungal infections at skin and mucosal membrane levels, as has been demonstrated with other KTs [48]. Further characterization of Wa1F1-KT enzymatic activity and the cloning of its encoding gene may represent the next step to investigate the feasibility to produce molecules with broader therapeutic activity, possibly including systemic infections.

The mechanism of action of Wa1F1-KT on *C. glabrata*appeared to be independent from the fluconazole-resistance pathway, as only slightly different effects were observed against the susceptible clinical isolate (DSY562) or its mutant derivative strains (SFY93, SFY105, SFY115, SFY116).

This phenomenon underlies the potential of Wa1F1-KT as a universally active anti-*C. glabrata* tool, likely not affected by drug-resistance phenotypes. On the contrary, the fact that Wa1F1-KT was active only on *C. albicans* DSY289 implies that the mechanism of action of the toxin may be dependent upon the specific mutations that confer resistance to fluconazole in this strain. *C. albicans* DSY289 was derived from the fluconazole-susceptible clinical strain DSY347 by mutations that confer combined resistance to azoles [27,29]. In particular, the point mutations S405F/Y132H in the ERG11 gene encoding the enzyme lanosterol 14-α-sterol demethylase, which is involved in converting lanosterol into ergosterol, an essential component of the fungal cell membrane, are associated with a conformational change of the target enzyme and reduced interaction or binding of azoles [49–51]. The gain-of-function A736V mutation in the transcriptional activator TAC1 causes the overexpression of the ATP binding cassette (ABC)-transporters CDR1 and CDR2, decreasing the concentration of azoles within the fungal cell [29,52]. It is not clear how this mutation may affect Wa1F1-KT activity, but it may be speculated that the overexpression of transport systems and associated extracellular loops could possibly alter the recognition of the cell surface target by the toxin or that the transcriptional activator TAC1 is involved in the regulation of transcription of toxin receptors. Further studies aimed at the biochemical characterization of properly purified Wa1F1-KT, the elucidation of its mechanism of action, and the reasons for its differential killing ability against different mutant strains can provide important information for developing new strategies to combat infections caused by azole-resistant *Candida* strains.

4. Materials and Methods

4.1. Yeast Strains

Strains belonging to the species *W. anomalus*, *W. saturnus* var. mrakii, *C. lusitaniae*, *C. albicans*, and *C. glabrata* were used in this study. *W. anomalus* 1F1 isolated from *P. perniciosus* [15], *W. anomalus* ATCC 96603 (a KT-producing strain formerly referred to as UP25F) [32], and *Williopsis saturnus* var. mrakii MUCL 41968 [34] were used for the production of Wa1F1-KT, Wa96603-KT, and Wm41986-KT, respectively. The KT non-producing, KT-susceptible, *W. anomalus* UM3 strain was also used in this study as a negative control for KT expression [32]. The activity of Wa1F1-KT was tested against the reference *C. albicans* strain SC5314, two wild-type *C. albicans* clinical isolates (DSY544 and DSY347) [27,30], two *C. albicans* mutant strains resistant to fluconazole (DSY775, derived from DSY544, and DSY289, derived from DSY347) [27,29,30], two wild-type *C. glabrata* clinical isolates susceptible (DSY562) and resistant (DSY565) to fluconazole [28], and four *C. glabrata* fluconazole-resistant strains derived from DSY562 by mutations in the gene CgPDR1 (SFY93, SFY105, SFY115, SFY116) [31]. The reference strain *C. lusitaniae* NEQAS 6208, known to be susceptible to the activity of *Wa*1F1-KT [15], was also used as a positive control for KT activity. Yeasts maintained in sterile distilled water were subcultured on Sabouraud Dextrose Agar plates.

4.2. Media

KT-producing strainswere grown in YPDmedium(1%yeast extract, 2%peptone, and 2%dextrose), then subcultured for KT production in YPD medium with 15% glycerol, buffered at pH 4.6 with 0.1 M citric acid and 0.2 M Na2HPO4. For the KT activity assay, YPD medium was added with 3% agar, and 0.003% methylene blue, and adjusted to pH 4.6 with 0.1 M citric acid and 0.2 M Na2HPO4.

4.3. Production of KTs

For the production of crude extracts, a seed culture of the *W. anomalus* and *W. saturnus* var. mrakii strains was incubated at 20 _C for 24 h with shaking at 150 rpm in YPD medium. Flasks (500-mL volume) containing 100 mL of YPD buffered at pH 4.6, with 15% glycerol, were inoculated with 1 mL of the seed culture and incubated at 20 _C for 72 h with shaking (150 rpm). After this period, the cells were removed by centrifugation (5000_ g, 10 min, 4 _C); the supernatant was filtered through 0.45 _m pore size membranes (Merck Millipore, Darmstadt, Germany) and concentrated (50-fold) through an Amicon Ultra-15 (10-kDa cutoff) filter unit

(Merck Millipore) by centrifugation at 4000X g, 4 °C. Accordingly, a concentrated extract of YPD medium used for KT production was prepared. The concentrated crude extracts were stored at 4 °C until use.

4.4. Western Blot Analysis

The crude extracts from *W. anomalus* ATCC 96603, UM3, and 1F1 were analyzed by non-continuous denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) in 7% polyacrylamide gel, at 100 Volts for 2 h in a minigel system (Bio-Rad Laboratories, Hercules, CA, USA). Concentrated YPD medium was also run as a control. Proteins were electrically transferred to a polyvinylidene difluoride (PVDF) membrane at 100 Volts for 1 h. The total protein content of the crude extracts was estimated by Ponceau staining prior to immunodetection. The PVDF membrane was then incubated for 1 h at room temperature with 5% bovine serum albumin in Tris-buffered saline (TBS) at pH 7.5 and 0.5% tween-20 (TBS-T). Subsequently, the membrane was incubated overnight at 4 °C with a 1:500 dilution in TBS-T of the monoclonal *W. anomalus* ATCC 96603 KT-neutralizing antibody mAbKT4 [32], known to cross-react with KTs from other *Wickerhamomyces* [14] and *Williopsis* [33] strains. After washing three times in TBS-T, the membrane was incubated for 1 h at room temperature with a secondary, peroxidase-conjugated anti-mouse antibody. The membrane was thoroughly washed with TBS-T, incubated for 1 min with the proper substrate (BM Chemiluminescence blotting substrate, Roche, Basel, Switzerland), and detected by ChemiDoc 2000R (Kodak, Rochester, NY, USA).

4.5. Characterization of Wa1F1-KT Using Size Exclusion Chromatography

The crude Wa1F1-KT was dialyzed against 0.01 M citric acid-Na2HPO4 buffer (pH 4.5) for 24 h at 4 °C using a membrane with a molecular mass cut-off of 10 kDa. Analytical gel filtration was performed on a HiPrep Sephacryl S-200 prepacked column (GE Healthcare Life Sciences, Marlborough, MA, USA), characterized by bed dimensions of 16 X 600 mm and an exclusion limit (for globular proteins) of about 400 kDa, connected to an AKTA purifier system (GE Healthcare Life Sciences). Dialyzed crude extract was applied to the column, equilibrated, and eluted with 1.2 column volume of citric acid-Na2HPO4 buffer, pH 4.5. Eluted fractions (1 mL) were combined according to chromatogram peaks, lyophilized, and re-solubilized in 1 mL of sterile distilled water. Total protein content was quantified with an infrared-based spectrometry system (Direct Detect™, Merck Millipore) and the concentrated fractions were assayed for killing activity against the C. lusitaniae NEQAS 6208 susceptible strain (see below).

4.6. Evaluation of Wa1F1-KT Activity

The activity of Wa1F1-KT was tested against the reference *C. lusitaniae* NEQAS 6208 strain and fluconazole-susceptible or -resistant clinical isolates as well as laboratory strains of *C. albicans* and *C. glabrata*. Each test strain, grown overnight on SDA plates, was resuspended in water to a final concentration of 0.5 McFarland and spread (100 µL) on the surface of YPD agar plates. Crude extracts from *W. anomalus* 1F1 were poured into wells of 8 mm (40 µL per well) cut into the agar plates. The plates were incubated for 48 h at 25, 30, or 37 _C and the diameter of the area of growth inhibition was measured.

