Addictive manipulation: a perspective on the role of reproductive parasitism in the evolution of bacteria-eukaryote symbioses

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Abstract

Wolbachia bacteria encompass noteworthy reproductive manipulators of their arthropod hosts. which influence host reproduction to favour their own transmission, also exploiting toxin-antitoxin systems. Recently, multiple other bacterial symbionts of arthropods have been shown to display comparable manipulative capabilities.

Here we wonder whether such phenomena are truly restricted to arthropod hosts. We focused on protists, primary models for evolutionary investigations on eukaryotes due to their diversity and antiquity, but still overall under-investigated.

After a thorough re-examination of the literature on bacterial-protist interactions with this question in mind, we conclude that such bacterial "addictive manipulators" of protists do exist, are probably widespread, and have been overlooked until now as a consequence of the fact that investigations are commonly host-centred, thus ineffective to detect such behaviour.

Additionally, we posit that toxin-antitoxin systems are crucial in these phenomena of addictive manipulation of protists, as a result of recurrent evolutionary repurposing. This indicates intriguing functional analogy and molecular homology with plasmid-bacterial interplays.

Finally, we remark that multiple addictive manipulators are affiliated to specific bacterial lineages with ancient associations with diverse eukaryotes. This suggests a possible role of addictive manipulation of protists in paving the way to the evolution of bacteria associated with multicellular organisms.

Keywords

Toxin-antitoxin systems, *Wolbachia*, intracellular bacteria, *Rickettsiales*, *Holosporales*, professional symbionts, protist

Overview and purposes

Multiple diverse bacteria live in association with a great phylogenetic and ecological variety of eukaryotic hosts (Rosenblueth and Martínez-Romero 2006; McFall-Ngai et al. 2013; Husnik et al. 2021). Such symbiotic associations are widespread, and exhibit different shades of reciprocal effects on the involved partners, ranging from mutualism to parasitism (Sapp 2004). Even the same partnership can vary along this spectrum, depending on physiological and life cycle states or on external conditions (Regus et al. 2015; Herrera et al. 2020). Along evolution, the functional properties of the symbiotic partners can be deeply influenced by the association (Moran 2007). Among this plethora of diverse associations, several systems have attracted the attention of researchers, mostly pathogens (Akira et al. 2006) and mutualists (Moran et al. 2008).

Another noteworthy and peculiar type of bacterial-host interaction is reproductive manipulation, exerted by some phylogenetically diverse bacteria (e.g., *Wolbachia*) on their arthropod hosts, with cytoplasmic incompatibility (CI) as the most distinctive instance (Werren et al. 2008; Shropshire et al. 2020). As a result, the new host generation from an infected male cannot survive unless it receives the bacterial symbiont from the female (Figure 1). This tight association might superficially seem to match an obligatory mutualism. However, rather than being powered by some benefit provided to the host, it is due to the ability of the bacterium to make the host unable to get rid of it, namely to "addict" the host (see below paragraph "*Wolbachia*, a prototypical addictive manipulator" for details).

A recent work explored the concept of "evolutionary addiction" from the perspective of the host (Hammer 2023). Therein, it was proposed that hosts having experienced prolonged associations with their microbiome may evolve some dependence on the bacteria, thus becoming secondarily addicted to them (see Box 1 for details).

Still, addiction may also be the direct and primary consequence of active mechanisms exerted by the bacteria on their hosts, as in the case of CI in *Wolbachia*. One could wonder whether such primary addictions are evolutionary oddities restricted to a few specific cases, or the phenomenon has wider evolutionary and ecological significance. Here we follow this line of thought, exploring

the presence and role of addiction in host-bacterial interactions from the perspective of the bacterial symbionts and their evolution, rather than sticking to a host-centric approach, the more "conventional" one in the study of bacterial-eukaryotic symbioses. In particular, with the goal to expand our view, we focus on unicellular eukaryotic hosts (i.e., protists). Protists constitute the vast majority of eukaryotic lineages including the most ancestral ones (Adl et al. 2019; Keeling and Burki 2019), thus being fundamental for understanding the eukaryotic features and their evolution (O'Malley et al. 2013). Moreover, protists host multiple diverse bacterial symbionts (Husnik et al. 2021), however, such symbioses are neglected, and in most cases their functional and evolutionary foundations still await to be understood. Given the distinctive and diverse life cycles, physiology and ecology of protists (Caron et al. 2012; Geisen et al. 2018; Burki et al. 2021), these associations rarely and only partly fit to some of the most widely used "reference" models in bacterial-host symbioses, such as nutritional bacterial mutualists of animal hosts (Husnik et al. 2021).

Here we reason on whether the evolutionary origin and maintenance of associations between bacteria and protists could be explained by considering another kind of interaction between bacteria and eukaryotes, already known in animals, namely "addictive manipulation" of host reproduction. Therefore, we carefully examine the literature on bacterial-protist associations in search of indications of potential addictive phenomena and mechanisms. According to several lines of evidence, we propose that addictive manipulation (Figure 1; Box 1) is widely diffused, though yet not properly recognised, among bacterial-protist associations, and is possibly a key feature in the evolution of many such interactions.