4.7. Laminarin Hydrolysis

The ability ofWa1F1-KT to hydrolyze the soluble β-1,3-glucan laminarin (Sigma-Aldrich, St. Louis, MO, USA) was assayed in comparison to Wa96603-KT, Wm41968-KT (with recognized β-glucanase activity [34]), and laminarinase (Sigma-Aldrich). The reaction mixtures contained 20 µL of 50-fold concentrated crude KTs or 10 _L of laminarinase (4.5 U/mL) and 2 mg/mL laminarin in 100 µL of citric acid-Na2HPO4 buffer (0.01 M, pH 4.5). After incubation at 25 °C for 2 and 4 h, the reaction was stopped by heating at 100 °C for 15 min. The activity on laminarin was estimated through observation of the end products of laminarin hydrolysis by thin layer chromatography [53]. Reaction mixtures containing crude KTs inactivated by heating at 100 °C for 15 min were used as controls.

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Author Contributions: L.G., S.E., and S.C. conceived and designed the experiments; L.G., C.S., E.F., and T.C. performed the experiments; L.G., E.F., S.E., and C.B. analyzed the data; E.M. and I.R. contributed materials; L.G. and S.C. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

1. Bevan, E.A.; Makower, M. The Physiological Basis of the Killer Character in Yeast; XIth Int Congr Genet, Leiden, The Netherlands; Pergamon Press: Oxford, England, 1963; pp. 202–203.

2. Magliani, W.; Conti, S.; Gerloni, M.; Bertolotti, D.; Polonelli, L. Yeast killer systems. Clin. Microbiol. Rev. **1997**, 10, 369–400. [PubMed]

3. Muccilli, S.; Restuccia, C. Bioprotective role of yeasts. Microorganisms **2015**, 3, 588–611. [CrossRef] [PubMed]

4. Buzzini, P.; Turchetti, B.; Vaughan-Martini, A.E. The use of killer sensitivity patterns for biotyping yeast strains: The state of the art, potentialities and limitations. FEMS Yeast Res. **2007**, 7, 749–760. [CrossRef] [PubMed]

5. Polonelli, L.; Conti, S. Biotyping of *Candida albicans* and other fungi by yeast killer toxins sensitivity. Methods Mol. Biol. **2009**, 499, 97–115. [CrossRef] [PubMed]

6. Polonelli, L.; Magliani,W.; Ciociola, T.; Giovati, L.; Conti, S. From *Pichia anomala* killer toxin through killer antibodies to killer peptides for a comprehensive anti-infective strategy. Antonie Van Leeuwenhoek **2011**, 99, 35–41. [CrossRef] [PubMed]

7. Walker, G.M. *Pichia anomala*: Cell physiology and biotechnology relative to other yeasts. Antonie Van Leeuwenhoek **2011**, 99, 25–34. [CrossRef] [PubMed]

8. Valzano, M.; Cecarini, V.; Cappelli, A.; Capone, A.; Bozic, J.; Cuccioloni, M.; Epis, S.; Petrelli, D.; Angeletti, M.; Eleuteri, A.M.; et al. A yeast strain associated to *Anopheles* mosquitoes produces a toxin able to kill malaria parasites. Malaria J. **2016**, 15, 21. [CrossRef] [PubMed]

9. Kagiyama, S.; Aiba, T.; Kadowaki, K.; Mogi, K. New killer toxins of halophilic *Hansenula anomala*. Agric. Biol. Chem. **1988**, 52, 1–7. [CrossRef]

10. Fredlund, E.; Druvefors, U.; Boysen, M.E.; Lingsten, K.-J.; Schnürer, J. Physiological characteristics of the biocontrol yeast *Pichia anomala* J121. FEMS Yeast Res. **2002**, 2, 395–402. [CrossRef] [PubMed]

11. Perez, M.F.; Contreras, L.; Garnica, N.M.; Fernández-Zenoff, M.V.; Farías, M.E.; Sepulveda, M.; Ramallo, J.; Dib, J.R. Native killer yeasts as biocontrol agents of postharvest fungal diseases in lemons. PLoS ONE **2016**, 11, e0165590. [CrossRef] [PubMed]

12. Wang, X.; Chi, Z.; Yue, L.; Li, J.; Li, M.;Wu, L. A marine killer yeast against the pathogenic yeast strain in crab (*Portunus trituberculatus*) and an optimization of the toxin production. Microbiol. Res. **2007**, 162, 77–85. [CrossRef] [PubMed]

13. Ricci, I.; Damiani, C.; Scuppa, P.; Mosca, M.; Crotti, E.; Rossi, P.; Rizzi, A.; Capone, A.; Gonella, E.; Ballarini, P.; et al. The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*. Environ. Microbiol. **2011**, 13, 911–921. [CrossRef] [PubMed]

14. Cappelli, A.; Ulissi, U.; Valzano, M.; Damiani, C.; Epis, S.; Gabrielli, M.G.; Conti, S.; Polonelli, L.; Bandi, C.; Favia, G.; et al. A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*. PLoS ONE **2014**, 9, e95988. [CrossRef] [PubMed]

15. Martin, E.; Bongiorno, G.; Giovati, L.; Montagna, M.; Crotti, E.; Damiani, C.; Gradoni, L.; Polonelli, L.; Ricci, I.; Favia, G.; et al. Isolation of a *Wickerhamomyces anomalus* yeast strain from the sandfly *Phlebotomus perniciosus*, displaying the killer phenotype. Med. Vet. Entomol. **2016**, 30, 101–106. [CrossRef] [PubMed]

16. Passoth, V.; Fredlund, E.; Druvefors, U.Ä.; Schnürer, J. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. **2006**, 6, 3–13. [CrossRef] [PubMed]

17. De Hoog, G.S. Risk assessment of fungi reported from humans and animals. Mycoses **1996**, 39, 407–417. [CrossRef] [PubMed]

18. Epis, S.; Capone, A.; Martin, E.; Paolucci, M.; Bazzocchi, C.; Valzano, M.; Bozic, J.; Novati, S.; Favia, G.; Ricci, I. A rapid qPCR method to investigate the circulation of the yeast *Wickerhamomyces anomalus* in humans. New Microbiol. **2015**, 38, 577–581. [PubMed]

19. ˙Izgü, F.; Altınbay, D.; Türeli, A.E. In vitro activity of panomycocin, a novel exo-β-1,3-glucanase isolated from *Pichia anomala* NCYC 434, against dermatophytes. Mycoses **2007**, 50, 31–34. [CrossRef] [PubMed]

20. Tay, S.-T.; Lim, S.-L.; Tan, H.-W. Growth inhibition of *Candida* species by *Wickerhamomyces anomalus* mycocin and a lactone compound of *Aureobasidium pullulans*. BMC Complement. Altern. Med. **2014**, 14, 439. [CrossRef] [PubMed]

21. Paris, A.P.; Persel, C.; Serafin, C.F.; de Cassia Garcia Simao, R.; Gandra, R.F. Susceptibility of *Candida albicans* isolated from blood to *Wickerhamomyces anomalous* mycocins. Curr. Microbiol. **2016**, 73, 878–884. [CrossRef] [PubMed]

22. Enoch, D.A.; Yang, H.; Aliyu, S.H.; Micallef, C. The changing epidemiology of invasive fungal infections. In Human Fungal Pathogen Identification: Methods and Protocols; Lion, T., Ed.; Springer: New York, NY, USA, 2017; pp. 17–65.

23. Guinea, J. Global trends in the distribution of *Candida* species causing candidemia. Clin. Microbiol. Infect. **2014**, 20, 5–10. [CrossRef] [PubMed]

24. Lockhart, S.R. Current epidemiology of *Candida* infection. Clin. Microbiol. Newsl. **2014**, 36, 131–136. [CrossRef]

25. Orasch, C.; Marchetti, O.; Garbino, J.; Schrenzel, J.; Zimmerli, S.; Mühlethaler, K.; Pfyffer, G.; Ruef, C.; Fehr, J.; Zbinden, R.; et al. *Candida* species distribution and antifungal susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing and new vs. old Clinical and Laboratory Standards Institute clinical breakpoints: A 6-year prospective *Candida*emia survey from the fungal infection network of Switzerland. Clin. Microbiol. Infect. **2014**, 20, 698–705. [CrossRef] [PubMed]

26. Tan, T.Y.; Hsu, L.Y.; Alejandria, M.M.; Chaiwarith, R.; Chinniah, T.; Chayakulkeeree, M.; Choudhury, S.; Chen, Y.H.; Shin, J.H.; Kiratisin, P.; et al. Antifungal susceptibility of invasive *Candida* bloodstream isolates from the Asia-Pacific region. Med. Mycol. **2016**, 54, 471–477. [CrossRef] [PubMed]

27. Sanglard, D.; Kuchler, K.; Ischer, F.; Pagani, J.L.; Monod, M.; Bille, J. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **1995**, 39, 2378–2386. [CrossRef] [PubMed]

28. Sanglard, D.; Ischer, F.; Calabrese, D.; Majcherczyk, P.A.; Bille, J. The ATP binding cassette transporter gene CgCDR1 from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob. Agents Chemother. **1999**, 43, 2753–2765. [PubMed]

29. Coste, A.; Selmecki, A.; Forche, A.; Diogo, D.; Bougnoux, M.-E.; d'Enfert, C.; Berman, J.; Sanglard, D. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. Eukaryot. Cell **2007**, 6, 1889–1904. [CrossRef] [PubMed]

30. Coste, A.T.; Crittin, J.; Bauser, C.; Rohde, B.; Sanglard, D. Functional analysis of cis- and trans-acting elements of the *Candida albicans* CDR2 promoter with a novel promoter reporter system. Eukaryot. Cell **2009**, 8, 1250–1267. [CrossRef] [PubMed]

31. Ferrari, S.; Ischer, F.; Calabrese, D.; Posteraro, B.; Sanguinetti, M.; Fadda, G.; Rohde, B.; Bauser, C.; Bader, O.; Sanglard, D. Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. PLoS Pathog. **2009**, 5, e1000268. [CrossRef] [PubMed]

32. Polonelli, L.; Séguy, N.; Conti, S.; Gerloni, M.; Bertolotti, D.; Cantelli, C.; Magliani, W.; Cailliez, J.C. Monoclonal yeast killer toxin-like Candidacidal anti-idiotypic antibodies. Clin. Diagn. Lab. Immunol. **1997**, 4, 142–146. [PubMed]

33. Guyard, C.; Evrard, P.; Corbisier-Colson, A.M.; Louvart, H.; Dei-Cas, E.; Menozzi, F.D.; Polonelli, L.; Cailliez, J. Immuno-crossreactivity of an anti-*Pichia anomala* killer toxin monoclonal antibody with a *Williopsis saturnus* var. mrakii killer toxin. Med. Mycol. **2001**, 39, 395–400. [CrossRef] [PubMed]

34. Guyard, C.; Dehecq, E.; Tissier, J.-P.; Polonelli, L.; Dei-Cas, E.; Cailliez, J.-C.; Menozzi, F.D. Involvement of beta-glucans in the wide-spectrum antimicrobial activity of *Williopsis saturnus* var. mrakii MUCL 41968 killer toxin. Mol. Med. **2002**, 8, 686–694. [PubMed]

35. López-Martínez, R. Candidosis, a new challenge. Clin. Dermatol. **2010**, 28, 178–184. [CrossRef] [PubMed]

36. Pfaller, M.A.; Diekema, D.J. Epidemiology of invasive candidiasis: A persistent public health problem. Clin. Microbiol. Rev. **2007**, 20, 133–163. [CrossRef] [PubMed]

37. Spampinato, C.; Leonardi, D. *Candida* infections, causes, targets, and resistance mechanisms: Traditional and alternative antifungal agents. Biomed. Res. Int. **2013**, 2013, 204237. [CrossRef] [PubMed]

38. Polonelli, L.; Archibusacci, C.; Sestito, M.; Morace, G. Killer system: A simple method for differentiating *Candida albicans* strains. J. Clin. Microbiol. **1983**, 17, 774–780. [PubMed]

39. Polonelli, L.; Conti, S.; Gerloni, M.; Campani, L.; Mantovani, M.P.; Morace, G. Production of yeast killer toxin in experimentally infected animals. Mycopathologia **1990**, 110, 169–175. [CrossRef] [PubMed]

40. Guo, F.-J.; Ma, Y.; Xu, H.-M.;Wang, X.-H.; Chi, Z.-M. A novel killer toxin produced by the marine-derived yeast *Wickerhamomyces anomalus* YF07b. Antonie Van Leeuwenhoek **2013**, 103, 737–746. [CrossRef] [PubMed]

41. ˙Izgü, F.; Altınbay, D. Isolation and characterization of the K5-type yeast killer protein and its homology with an exo-beta-1,3-glucanase. Biosci. Biotechnol. Biochem. **2004**, 68, 685–693. [CrossRef] [PubMed]

42. Wang, L.; Yue, L.; Chi, Z.; Wang, X. Marine killer yeasts active against a yeast strain pathogenic to crab *Portunus trituberculatus*. Dis. Aquat. Organ. **2008**, 80, 211–218. [CrossRef] [PubMed]

43. Mathews, H.L.; Conti, S.; Witek-Janusek, L.; Polonelli, L. Effect of *Pichia anomala* killer toxin on *Candida albicans*. Med. Mycol. **1998**, 36, 199–204. [CrossRef] [PubMed]

44. Polonelli, L.; Beninati, C.; Teti, G.; Felici, F.; Ciociola, T.; Giovati, L.; Sperindè, M.; Passo, C.L.; Pernice, I.; Domina, M.; et al. Yeast killer toxin-like Candidacidal Ab6 antibodies elicited through the manipulation of the idiotypic cascade. PLoS ONE **2014**, 9, e105727. [CrossRef] [PubMed]

45. Gopal, P.; Sullivan, P.A.; Shepherd, M.G. Isolation and structure of glucan from regenerating spheroplasts of *Candida albicans*. Microbiology **1984**, 130, 1217–1225. [CrossRef] [PubMed]

46. ˙Izgü, F.; Altınbay, D.; Acun, T. Killer toxin of Pichia anomala NCYC 432; purification, characterization and its exo-β-1,3-glucanase activity. Enzyme Microb. Technol. **2006**, 39, 669–676. [CrossRef]

47. Chi, Z.; Chi, Z.; Zhang, T.; Liu, G.; Li, J.; Wang, X. Production, characterization and gene cloning of the extracellular enzymes from the marine-derived yeasts and their potential applications. Biotechnol. Adv. **2009**, 27, 236–255. [CrossRef] [PubMed]

48. Polonelli, L.; Lorenzini, R.; De Bernardis, F.; Morace, G. Potential therapeutic effect of yeast killer toxin. Mycopathologia **1986**, 96, 103–107. [CrossRef] [PubMed]

49. Franz, R.; Kelly, S.L.; Lamb, D.C.; Kelly, D.E.; Ruhnke, M.; Morschhäuser, J. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob. Agents Chemother. **1998**, 42, 3065–3072. [PubMed]

50. Kanafani, Z.A.; Perfect, J.R. Resistance to antifungal agents: Mechanisms and clinical impact. Clin. Infect. Dis. **2008**, 46, 120–128. [CrossRef] [PubMed]

51. Xu, Y.; Sheng, F.; Zhao, J.; Chen, L.; Li, C. ERG11 mutations and expression of resistance genes in fluconazole-resistant *Candida albicans* isolates. Arch. Microbiol. **2015**, 197, 1087–1093. [CrossRef] [PubMed]

52. Coste, A.T.; Karababa, M.; Ischer, F.; Bille, J.; Sanglard, D. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters CDR1 and CDR2. Eukaryot. Cell **2004**, 3, 1639–1652. [CrossRef] [PubMed]

53. Gong, F.; Sheng, J.; Chi, Z.; Li, J. Inulinase production by a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the crude inulinase. J. Ind. Microbiol. Biotechnol. **2007**, 34, 179–185. [CrossRef] [PubMed]

2.5. Article 5

A rapid qPCR method to investigate the circulation of the yeast *Wickerhamomyces anomalus* in humans

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A rapid qPCR method to investigate the circulation of the yeast *Wickerhamomyces anomalus* in humans

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Summary

The yeast *Wickerhamomyces anomalus* has been proposed for many biotechnological applications in the food industry. However, a number of opportunistic pathogenic strains have been reported as causative agents of nosocomial fungemia. Recognition of potentially pathogenic isolates is an important challenge for the future commercialization of this yeast. The isolation of *W. anomalus* from different matrices and, recently, from mosquitoes, requires further investigations into its circulation in humans. Here we present a qPCR protocol for the detection of *W. anomalus* in human blood samples and the results of a screening of 525 donors, including different classes of patients and healthy people.