Below, we will start with a presentation of the most relevant features of well-studied addictive manipulators in arthropods, employing *Wolbachia* as a main example. Then, we will move to symbioses between bacteria and protists, reasoning on which would be the expected features of addictive manipulation in those associations, and on why, in our view, available clues have not been properly recognised to date. Subsequently, we will focus on selected cases of bacterial-protist symbioses in which we could find convincing signs of addictive manipulation taking place. We will show how re-interpreting those previous findings allowed us to draw a framework on the evolution

of addictive manipulation in protists and other eukaryotic hosts, also accounting for possible underlying molecular mechanisms.

We will then conclude with a general evolutionary perspective on addictive manipulation and its role in the evolution of bacterial lineages with evolutionarily conserved interactions with protists and other eukaryotes. Considering that several bacteria hosted by protists are phylogenetically close to others hosted by multicellular organisms (Duron et al. 2018; Dharamshi et al. 2020; Castelli et al. 2024), the herein presented framework may reinforce and shed novel light on previous notions of protists as "Trojan horses" or "melting pots" for the evolution of eukaryote-associated bacteria (Barker and Brown 1994; Wang and Wu 2017).

Wolbachia, a prototypical addictive manipulator

As outlined above, reproductive manipulation is a quite well known phenomenon in arthropod hosts, which can be made addicted by multiple phylogenetically diverse bacterial symbionts. These include *Wolbachia* (*Rickettsiales*) (Taylor et al. 2005; Werren et al. 2008; Hurst and Frost 2015; Chen et al. 2020; Shropshire et al. 2020), *Spiroplasma* (*Mollicutes*) (Pollmann et al. 2022), *Cardinium* (*Cytophagales*) (Nguyen et al. 2017), *Rickettsiella* (*Legionellales*) (Rosenwald et al. 2020), *Rickettsia* (*Rickettsiales*) (Perlman et al. 2006; Gillespie et al. 2018), and the recently discovered *Mesenetia* (*Rickettsiales*) (Takano et al. 2021).

Wolbachia is the most studied, and noteworthy enough to deserve the title of "master manipulator of invertebrate biology" (Werren et al. 2008). Here we will use this symbiont as a main example to delineate the major features of addictive manipulators. *Wolbachia* is widespread in insects and other arthropods (Weinert et al. 2015; Sazama et al. 2017), thanks to multiple strategies enhancing its vertical transmission through host generations, namely feminisation, parthenogenesis, male killing, and CI (Werren et al. 2008). CI is an intriguing process, which makes crosses between infected males and non-infected females non-viable, thus indirectly favouring the fitness of infected females.

Since the symbionts' vertical inheritance relies solely on transovarial transmission from the mother to the offspring, the bacteria increase their own fitness, often massively, by favouring the reproduction of infected females (Figure 1). The effect of CI is so powerful that it is being used for biocontrol of arthropod vectors of pathogens, with great success (Hoffmann et al. 2011; Utarini et al. 2021).

While reproductive manipulation has been known for a long time, its molecular mechanisms have been elusive until recently (Harumoto and Lemaitre 2018; Chen et al. 2020; Shropshire et al. 2020; McNamara et al. 2024). A generalised modification-rescue model had been proposed for CI (Werren 1997), under which some bacterial-derived factor "poisons" the male gametes, leading to the unsuccessful development of the zygote, and can be counteracted only by the presence of a rescue factor in the infected female gametes. Two *Wolbachia* proteins responsible for these mechanisms were recently discovered (Beckmann et al. 2017; LePage et al. 2017), and shown to form a molecular complex, which can act by a toxin-antitoxin (or "toxin-antidote") regulation (Hochstrasser 2022) (Figure 1). The toxic effect is probably dysregulation of the ubiquitination (Beckmann et al. 2017; Harumoto 2023; Terretaz et al. 2023), linked to observed cytological defects in condensation of the male pronuclei (Tram et al. 2003; Shropshire et al. 2020). Interestingly, the two involved genes are adjacent in the *Wolbachia* genome, located in a putative phage-derived region, and their conditional expression appears to be linked to prophage induction (LePage et al. 2017). Several paralogs to these genes are present in different *Wolbachia* strains, and may account for mechanisms of reproductive manipulation other than CI, host specificities, and/or competition between *Wolbachia* strains (LePage et al. 2017; Harumoto and Lemaitre 2018; Lindsey et al. 2018). Among the very few homologs of these genes outside *Wolbachia*, notable are those found in *Rickettsia* and *Spiroplasma* (Gillespie et al. 2018). Taken together, these data indicate a spread of factors inducing CI by horizontal gene transfer (HGT), possibly driven by phages, suggesting that other symbionts could, by molecularly homologous mechanisms, be in fact analogous "master manipulators".