KEY WORDS: Wickerhamomyces anomalus, opportunistic pathogen, fungemia, qPCR, mosquito vectors.

Wickerhamomyces anomalus (also designated as *Pichia anomala*, *Hansenula anomala* and *Candida pelliculosa*) is an ascomycete yeast well known for its antimicrobial properties, that have led to this microorganism being considered a biocontrol agent (Walker, 2011). Some examples of applications of *W. anomalus* are: in food/feed biopreservation (Olstorpe and Passoth, 2011), as a probiotic additive (Zuo *et al*., 2013) and as a volatile aroma enhancer in wine making (Swangkeaw *et al*., 2009). This yeast has been isolated from different habitats or

matrices like food and feed systems (Walker, 2011) and even from insects (Toki *et al*., 2012, Ricci *et al*., 2011a). The identification of antimicrobial *W. anomalus* strains in *Anopheles* and *Aedes* mosquitoes have led this yeast being proposed also in the biocontrol of malaria (Cappelli *et al*., 2014, Ricci *et al*., 2011b) and other mosquito- borne diseases (Ricci *et al*., 2011a). With regard to food safety aspects, *W. anomalus* is classed at biosafety level 1 by the European Food Safety Authority (De Hoog, 1996). In addition, there are no reports in the literature on hazardous mycotoxin formation or allergic reactions to the spores from this yeast. However, a number *W. anomalus* strains have been isolated from humans, raising the possibility that this yeast is an emerging opportunistic pathogen (Hazen, 1995, Kalkanci *et al*., 2010). In particular, reports over the last decades have highlighted an increase in nosocomial yeast bloodstream infections, particularly pediatric outbreaks, due to uncommon species, including *W. anomalus* (Taj-Aldeen *et al*., 2014). *W. anomalus* infections have been reported in cancer patients (Thuler *et al.*, 1997), infants and premature neonates (Aragão *et al*., 2001, Oliveira *et al*., 2014), and in patients in surgical intensive care units (Kalenic *et al*., 2001). Possible explanations for the emergence of opportunistic pathogenic yeasts could be the increase in the number of patients at risk, such as immunocompromised persons, and the contribution of more sensitive diagnostic methods. However, the reasons for the increased incidence of fungemia from uncommon pathogenic yeasts are not completely clear. Differentiating non-pathogenic strains from potential opportunistic pathogens is necessary for future biotechnological applications of *W. anomalus* (Passoth *et al*., 2011). In addition, the recent isolation of a *W. anomalus* strain from different mosquito species (Ricci *et al*., 2011a) has set a new alert, considering that hematophagous insects might play a role in spreading this microorganism.

Here, we present a qPCR protocol for the detection of *W. anomalus* using species-specific primers previously designed (Huang *et al*., 2012), aimed at improving the current procedures for the rapid diagnosis of infections by this yeast. This optimized qPCR protocol was applied in a screening study to estimate the circulation of *W. anomalus* in human blood samples from different classes of patients (including immunocompromised) and from healthy donors.

We carried out a retrospective analysis using previously collected blood samples. A total of 525 donors were examined by qPCR (Table 1): 243 blood samples, collected between January 2011 and November 2012 from critically ill patients (adult and pediatric patients with suspicion of sepsis) admitted to the Intensive Care Units and Haematology Units (ICUs & HU) at the St. Orsola Malpighi University Hospital of Bologna; 183 blood samples, collected at the S. Matteo Hospital of Pavia in the context of another study, from 20 HIV, 61 HCV, and 19 HIV/ HCV

positive patients, and 83 healthy donors; 99 blood samples from additional 81 healthy donors collected at St. Orsola Malpighi, 10 malaria patients collected at Spedali Civili di Brescia (Institute for Infectious and Tropical Diseases, IITD) and 8 workers at the insectary of the University of Camerino (Unicam). All of the samples included in the present study were obtained and analysed in strict accordance with the current European and Italian rules on informed consent.

The results of this screening for *W. anomalus* (*Wa*) in blood samples required the development of a highly specific and sensitive qPCR assay. For the protocol optimization 12 yeast isolates (eight representing *W. anomalus*; four representing other yeast species) were used as control strains: *Wa*F17.12, *Wa*M9.11, *Candida* sp. and *Pichia* sp. isolated from the malaria vector mosquito *An. stephensi* (Ricci *et al*., 2011b); the environmental strains *Wa*ATCC 996603 and *Wa*UM3 (Polonelli *et al*., 1997), *Saccaromyces cerevisiae* ATCC 2601 and *Williopsis saturnus* var. *mrakii* (Guyard *et al*., 2002); four clinical isolates of *W. anomalus* from four neonates hospitalized in an intensive care unit (Kalkanci *et al*., 2010). In the qPCR standard curve setup, a target sequence of 218 bp of β-tubulin gene was amplified using the specific primers SpWanom-170F (5'TTATCCATCCACCAATTG3') and SpWanom-374R (5'GGAACTAAGTTCACAGCTA3') (Huang *et al*., 2012). The primers were previously tested against yeast genomic DNAs from all of 12 isolates listed above.

TABLE 1 - *Summary of the screening for Wickerhamomyces anomalus (Wa) in human blood samples.*

Human samples and health or disease status	*Samples origin*	*Wa-qPCR assay results positive/total*
Critically ill patients	St. Orsola Malpighi/ICUs & HU, University Hospital of Bologna	0/243* (36 pediatric cases; 207 adults cases)
HIV positive patients	S. Matteo, Hospital of Pavia	0/20
HCV positive patients	S. Matteo, Hospital of Pavia	1/61
HIV/HCV positive patients	S. Matteo, Hospital of Pavia	0/19
Malaria patients	Spedali Civili di Brescia/ IITD	0/10
Donors exposed to mosquito bites at insectary	Unicam	0/8
Healthy blood donors	S. Matteo, Hospital of Pavia	0/83
Healthy blood donors	St. Orsola Malpighi, University Hospital of Bologna	0/81
Total		1/525

*All of these patients displayed at least two signs among the criteria of the systemic inflammatory response syndrome (Kaukonen *et al.*, 2015).

The expected amplicon was obtained from all eight *W. anomalus* isolates tested (two isolated from mosquito, two environmental and four clinical isolates), while no amplification was

obtained from the other four yeast isolates, representing other species (*Candida* sp., *Pichia* sp., *Saccaromyces cerevisiae* ATCC 2601 and *Williopsis saturnus* var. *mrakii*).

To evaluate the amplification efficiency of the selected primers, the PCR product from *Wa*F17.12 was cloned into a plasmid vector (pGEM-T Easy Vector System, Promega, Wisconsin, USA) and the amount of the recombinant plasmid was determined. We carried out a standard curve, using as DNA templates eight serial dilutions (10^{-1}) of the recombinant plasmid, corresponding to around $2.3x10^7$ – 2.3 gene copies per µl of reaction. qPCR amplifications were carried out with the β-tubulin gene primers (200 nM each) using Sybr Green Master Mix (Fermentas, Burlington, Canada) in a final volume of 25 µl. Amplification cycling conditions were as follows: 10' at 95°C; 1' at 95°C, 1' at 52°C, 30'' at 72°C for 40 cycles. A final step for the melting curve analysis from 65°C to 95°C (increment of +0.5°C) was performed and the relative dissociation curve displayed a single specific dissociation peak at 74.50°C. The obtained standard curve showed high sensitivity, up to around 2.3 gene copies per µl of PCR reaction were detected.

After the validation The qPCR protocol was then validated mixing *W. anomalus* cells with human blood samples. We tested the strain *Wa*F17.12 and the four clinical isolates of *W. anomalus*. The yeasts were grown in YPD medium and incubated overnight at 28°C at 110 rpm to obtain an optical density 1.0 (3.2x107 cells/ml). Yeast cells were collected by centrifugation at 3500 rcf, 4°C for 10' and diluted in sterile water. Eight serial dilutions from 3.2 x107 cells to 3.2 cells were obtained in a final volume of 200 µl of human blood samples from a healthy donors (in triple replicates for each isolates). Total DNAs were extracted using the Jet Quick Blood DNA Spin Kit (Gentaur, Löhne, Germany) and suspended in 50 µl of sterile water. Fifty ng of the purified DNAs were used as templates for qPCR analysis at the same conditions described for the standard curve set-up. The relative dissociation curves confirmed the same melting temperature as the standard curve, while no specific peak was detected in the negative control reactions carried out with pure blood samples. The sequence analysis of the amplified product confirmed 100% homology with the GeneBank *W. anomalus* partial sequence of ß-tubulin gene available in GeneBank (JQ734945.1). The qPCR assay was able to detect up to around 16 yeast cells per ml of blood sample, for all the tested isolates (*Wa*F17.12 and four *W. anomalus* clinical isolates).

After the validation of the *Wa*-qPCR assay, we proceeded to screen the 525 selected blood samples. The DNA from these samples was obtained as described in Epis *et al*. (2012) and

quantity and quality of the extracted samples were checked by spectrophotometer. Each sample was tested in triplicate (both undiluted and at a 1:10 dilution) and the positive control (DNA from *Wa*F17.12 strain) was included. The template DNAs were subjected to the *Wa*-qPCR assay at the amplification conditions previously described. In addition, an amplification control was performed by running a parallel PCR test targeting the human Beta-2-microglobulin. Beta-2-microglobulin amplification was carried out using a new primer set, Bmicr2F (5'CTCCGTGGCCTTAGCTGTG3') and Bmicr2R (5'TTTGGAGTACGCTGGATAGCCT3') (250 nM each) that amplifies a 69 bp fragment, following the conditions described for *W. anomalus* detection except for the annealing step (60°C for 30'').

The present study analysed a heterogeneous group, including healthy donors and different types of patients (critically ill adults and children, HIV and HCV patients, malaria patients), blood donors exposed to mosquito bites and healthy donors, in order to have an overview of the yeast circulation in humans exposed at different types of risk. The screening revealed an almost complete negativity of the analysed samples, with the exception of a single HCV patient that tested positive (3.9 copies of ß-tubulin gene per µl of reaction) at the *Wa*-qPCR assay (Table 1). Sequence analysis of the amplified product revealed the perfect match of the amplified fragment with the β-tubulin gene from *W. anomalus* (JQ734945.1). This newly obtained sequence was deposited in EMBL-EBI under the accession number LN680997. In addition, to confirm the positivity of this sample and the negativity of 30 samples (a sub-sample, casually selected), we performed a nested-PCR targeted at 18S rRNA gene, for the specific *W. anomalus* detection following the protocol published in Ricci *et al.,* 2011b. Application of this further method to the positive sample and to 30 samples that were negative at the first PCR confirmed our results.

Our results support the view of *W. anomalus* as an organism considered safe for healthy individuals and are coherent with the evidence of an uncommon opportunistic yeast (Taj-Aldeen *et al*., 2014). As stated in a recent review of the literature, *W. anomalus* infections have been reported occasionally and the occurrence of a few hundred positive cases (single cases and outbreaks) worldwide is estimated over a period longer than twenty years (Kalkanci *et al*., 2010). We found a single case out of 353 immunocompromised patients (St. Orsola Malpighi, 243; Spedali Civili di Brescia, 10; S. Matteo Hospital, 100) and 164 healthy donors screened. Interestingly, no positivity was found in the malaria patients (exposed to malaria vector mosquito bites in Burkina Faso) and in the workers at the insectary (exposed to *Anopheles* and

Aedes mosquitoes bite and to *W. anomalus* cultures). Nevertheless, the recognition of potentially pathogenic isolates is required for any future biotechnological applications of this yeast as a biocontrol agent. Our quantitative assay aimed at performing rapid mass screenings may effectively contribute to further investigations necessary for a better understanding of the epidemiology of *W. anomalus* infections.

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REFERENCES

Aragao P.A., Oshiro I.C., Manrique E.I., Gomes C.C., Matsuo L.L., Leone C., Moretti-Branchini M.L., Levin A.S. (2001). *Pichia anomala* outbreak in a nursery: exogenous source? *Pediatr. Infect. Dis. J.* **20**, 843-848.

Cappelli A., Ulissi U., Valzano M., Damiani C., Epis S., Gabrielli M.G., Conti S., Polonelli L., Bandi C., Favia G., Ricci I. (2014). A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*. *PLoS One*. **9**, e95988.

De Hoog G.S. (1996). Risk assessment of fungi reported from humans and animals. *Mycoses*. **39**, 407-417.

Epis S., Gaibani P., Ulissi U., Chouaia B., Ricci I., Damiani C., Sambri V., Castelli F., Buelli F., Daffonchio D., Bandi C., Favia G. (2012). Do mosquito-associated bacteria of the genus *Asaia* circulate in humans? *Eur. J. Clin. Microbiol. Infect. Dis.* **31**, 1137-1140.

Guyard C., Seguy N., Cailliez J.C., Drobecq H., Polonelli L., Dei-Cas E., Mercenier A., Menozzi F.D. (2002). Characterization of a *Williopsissaturnus* var. *mrakii* high molecular

weight secreted killer toxin with broad-spectrum antimicrobial activity. *J. Antimicrob. Chemother.* **49**, 961-971.

Hazen K.C. (1995). New and emerging yeast pathogens. *Clin. Microbiol. Rev*. **8**, 462-478. Huang C.H., Chang M.T., Huang L. (2012). Species identification of *Wickerhamomyces anomalus* and related taxa using β-tubulin (β-tub) DNA barcode marker. *Yeast*. **29**, 531-535.

Kalenic S., Jand rlic M., Vegar V., Zuech N., Sekulic A., Mlinaric-Missoni E. (2001). *Hansenula anomala* outbreak at a surgical intensive care unit: a search for risk factors. *Eur. J. Epidemiol.* **17**, 491-496.

Kalkanci A., Dizbay M., Turan O., Fidan I., Yalcin B., Hirfanog˘lu I., Kus¸ timur S., Aktas¸ F., Sugita T. (2010). Nosocomial transmission of *Candida pelliculosa* fungemia in a pediatric intensive care unit and review of the literature. *Turk. J. Pediatr.* **52**, 42-49.

Kaukonen K.M., Bailey M., Pilcher D., Cooper D.J., Bellomo R. (2015). Systemic inflammatory response syndrome criteria in defining severe sepsis. *N. Engl. J. Med.* **23,** 1629-1638.

Oliveira V.K., Ruiz Lda S., Oliveira N.A., Moreira D., Hahn R.C., Melo A.S., Nishikaku A.S., Paula C.R. (2014). Fungemia caused by *Candida* species in a children's public hospital in the city of São Paulo, Brazil: study in the period 2007-2010. *Rev. Inst. Med. Trop. Sao Paulo*. **56**, 301-305.