Addictive manipulation of unicellular eukaryotic hosts

Wolbachia and the other cases listed above show that addictive manipulation is not uncommon in arthropod-bacteria symbioses. Drawing an ideal parallel, one could wonder whether some bacterial symbionts associated with protists could exert addictive manipulation on their hosts, possibly exploiting analogous processes of modification-rescue involving toxin-antitoxin systems. We will start here with some preliminary thoughts and considerations as premise and basis for interpreting the in-depth cases analysed and presented below.

At first glance, it might seem surprising that, despite the diversity and abundance of protists and their bacterial symbionts, an actual addictive manipulation has never been clearly recognised and demonstrated before among those associations. However, in our view, several aspects should be taken into account, first of all, the strong bias in the hosts chosen as subjects of most studies. Indeed, despite valuable investigations from the past decades (e.g., (Jeon and Lorch 1967; Quackenbush and Burbach 1983; Jeon 1987; Pond et al. 1989; Jeblick and Kusch 2005; Fokin 2012)) and a number of studies conducted in recent years (e.g., (Hess 2017; Maita et al. 2018; Boscaro et al. 2019, 2022; Castelli et al. 2019, 2021; Lanzoni et al. 2019; Herrera et al. 2020; Midha et al. 2021; Arthofer et al. 2022; Paight et al. 2022; Davison et al. 2023; Dharamshi et al. 2023)), partnerships between bacteria and protists are still profoundly under-investigated when compared to symbioses involving bacteria and arthropods (or other multicellular hosts).

Moreover, the study of such associations in search for addiction presents multiple inherent limitations. Vertical transmission, which is a major "target" for any host-dependent bacterial symbiont to ensure its own persistence, is accomplished during sexual reproduction in metazoan hosts. This allows researchers a clear observation of the effects of potential addictive manipulation exerted by the symbionts at each new host generation (particularly, distortion of sex ratio in the progeny). On the other hand, unicellular eukaryotes most frequently reproduce asexually, in particular by cell division, which may nuance and completely "hide" the effect of addiction. Indeed, a plausible outcome would be the death of daughter cells that did not receive the bacteria. However,

this is inherently hard to distinguish from the case of a primary obligatory mutualism, in which the host is simply dependent on the bacteria (see Box 2 for indications on potential proof-of-principle experiments). Thus, hints of addictive manipulation could be harder to detect, and in general disregarded.

As a matter of fact, several partnerships between protists and bacteria have been stably maintained in the laboratory, even for decades (Schweikert and Meyer 2001; Potekhin et al. 2018; Lanzoni et al. 2019), with targeted attempts to remove the bacteria frequently unsuccessful (Mironov and Sabaneyeva 2020; Flemming et al. 2021; Midha et al. 2021; Mironov et al. 2022). These data clearly indicate the presence of a "bond" between the bacteria and their hosts, which in some cases could be assimilated to "true" mutualisms, such as for *Polynucleobacter* (*Burkholderiales*) with the ciliate *Euplotes* (Boscaro et al. 2019, 2022).

However, several other cases display additional and differential features, which, we argue, are suggestive of an ongoing addictive manipulation. Closely related bacteria, even belonging to the same species (Schweikert and Meyer 2001; Senra et al. 2016; Potekhin et al. 2018), may be hosted by phylogenetically, physiologically and ecologically diverse hosts. For instance, *Megaera polyxenophila* (*Rickettsiales*) can be associated with heterotrophic protists such as ciliates, multiple lineages of photoautotrophic algae, and even cnidarians (Schrallhammer et al. 2013; Hess 2017; Lanzoni et al. 2019; Davison et al. 2023). In principle, it is possible that the bacteria are able to provide universal mutualistic benefits to such arrays of hosts. At the same time, it seems meaningful to consider a potential involvement of addictive manipulation, which could enable tight associations to diverse hosts thanks to effector molecules with broad specificity on eukaryotic targets (see also below the paragraph "Mechanisms and evolution of addictive manipulation").

On the other hand, the same protists that have been repeatedly found as hosts for stably-associated bacteria (e.g., *Paramecium aurelia*, *Paramecium caudatum*, *Acanthamoeba*) are also commonly found devoid of any those (Fokin 2012; Flemming et al. 2021). These data are reminiscent of *Wolbachia* present in multiple diverse arthropod species, with variable prevalence (Weinert et al. 2015). Eventually, many bacteria could be experimentally removed from their protist hosts by

elaborate but potentially fluky approaches (Bella et al. 2016; Pasqualetti et al. 2020), with the hosts then surviving and often thriving (Pasqualetti et al. 2020; Flemming et al. 2021). This is a sharp difference from dependence on obligatory symbionts, and is instead reminiscent of addictive manipulators, which are not required by their hosts inherently.

We should also consider that addictive manipulative mechanisms are unlikely to be "all-or-nothing" phenomena in every condition (Figure 2; Box 2). Even in the case of *Wolbachia*, it is known that reproductive manipulation does not show full penetrance, and is dependent on host genetic background (Walker et al. 2011) and age (Layton et al. 2019), as well as on external factors such as temperature (Ross et al. 2019), so that in some host it was initially completely overlooked (Shropshire et al. 2020).

Thus, while typical investigations are focused on single partnerships (e.g., (Yurchenko et al. 2018; George et al. 2020; Castelli et al. 2021)), the best indications for an "elusive" trait such as addictive manipulation in protist hosts would most probably come from comprehensive comparative studies aimed at evidencing general trends, as herein.

Bacteria addictively manipulating protist hosts

Here we highlight the cases which, in our view, show the most distinctive and convincing signs of addictive manipulation of protist hosts exerted by associated bacteria.

The first instance pertains to *Legionella jeonii* (initially termed "X-bacteria" (Jeon 1987)), on which an interesting set of experiments was performed some decades ago (Park et al. 2004). When introduced in symbiont-free *Amoeba* cells, *L. jeonii* repeatedly produced harmful effects (reduced size, fragility, poor clonability, slower growth, or even death) (Jeon and Lorch 1967). However, after some time, surviving subpopulations of amoebas became healthier and, surprisingly, dependent on the symbiont (Jeon 1972), so that antibiotic treatments led not only to bacterial death, but also to demise of the host (Jeon and Hah 1977). In principle, these findings could be interpreted

as the consequence of an experimentally induced obligatory mutualism (or an evolutionary addiction *sensu* Hammer (Hammer 2023)).

The observed effects were partly correlated with specific pairings of nucleus and cytoplasm (containing the bacteria), as experimental combinations of nuclei from infected cells with cytoplasms from non-infected ones were mostly unviable. However, such combinations survived in a minority of cases, thus not presenting an absolute "all or nothing" outcome, as would be most probable in an "idealised" necessary mutualism.

Even more remarkably, the same series of effects were observed when *L. jeonii* was transferred to other amoeba cells, which in turn eventually became dependent on the bacteria (Jeon 1972; Jeon and Ahn 1978). These data strongly indicate that the factor(s) leading to the stability and non-breakability of the association are derived from *L. jeonii*. The mechanism for the apparent dependence of *Amoeba* on *L. jeonii* is unknown, but was tentatively linked to a plasmid-encoded 29 kDa protein (Jeon 1987), which is translocated to the host cytoplasm and nucleus (Pak and Jeon 1997), where it can influence host gene expression (Jeon and Jeon 2004).

To summarise, available data point to *L. jeonii* possessing the ability to manipulate its *Amoeba* host, making it addicted through context-dependent gene regulation involving plasmids, and resulting in epigenetic mechanisms in the host (Figure 1).

Other noteworthy and long-time known cases are those of *Caedibacter taeniospiralis* and *Caedimonas varicaedens (Kusch and Görtz 2006; Schrallhammer and Schweikert 2009)*. These bacteria share many traits (see below), and were originally grouped together in the single genus *Caedibacter*, but are now recognised as phylogenetically unrelated, belonging respectively to the *Holosporales* and to the *Thiotrichales* (Schrallhammer et al. 2018). Both of these bacteria are typically intracellularly hosted by ciliate protists of the genus *Paramecium*, and are able to confer them a "killer trait".

Under certain conditions such as starvation, a portion of bacteria arrest their replication and produce R-bodies, i.e. large proteinaceous elements shaped as coiled ribbons (Pond et al. 1989). Some bacteria are released extracellularly, and, if they are endocytosised by *Paramecium* cells that lack

the symbiont, the acidification of the digestive vacuoles causes the unrolling of the R-bodies and the release of a still uncharacterised toxin (Schrallhammer and Schweikert 2009). This leads to *Paramecium* cell death by multiple alternative mechanisms, depending on the bacterial and host strain/species, namely hump killing, spin killing, vacuolisation, and paralysis (Pond et al. 1989; Schrallhammer and Schweikert 2009). These multifaceted lethal effects are reminiscent of the multiple phenomena of reproductive manipulation of *Wolbachia* in arthropods. The *Caedimonas*/*Caedibacter* bacteria are assumed to produce an antitoxin that rescues the toxicity, thus protecting their natural hosts. Interestingly, R-bodies and possibly also toxin-antitoxin genes are encoded into plasmids that also bear phagic genes (Quackenbush and Burbach 1983; Jeblick and Kusch 2005), and the presence of R-bodies was associated with prophage induction (Preer et al. 1974).

The killer trait has been proposed to provide a competitive advantage to the *Paramecium* hosts towards non-infected conspecifics, thus being indicative of mutualism (Schrallhammer and Schweikert 2009). In addition, we propose that it is a variation of an addictive manipulation phenomenon, in which the host that loses the symbionts is "punished" indirectly, thanks to the probable close presence of "sister cells" still bearing the bacteria (Figure 1). One could say that *Caedimonas*/*Caedibacter* kills paramecia that have lost it pretty much as *Wolbachia* sterilises females that do not have it. From an evolutionary perspective, competitive advantages would then represent an exaptation of a pre-existing control mechanism acting on the host cells, and a way to further strengthen the association.