Olstorpe M., Passoth V. (2011). *Pichia anomala* in grain biopreservation. *Antonie Van Leeuwenhoek*. **99**, 57-62. Passoth V., Olstorpe M., Schnurer J. (2011). Past, present and future research directions with *Pichia anomala*. *Antonie Van Leeuwenhoek*. **99**, 121-125.

Polonelli L., Seguy N., Conti S., Gerloni M., Bertolotti D., Cantelli C., Magliani W., Cailliez J.C. (1997). Monoclonal yeast killer toxin-like Candidacidal anti-idiotypic antibodies. *Clin. Diagn. Lab. Immunol.* **4**, 142-146.

Ricci I., Mosca M., Valzano M., Damiani C., Scuppa P., Rossi P., Crotti E., Cappelli A., Ulissi U., Capone A., Esposito F., Alma A., Mandrioli M., Sacchi L., Bandi C., Daffonchio D., Favia G. (2011a). Different mosquito species host *Wickerhamomyces anomalus* (*Pichia anomala*): perspectives on vector- borne diseases symbiotic control. *Antonie Van Leeuwenhoek*. **99**, 43-50.

Ricci I., Damiani C., Scuppa P., Mosca M., Crotti E., Rossi P., Rizzi A., Capone A., Gonella E., Ballarini P., Chouaia B., Sagnon N., Esposito F., Alma A., Mandrioli M., Sacchi L., Bandi C., Daffonchio D., Favia G. (2011b). The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*. *Environ. Microbiol.* **13**, 911-921.

Swangkeaw J., Vichitphan S., Butzke C.E., Vichitphan K. (2009). The characteristics of a novel *Pichia anomala* b-glucosidase with potentially aroma- enhancing capabilities in wine. *Ann. Microbiol.* **59**, 335-343.

Taj-Aldeen S.J., Abdulwahab A., Kolecka A., Deshmukh A., Meis J.F., Boekhout T. (2014). Uncommon opportunistic yeast bloodstream infections from Qatar. *Med. Mycol.* **52**, 552-556.

Thuler L.C., Faivichenco S., Velasco E., Martins C.A., Nascimento C.R., Castilho I.A. (1997). Fungaemia caused by *Hansenula anomala*: an outbreak in a cancer hospital. *Mycoses*. **40**, 193-196.

Toki W., Tanahashi M., Togashi K., Fukatsu T. (2012). Fungal farming in a non-social beetle. *PLoS One*. **7**, e41893. Walker G.M. (2011). *Pichia anomala*: cell physiology and biotechnology relative to other yeasts. *Antonie Van Leeuwenhoek*. **99**, 25-34.

Zuo R.Y., Chang J., Yin Q.Q., Wang P., Yang Y.R., Wang X., Wang G.Q., Zheng Q.H. (2013). Effect of the combined probiotics with aflatoxin B1-degrading enzyme on aflatoxin detoxification, broiler production performance and hepatic enzyme gene expression. *Food. Chem. Toxicol*. **59**, 470-475.

3. CONCLUSIONS

3.1. The mycobiota of sand flies.

In this thesis, I reported my study of the mycobiota associated with the sand fly *Phlebotomus perniciosus*, with the main goal to identify microoorganisms useful for developing new strategies for the control of vector-borne diseases (VBDs). Sand flies are the only proven vectors of leishmaniasis (Killick-Kendrick, 1999), a re-emerging parasitic disease that affects people living in 98 countries worldwide. Traditional control methods for fighting VBDs, such as leishmaniasis, have focused on the control of the vector population, mainly with insecticides. For VBDs control, World Health Organization (WHO) encourages an integrated approach in which chemicals and biological methods for the control of vectors are combined. Among these methods, the symbiotic control, a biological approach that exploits microorganisms that live in symbiosis with vectors of parasites to reduce their vectorial capacity or interfere with the transmission of the pathogen, is attracting interest.

The aim of my work was to investigate the fungal community of a sand fly in order to identify yeasts that could be proposed as candidates for the development of strategies of symbiotic control.

The first research article I presented (Martin et al., 2018, see Article 1) is the description of the fungal community of the sand fly *P. perniciosus*, the main vector of leishmaniasis in the western Mediterranean area and the most widespread sand fly in Italy (Busani et al., 2012), with a focus on the dominant yeast species *Meyerozyma guilliermondii*. This yeast was also the focus of the study reported in the second article (De Marco et al., 2018, see Article 2), in which yeast strains of the *M. gilliermondii* complex isolated from arthropods were subjected to whole-genome sequencing, *de novo* assembly and phylogenomic analysis. *M. gilliermondii* is an ascomycetous yeast that appeals for biotechnological applications thanks to its ability to produce riboflavin, convert xylose in xylitol, be source of enzymes, biofuel and aromas. Furthermore, it has been used as a biocontrol agent in agriculture and food industry because it showed antimicrobial activity against bacteria, fungi, and even nematodes and protozoa (Papon et al., 2013). The result of the study of the prevalence of this yeast in *P. perniciosus* populations leads us to hypothesize that, under laboratory conditions, *M. guilliermondii* can contributes to the diet of reared sand flies with nutrients which are on the other side easily available in nature. Moreover, the FISH and microscopic examination, in addition to the sequencing and analysis of the genome of *M. guilliermondii*, let us speculate that the yeast could also contribute to the removal of the excess of nitrogenous wastes after the blood meal of the insect host. Our investigation of the mycobiota of *P. perniciosus* can have a great impact in the understanding of some aspects

of the sand fly biology, such as the role of yeasts associated with the insect. The analysis carried out in the second work lead to publish the first four genomes of the species *M. caribbica* and to provide novel insights into the evolutionary history of yeasts of *M. guilliermondii* complex.

The last three articles (Martin et al., 2016, see Article 3; Giovati et al., 2018, see Article 4; Epis et al., 2015, see Article 5) are focused on another yeast species that was isolated from sand flies, *Wickerhamomyces anomalus*, with the aim of evaluate if it could be a good candidate for VBDs control and for the purification of molecules with potential therapeutic applications. This ascomycetous ubiquitous yeast is renowned for its biotechnological potential and has been studied as a promising agent of biocontrol and environmental bioremediation (Passoth et al., 2011). In fact, as *M. guilliermondii*, *W. anomalus* possesses antimicrobial activity towards microorganisms of different phylogenetic groups such as viruses, bacteria, fungi and yeasts (Passoth et al., 2011). The mechanisms of this activity include the production of β-1-3-glucanase, ethanol and ethyl acetate (Passoth et al., 2011). Recently this yeast was isolated from different mosquitoes of several genera (Ricci et al., 2011). Moreover, a strain isolated from the vector *Anopheles stephensi* is able to produce killer toxins active against sensitive yeast stains, bacteria (Cappelli et al., 2014) and malaria parasites (Valzano et al., 2016). In Article 3, I reported the isolation and phylogenetic characterization of a killer strain of *W. anomalus* from the sand fly *P. perniciosus*. In the study we hypothesized the production of a killer toxin and we speculated about the facultative nature of the symbiosis between *W. anomalus* and *P. perniciosus,* in which the yeast could provide benefices to the host, maybe exerting anti-*Leishmania* activity. The production of the killer toxin by the *W. anomalus* killer strain isolated from the sand fly was verified in the study presented in Article 4. The fourth article, indeed, is focused on the purification and characterization of the β-1,3-glucanase killer toxin from the stain isolated from *P. perniciosus* (*Wa*1F1-KT). Its antimicrobial activity *in vitro* was investigated against antimicrobial-resistant pathogenic yeast strains responsible for candidiasis, showing that *Wa*1F1-KT is active against *C. glabrata* isolates, independently from their drug-resistance phenotypes. The results obtained in *Wa*1F1-KT characterization suggest a potential application of this toxin against fungal infections at skin and mucosal membranes levels provoked by *C. glabrata* and may be useful for the design of new therapeutic molecules and the study of yeast resistance mechanisms. Finally, a biological control approach must take into consideration the assessment of the potential impact on human health of the biocontrol agent. This was the reason behind the study reported in Article 5, that was carried out to evaluate the yeast circulation in humans exposed at different types of risk. The screening revealed an almost

complete negativity of the analyzed samples, thus letting us hypothesize that it a yeast very rarely found in humans, even though potentially opportunistic, as demonstrated by a few clinical cases (Taj-Aldeen et al., 2014; Thuler et al., 1997; Aragão et al., 2001, Oliveira *et al*., 2014; Kalenic et al., 2001). This result is important in view of the possible application of *W. anomalus* for VBDs control.