It is interesting to observe that in the past decades several other bacteria were found to cause killer effects in protists hosts (Görtz and Fokin 2009). Among them, more recent molecular and phylogenetic characterisations revealed that *Lyticum* spp. are part of the same bacterial order encompassing *Wolbachia* and other addictive manipulators of arthropods, namely the *Rickettsiales* (Boscaro et al. 2013).

Mechanisms and evolution of addictive manipulation

The cases of *L. jeonii*, *Caedibacter*, and *Caedimonas* present some common traits at the molecular level, as they all involve modification/rescue mechanisms and mobile elements, such as plasmids and phages. These features also equate them to *Wolbachia* and other addictive manipulators of arthropods (Figure 1).

Accordingly, we posit that modification/rescue mechanisms, mediated by toxin-antitoxin systems, could lie behind these and potentially many other cases of addictive manipulation of protist hosts. In the broadest sense (Jurėnas et al. 2022), multiple types of molecules could be involved (including proteins and RNA) exerting or rescuing toxicity through various mechanisms, such as post-transcriptional regulation, or post-translational direct and indirect interactions. Besides additional functions in bacterial physiology (Harms et al. 2018), conventional toxin-antitoxin systems are involved in the addictive control exerted by plasmids on bacterial cells (Jurėnas et al. 2022). Moreover, they were also shown to be active on eukaryotic cells (Yeo et al. 2016; You et al. 2023), and are thus reasonable candidates for "exaptation" towards addictive manipulation of eukaryotic host cells in general, as already hypothesised for some of the specific cases presented above (Schrallhammer and Schweikert 2009; Beckmann et al. 2019). Multiple independent events of development/exaptation of distinct molecular determinants of addictive manipulation could be envisioned in different bacterial symbionts of protists. Noteworthy is the *Holosporales* bacterium *Bodocaedibacter*, which actively transcribes toxin and antitoxin genes, and its suppression by antibiotics leads to death of its host, the kinetoplastid flagellate *Bodo saltans*, thus suggesting an addictive role and its molecular determinants (Midha et al. 2021).

Under this framework, mobile elements such as plasmids and phages could play a fundamental part, due to their well-recognised role in HGT (Haudiquet et al. 2022). As a matter of fact, multiple bacterial symbionts of protists were shown to bear mobile elements (Pond et al. 1989; Wang and Wu 2015; George et al. 2020; Castelli et al. 2024). In the case of the *Paramecium* symbiont

Trichorickettsia (*Rickettsiales*), putatively plasmid-encoded (Castelli et al. 2024) phage particles were also observed (Mironov and Sabaneyeva 2020), reminiscent of *Caedibacter*/*Caedimonas*. The same protist cell is frequently co-infected by different bacteria, which could easily exchange genes (Wang and Wu 2017; Gomez-Valero and Buchrieser 2019), thereby acquiring factors that confer/refine the capability to addictively manipulate their hosts. Accordingly, the impact of HGT in the evolution of bacterial symbionts of protists is more and more recognised (e.g., (Castelli et al. 2021; George et al. 2022)).

Thanks to HGT driven by mobile elements, we can expect the presence of multiple alternative determinants in the same bacterium, with even significant variations between closely related bacteria. Such patterns could account for broad host ranges and their variation (which may be also explained by the molecular specificity of toxins and antitoxins in relation with host targets), as well as for competition among symbionts, such as in the case of *Wolbachia* (Beckmann et al. 2019). Considering all the above, it seems highly intriguing the discovery of plasmid-encoded R-bodies, possibly linked with an addictive killer trait, in several protist-associated *Holosporales* bacteria other than *Caedimonas* (Giovannini et al. 2024).

Evolution of addictive manipulators

From the perspective of bacterial evolution, it is interesting to observe that many of the bacteria with well-demonstrated or presumed capabilities of addictive manipulation of different eukaryotes are phylogenetically akin. Particularly, it is remarkable to find multiple representatives of the *Rickettsiales* (e.g., *Wolbachia*, *Rickettsia*, *Mesenetia*, *Megaera, Lyticum*), the *Legionellales* (e.g., *Rickettsiella*, *L. jeonii*), and the *Holosporales* (e.g., *Caedimonas*, *Bodocaedibacter*). Along with other independent phyletic lines (chiefly *Chlamydiae*), these phylogenetically unrelated lineages share some peculiar functional and evolutionary traits that make them noteworthy for the study of bacterial-eukaryotic symbioses in general, and which also led some authors to categorise them as

"professional symbionts" (Husnik et al. 2021). The recurrent involvement of addictive manipulation within these lineages suggests to examine them further.