3.2. Yeast as candidates for VBD control strategies.

After the study of the mycobiota associated with *P. perniciosus,* I took under consideration two yeast candidates for the development of VBD control strategies. Desirable characteristics of candidates for symbiotic control are: stable association with the host, dominance in the microbial community, localization in the same organs of the pathogen, easiness of cultivation and *in vitro* manipulation and vertical/horizontal transmission routes (Alma et al., 2010). Both of the considered yeasts (*M. guilliermondii* and *W. anomalus*) are easily cultivable *in vitro*. The first, *M. guilliermondii*, localizes in the midgut of the sand fly (place in which promastigote development occurs) and seems to be the dominant yeast symbiont of the examined reared sand fly samples, so it makes us suppose that can efficiently colonize/re-colonize wild populations after an eventual release. The second, *W. anomalus*, seems to be a promising organism for the development of symbiotic strategies aimed at reducing vectorial capacity and/or inhibiting the transmission of *Leishmania* and other pathogens and for the development of new therapeutics. In fact, in this thesis I showed that: i) a killer strain of the yeast is present in the sand fly *P. perniciosus*; ii) the same strain isolated from the sand fly produces a killer toxin active against etiological agents of non-*albicans* candidiasis; iii) the circulation of this yeast in humans is minimal.

3.3. Future developments.

The association between *P. perniciosus* and *W. anomalus* deserves further investigation in order to explore the possibility that this yeast may exert inhibitory/killing activity against *Leishmania* spp., as reported in the analysis of the purified yeast killer toxin against another protozoa, the rodent malaria parasite *Plasmodium berghei* (Valzano et al., 2016). Additional studies should also investigate whether killer toxin-producing strains of *W. anomalus* are commonly found in wild populations of *P. perniciosus* and the route of yeast transmission in sand flies, to better understand their role as non-obligate symbionts. In order to develop new therapeutic molecules based on *Wa*1F1-KT to combat infections caused by azole-resistant *Candida* strains, further studies aimed at its biochemical characterization and the elucidation of its mechanism of action are needed.

3.4. References.

- Alma A, Daffonchio D, Gonella E, Raddadi N. (2010). Microbial symbionts of *Auchenorrhyncha* transmitting phytoplasmas: a resource for symbiotic control of phytoplasmoses. In: Phytoplasmas: Genomes, Plant Hosts and Vectors, Weintraub P. & Jones P. Ed., CAB International, Wallingford, UK, pp. 272-292.
- Aragao PA, Oshiro IC, Manrique EI, Gomes CC, Matsuo LL, Leone C, Moretti-Branchini ML, Levin AS. (2001). *Pichia anomala* outbreak in a nursery: exogenous source? Pediatr Infect Dis J. 20, 843-848.
- Busani L, Mughini Gras L, Romi R, Boccolini D, Severini F, Bongiorno G, Khoury C, Bianchi R, Gradoni L, Capelli G. (2012). Zanzare, flebotomi e zecche: atlante bibliografico delle specie di interesse sanitario in Italia (1985-1989). Rapporti ISTISAN 12/22.
- Cappelli A, Ulissi U, Valzano M, Damiani C, Epis S, Gabrielli MG, Conti S, Polonelli L, Bandi C, Favia G, Ricci I. (2014). A *Wickerhamomyces anomalus* killer strain in the malaria vector *Anopheles stephensi*. PLoS One. 9(5).
- Kalenic S, Jand rlic M, Vegar V, Zuech N, Sekulic A, Mlinaric-Missoni E. (2001). *Hansenula anomala* outbreak at a surgical intensive care unit: a search for risk factors. Eur J Epidemiol. 17, 491-496.
- Killick-Kendrick R. (1999). The biology and control of Phlebotomine sandflies. Clin Dermatol. 17, 279-289.
- Oliveira VK, Ruiz Lda S, Oliveira NA, Moreira D, Hahn RC, Melo AS, Nishikaku AS, Paula CR. (2014). Fungemia caused by *Candida* species in a children's public hospital in the city of São Paulo, Brazil: study in the period 2007-2010. Rev Inst Med Trop Sao Paulo. 56, 301-305.
- Papon N, Savini V, Lanoue A, Simkin AJ, Crèche J, Giglioli-Guivarc'h N, Clastre M, Courdavault V, Sibirny AA. (2013). *Candida guilliermondii*: biotechnological applications, perspectives for biological control, emerging clinical importance and recent advances in genetics. Curr Genet. 59(3), 73-90.
- Passoth V, Olstorpe M, Schnürer J. (2011). Past, present and future research directions with *Pichia anomala*. Antonie van Leeuwenhoek. 99, 121-125.

- Ricci I, Damiani C, Scuppa P, Mosca M, Crotti E, Rossi P, Rizzi A, Capone A, Gonella E, Ballarini P, Chouaia B, Sagnon N, Esposito F,Alma A,Mandrioli M, Sacchi L, Bandi C, Daffonchio D, Favia G. (2011). The yeast *Wickerhamomyces anomalus* (*Pichia anomala*) inhabits the midgut and reproductive system of the Asian malaria vector *Anopheles stephensi*. Environ Microbiol. 13(4), 911-21.
- Taj-Aldeen SJ, Abdulwahab A, Kolecka A, Deshmukh A, Meis JF, Boekhout T. (2014). Uncommon opportunistic yeast bloodstream infections from Qatar. Med Mycol. 52, 552-556.
- Thuler LC, Faivichenco S, Velasco E, Martins CA, Nascimento CR, Castilho IA. (1997). Fungaemia caused by *Hansenula anomala*: an outbreak in a cancer hospital. Mycoses. 40, 193-196.
- Valzano M, Cecarini V, Cappelli A, Capone A, Bozic J, Cuccioloni M, Epis S, Petrelli D, Angeletti M, Eleuteri AM, Favia G, Ricci I. (2016). A yeast strain associated to *Anopheles* mosquitoes produces a toxin able to kill malaria parasites. Malar J. 15, 21.

4. OTHER PUBLISHED PAPERS

Environ Microbiol Rep. 2017 Oct;9(5):642-648. doi: 10.1111/1758-2229.12569. Epub 2017 Jul 21.

Mosquitoes can harbour yeasts of clinical significance and contribute to their environmental dissemination.

Bozic J[1], Capone A[1], Pediconi D[1], Mensah P[1], Cappelli A[1], Valzano M[1], Mancini MV[1], Scuppa P[1], Martin E[2], Epis S[2], Rossi P[1], Favia G[1], Ricci I[1].

Abstract

There is still a lack of studies on fungal microbiota in mosquitoes, compared with the number available on bacterial microbiota. This study reports the identification of yeasts of clinical significance in laboratory mosquito species: *Anopheles gambiae*, *Anopheles stephensi*, *Culex quinquefasciatus*, *Aedes albopictus* and *Aedes aegypti*. Among the yeasts isolated, they focused on the opportunistic pathogen *Candida parapsilosis*, since there is a need to better understand breakthrough candidaemia with resistance to the usual antifungals, which requires careful consideration in the broad-spectrum therapy, as documented in many clinical reports. *C. parapsilosis* occurs widely and has been isolated from diverse sources, including insects, which may contribute to its dissemination. In this study, it was isolated from the gut of *An. gambiae* and its presence in developmental stages and organs of different mosquito species was studied. Our results indicated that there was a stable association between *C. parapsilosis* and reared mosquitoes during the entire life cycle, and in adult male and female gut and gonads. A wide occurrence of *C. parapsilosis* was also documented in several populations of wild mosquitoes. Based on these findings, it can be said that mosquitoes might participate in the spreading of this opportunistic pathogen, not only as a carrier.