The representatives of such "professional symbionts" typically live in association with eukaryotes, most likely since extremely ancient times (even over 1 bya) (Wang and Luo 2021; Hugoson et al. 2022; Dharamshi et al. 2023). Each lineage collectively displays a broad host range, colonising diverse protists, such as ciliates, amoebae and algae, as well as multicellular organisms, including arthropods and vertebrates (Castelli et al. 2016; Szokoli et al. 2016; Duron et al. 2018; Galindo et al. 2019; Gruber-Vodicka et al. 2019; Muñoz-Gómez et al. 2019; Guidetti et al. 2020; Carrier et al. 2021; Köstlbacher et al. 2021; Midha et al. 2021; Potekhin et al. 2021; Halter et al. 2022; Paight et al. 2022; Davison et al. 2023; Dharamshi et al. 2023; Dittmer et al. 2023). The most thoroughly investigated (and eponymous) representatives of each lineage are pathogens vectored by arthropods (Renvoisé et al. 2011; van Schaik et al. 2013; Elwell et al. 2016; Chauhan and Shames 2021). However, the majority are hosted by aquatic protists, which are considered the most probable ancestral hosts, with multiple independent secondary adaptations to multicellular hosts (Castelli et al. 2016; Szokoli et al. 2016; Duron et al. 2018; Dharamshi et al. 2020).

Consistently with such wide and variable host ranges, and despite being host-dependent (i.e., unable to multiply in the absence of host cells, though with few possible exceptions (Singh et al. 2013; Castelli et al. 2024; Schön et al. 2022)), "professional symbionts" are not strictly host-confined. Indeed, along with vertical transmission over host generations, many of them can also perform horizontal transmission (Huigens et al. 2004; Kocan et al. 2010; Dantas-Torres et al. 2012; Rizzoli et al. 2014), even shifting between very different host species (Duron et al. 2018; Modeo et al. 2020).

Consistently with the complex lifestyles, "professional symbionts" bear rich repertoires of effectors (Merhej et al. 2009; Betts-Hampikian and Fields 2010; Gillespie et al. 2015, 2016; George et al. 2020; Meir et al. 2020), enabling them to actively modulate, and possibly even "control" (Husnik et al. 2021) those multifaceted interactions with their diverse hosts. These molecular repertoires and their activities are still largely undisclosed.

In light of the above presented examples and considerations, it seems intriguing to speculate that, among those molecular mechanisms, some capable of inducing addictive manipulation could be significant and widespread. Along this line of thought, variegated interactions with a wide array of eukaryotic hosts, as in the lineages of "professional symbionts", would indeed be a plausible outcome for the descendants of hypothetical ancestral bacteria capable of addictive manipulation. Accordingly, addictive manipulation could have taken an active part in the evolution of such lineages, possibly even "determining" it. Variations in the repertoire and/or specificity of toxin-antitoxin modules (see above "Mechanisms and evolution of addictive manipulation") would be a reasonable mean to achieve such a breadth and evolutionary variability of host ranges, including in particular evolutionary shifts from protist to multicellular hosts.

Addictive manipulation and other kinds of interactions might concur in the establishment and maintenance of tight bacterial-host associations. At an evolutionary scale, each interaction might supersede others, likely with multiple replacements over time. Those other interactions include seemingly more conventional mutualisms, as exemplified by some *Wolbachia*, which have become necessary for filarial nematodes (Taylor et al. 2005; Werren et al. 2008) and for some insects, including for nutrient provision (Dedeine et al. 2001; Kremer et al. 2009; Hosokawa et al. 2010; Jaenike et al. 2010; Mahmood et al. 2023).

Concluding remarks and perspectives

Through the targeted literature review and re-interpretation presented above, here we propose a novel framework to explain the evolution and persistence of the associations between bacterial symbionts and protist hosts. Namely, many of those bacteria could be able to maintain such associations thanks to addictive manipulation mechanisms (Box 1), comparable to the reproductive manipulation exerted by *Wolbachia* and other bacteria on arthropods (Hurst and Frost 2015; Gillespie et al. 2018; Chen et al. 2020; Pollmann et al. 2022). Specifically, this would result in the

(most frequent) death of those hosts that have recently lost the symbionts, through direct or indirect toxic activity exerted by the bacteria under those specific circumstances, rather than due to some inherent inability of the hosts to cope with the lack of the symbionts.

The outlined behaviour of such addictive manipulators, in particular in protists and other asexual hosts (Figure 2), indicates them as selfish addictive elements. This allows intriguing analogies with the interplay between plasmids and bacterial cells (Rankin et al. 2012; Rodríguez-Beltrán et al. 2021). Such parallel becomes even more intriguing when considering the available indications that the toxin-antitoxin systems involved in addictive control exerted by plasmids on bacteria have been repurposed as molecular determinants for addictive manipulation by bacteria on eukaryotes (Jeon 1987; Schrallhammer and Schweikert 2009; Beckmann et al. 2019; Hochstrasser 2022), as well as the probable involvement of plasmids and other mobile elements in spreading such molecular determinants among eukaryote-associated bacteria.

Available data are still insufficient to provide a clear picture on addictive manipulation among bacteria-protists associations. Considering the inherent difficulties in distinguishing from other interactions such as obligatory mutualisms, we posit that the herein presented examples (Jeon 1987; Schrallhammer and Schweikert 2009) represent only the “tip of the iceberg” of a widespread phenomenon. Thus, we underline the need for dedicated research to elucidate the diffusion, mechanisms, impact, and evolutionary significance of those interactions, in particular targeted experimental analyses (Box 2).

Given the fundamental roles of protists in a broad range of ecosystems (Caron et al. 2012; Geisen et al. 2018; Burki et al. 2021), addictive manipulation by their symbionts likely has deep ecological impacts as well. As exemplified by the case of *Wolbachia*, fundamental insights on the eco-physiology and evolution of each host could be obtained by studying its addictive manipulators (Werren et al. 2008), which could even become the basis for innovative applications (Hoffmann et al. 2011; Utarini et al. 2021).

It is a quite accepted notion that, due to their antiquity, diversity and environmental diffusion, protists may act as “Trojan horses” or “melting pots” for the evolution of bacteria associated with

multicellular hosts (Barker and Brown 1994; Wang and Wu 2017). Under this framework, it seems also thought-provoking to examine the evolutionary significance of addictive manipulation of protists, in particular when considering the recurrent occurrence of (putative) addictive manipulators within lineages (e.g. *Rickettsiales*, *Legionellales*, *Holosporales*) that encompass bacteria associated both with protists and with multicellular organisms (Duron et al. 2018; Dharamshi et al. 2020; Castelli et al. 2024).

Figures legends

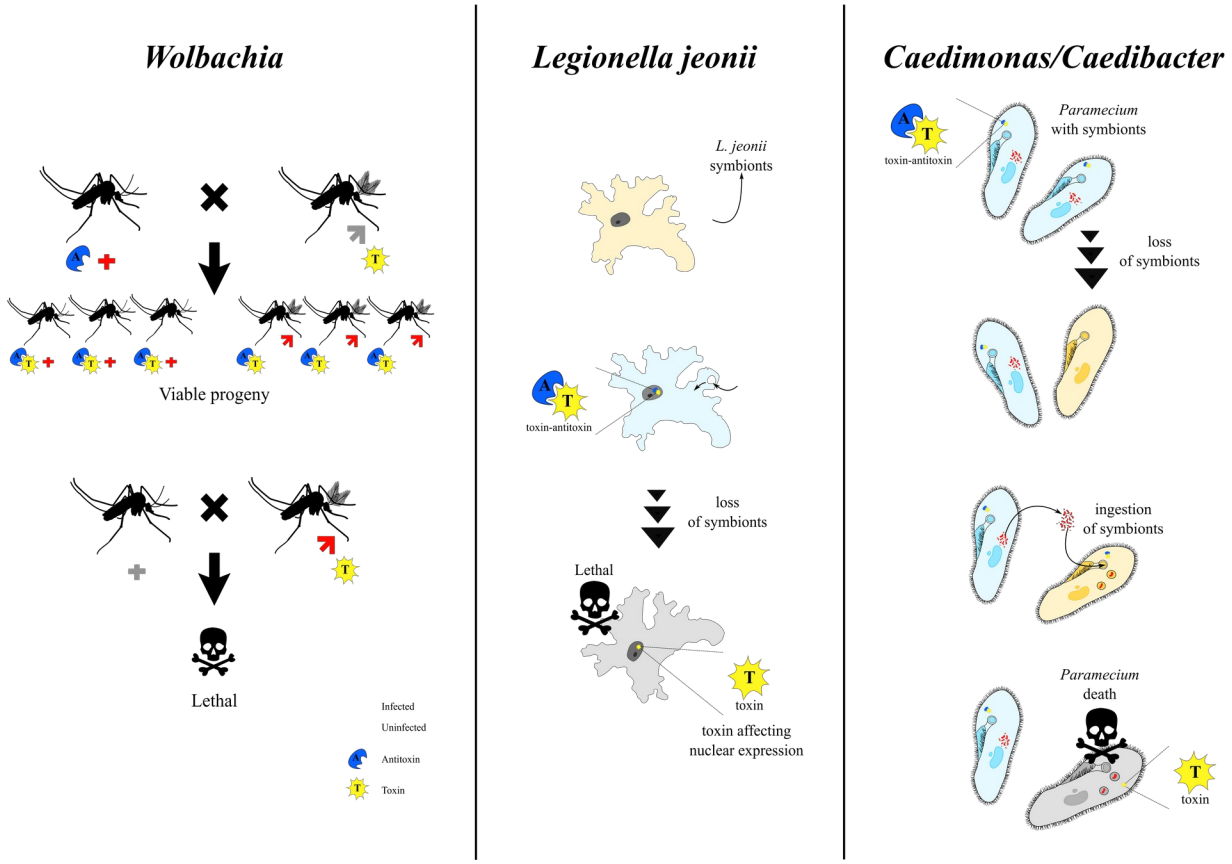

Figure 1. Addictive manipulation mechanisms exerted by bacterial symbionts on their diverse eukaryotic hosts, involving molecular determinants linked to mobile genetic elements. This way, the bacteria ensure their own proliferation by promoting their vertical transmission. *Wolbachia* (*Rickettsiales*) manipulates the reproduction of its vertebrate hosts by CI (and other mechanisms). The bacterium is vertically transmitted to the offspring only by the females. Gametes from infected males carry a prophage-linked toxin that kills the embryos, unless female gametes carry the bacterium with a cognate antitoxin, thus favouring the spread and maintenance of the bacterium in the host populations. Similarly, *L. jeonii* (*Legionellales*) manipulates the asexual life cycle of its unicellular eukaryotic hosts. When healthy amoebas get infected, they become unable to get rid of the bacteria. Most likely, a plasmid-encoded toxin by the bacteria epigenetically acts on host gene expression, a modification that persists after bacterial loss, and that can be rescued only in presence of live bacteria. *Caedimonas* (*Holosporales*) and *Caedibacter* (*Thiotrichales*) counteract their loss by *Paramecium* hosts by an indirect mechanism. The bacteria produce a plasmid-encoded toxin,

against which their hosts are protected by the cognate antitoxin. If a host loses the symbiont, it becomes sensitive to the toxin, and will be killed when ingesting symbionts released by its, still infected, sister cells.

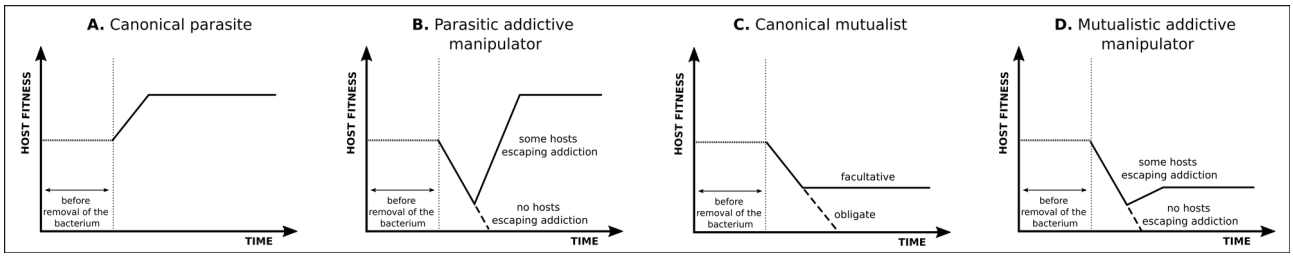