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

Varotto Boccazzi I[1], Ottoboni M[2], Martin E[1], Comandatore F[3], Vallone L[2], Spranghers T[4], Eeckhout M[5], Mereghetti V[6], Pinotti L[2], Epis S[1,3].

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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Anim Genet. 2015 Jun;46(3):333-6. doi: 10.1111/age.12290.

An ancient interlocus recombination increases class II MHC DQA diversity in sheep and other Bovidae.

Ballingall KT[1], Steele P, Lantier I, Cotelli M, Todd H, Lopez G, Martin E, Lantier F.

Abstract

Animals with fully characterised major histocompatibility complex (MHC) regions are often used to explore the molecular interactions that control the induction of adaptive immunity. The ovine MHC includes two DQA loci, termed DQA1 and DQA2. However, in a minority of haplotypes the DQA1 locus appears absent (DQA1 null) and is replaced by a second locus termed, DQA2-like. This raises a number of questions regarding the origins and function of the DQA2-like sequences. To address this, we have analysed DQA diversity associated with 10 MHC haplotypes, including two classified as DQA1 null. Pair-wise comparison between full-length DQA transcripts from each haplotype identified unique diversity throughout the DQA2-like sequences. Conserved orthologues of the DQA2-like sequences were identified in cattle and goat, and phylogenetic analysis clustered exons 1 and 2 with DQA2 whereas the remainder of the sequence clustered with DQA1. The DQA2-like allelic lineage appears functional and to have arisen from an ancient interlocus recombination between DQA1 and DQA2 loci which predates Bovidae speciation.

KEYWORDS: DQA2-like; MHC class II; cattle; goat; ovine

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J Dairy Sci. 2014 Oct;97(10):6326-33. doi: 10.3168/jds.2014-8351. Epub 2014 Aug 6.

Comparison of bacteriological culture and PCR for detection of bacteria in ovine milk--sheep are not small cows.

Zadoks RN[1], Tassi R[2], Martin E[2], Holopainen J[3], McCallum S[4], Gibbons J[5], Ballingall KT[2].

Abstract

Mastitis, inflammation of the mammary gland, is an important cause of disease, mortality, and production losses in dairy and meat sheep. Mastitis is commonly caused by intramammary infection with bacteria, which can be detected by bacterial culture or PCR. PathoProof (Thermo Fisher Scientific Ltd., Vantaa, Finland) is a commercially available real-time PCR system for the detection of bovine mastitis pathogens. Sheep differ from cattle in the bacterial species or bacterial strains that cause mastitis, as well as in the composition of their milk. The aim of this study was to evaluate whether the PathoProof system was suitable for detection of mastitis pathogens in sheep milk. Milk samples were collected aseptically from 219 udder halves of 113 clinically healthy ewes in a single flock. Aliquots were used for bacteriological culture and real-time PCR-based detection of bacteria. For species identified by culture, the diagnosis was confirmed by species-specific conventional PCR or by sequencing of a housekeeping gene. The majority of samples were negative by culture (74.4% of 219 samples) and real-time PCR (82.3% of 192 samples). Agreement was observed for 138 of 192 samples. Thirty-four samples were positive by culture only, mostly due to presence of species that are not covered by primers in the PCR system (e.g., *Mannheimia* spp.). Two samples were positive for *Streptococcus uberis* by culture but not by PCR directly from the milk samples. This was not due to inability of the PCR primers to amplify ovine *Streptococcus uberis*, as diluted DNA extracts from the same samples and DNA extracts from the bacterial isolates were positive by real-time PCR. For samples containing *Staphylococcus* spp., 11 samples were positive by culture and PCR, 9 by culture only, and 20 by PCR only. Samples that were negative by either method had lower bacterial load than samples that were positive for both methods, whereas no clear relation with species identity was observed. This study provides proof of principle that real-time PCR can be used for detection of mastitis pathogens in ovine milk. Routine use in sheep may require inclusion of primer sets for sheep-specific mastitis pathogens.

KEYWORDS: PCR; culture; mastitis; sheep

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Environ Entomol. 2014 Aug;43(4):913-22. doi: 10.1603/EN13228.

A new strain of *Wolbachia* in an alpine population of the viviparous *Oreina cacaliae* (Coleoptera: Chrysomelidae).

Montagna M[1], Chouaia B, Sacchi L, Porretta D, Martin E, Giorgi A, Lozzia GC, Epis S.

Abstract

Microbial symbionts played a central role in insect evolution. *Oreina cacaliae* (Schrank, 1785) (Coleoptera: Chrysomelidae) is a rare example of a viviparous insect, able to feed on toxic plants and sequester toxic compounds. In the current study, the microbiota associated with O. cacaliae was characterized using a culture-independent approach, targeting the 16S rRNA bacterial gene. The obtained 16S rRNA gene sequences were analyzed and identified at different taxonomic levels. Wolbachia was the dominant bacterium, both in male and female (100 and 91.9%, respectively) individuals; the detected Wolbachia was described as a new sequence type based on multilocus sequence typing (*Wolbachia* ST375 Ocac_A_wVdO). After phylogenetic analyses, *Wolbachia* ST375 Ocac_A_wVdO was attributed to the supergroup A. Immunofluorescence assays and electron microscopy confirmed the presence of *Wolbachia* within *O. cacaliae* oocytes, confirming its transovarial transmission in this species. Representatives of six species of *Oreina* were tested for the presence of *Wolbachia* through specific polymerase chain reaction, and a dendrogram was generated for these species based on coxI gene sequences. The *Wolbachia* harbored by different species of *Oreina* were characterized by multilocus sequence typing. Five out of the six examined Oreina species were positive for *Wolbachia*, with four of these harboring the same sequence type.

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Molecular and serological evidence for the circulation of the tick symbiont *Midichloria* (Rickettsiales: Midichloriaceae) in different mammalian species.

Bazzocchi C, Mariconti M, Sassera D, Rinaldi L, Martin E, Cringoli G, Urbanelli S, Genchi C, Bandi C, Epis S[1].

Abstract

BACKGROUND:

The Midichloriaceae is a novel family of the order Rickettsiales, that encompasses intracellular bacteria associated with hard ticks (Ixodidae) and other arthropods. The most intensively investigated member of this family is *Midichloria mitochondrii*, a symbiotic bacterium of the sheep tick *Ixodes ricinus*, characterized by the capacity of multiplying inside the mitochondria. A recent study suggested that these bacteria might be inoculated into the human host during the tick bite. The purpose of this study was to determine the potential infectivity of *Midichloria* bacteria for non-human animals exposed to the risk of tick bite.

METHODS:

Blood from horses, cattle, sheep and dogs exposed to the risk of tick bite was included in this study. DNAs were extracted, and amplified using 16S ribosomal RNA primers conserved in the *Midichloria* genus. Furthermore, sera from dogs exposed to the risk of tick bite were analyzed in order to evaluate the presence of antibodies against the recombinant flagellar protein (rFliD) from *M. mitochondrii* using an ELISA test.

RESULTS:

Here we present two lines of evidence that support the possibility that bacteria from the genus *Midichloria* are inoculated into vertebrate hosts during a tick bite: (i) a direct evidence, i.e. the detection of circulating DNA from bacteria related with *M. mitochondrii*, in the blood of vertebrates exposed to tick parasitism; (ii) a further indirect evidence, i.e. the presence of antibodies against an antigen from *M. mitochondrii* in dogs exposed to the risk of tick bite. It is interesting to note that variability was detected in the *Midichloria* gene sequences recovered from positive animals, and that some of these sequences were identical to those generated from tick-associated *Midichloria*.

CONCLUSIONS:

Based on the results, and on the overall information so far published on the genus *Midichloria*, we suggest that these bacteria are likely to represent a novel group of vector-borne agents, with the potential of infecting mammalian hosts. Whether inoculation of *Midichloria* bacteria could cause a true infection and pathological alteration in mammalian hosts is still to be determined. Surely, results emphasize the relevance of *Midichloria* bacteria in investigations on tick immunology and tick-bite markers.

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