Figure 2. Comparisons of idealised fitness responses of a protist (or another asexually reproducing host) to the removal (dotted vertical line) of an addictive manipulator (B, D) in comparison to the removal of a canonical parasite (A) or a canonical mutualist (C). In turn, depending on other potential concomitant interactions, a manipulator may have overall detrimental or beneficial effects, respectively behaving as a parasitic (B) or mutualistic (D) addictive manipulator.

Box 1. Addictive symbiont-host interactions

Addictive symbiont-host interactions imply that the host receives damage, up to potential death, if symbionts are lost, regardless of direct benefits provided by the symbionts. As a consequence, the association results tightened, with potential advantages for the symbionts. The most thoroughly studied cases are those of reproductive manipulation exerted by *Wolbachia*, *Spiroplasma* and other bacteria on arthropods, through CI, male killing, feminisation, or parthenogenesis.

Additionally, the concept of evolutionary addiction was recently proposed, namely that coexistence with the symbionts will cause different evolutionary processes in the host, which would eventually result in dysregulation in case the bacteria are removed (Hammer 2023). Specifically, according to Hammer, "adaptive accommodation" implies the irreversible accommodation of host regulatory mechanisms in the presence of bacteria, while "compensated trait loss" implies that the redundancy of certain metabolic and functional features in host and symbionts may result in the loss of the respective genes in the host, which would need compensation by the symbionts.

On the other hand, in case of reproductive manipulation, the addiction would depend directly on active properties exerted by the bacteria, specifically, in the experimentally validated cases of *Wolbachia* and *Spiroplasma*, by the action of toxins and antitoxins (Harumoto and Lemaitre 2018; Chen et al. 2020; Shropshire et al. 2020).

Here we propose the concept of "addictive manipulation", by generalising the case of reproductive manipulation of arthropods to other eukaryotes, in particular protists. Under this condition, the hosts are addicted to bacterial symbionts as a result of some active property evolved and exerted by the symbionts themselves, without directly implying any evolutionary change in the hosts. As in the specific cases of reproductive manipulators of arthropods, addictive manipulation likely takes place thanks to molecular toxin-antitoxin systems, and may consist in different phenomena depending on the physiology and ecology of host and symbionts (see also Box 2 "How to test addictive manipulation").

Accordingly, host-symbionts interactions in case of addictive manipulation expectedly result in complex interplays, which, to be fully delineated, should require accounting for several other features, such as the potential capability of symbionts to spread horizontally, and the interaction of host and/or professional symbionts with other organisms, including non-infected hosts (see the case of *Wolbachia* or *Caedimonas*/*Caedibacter*) (Werren et al. 2008; Schrallhammer and Schweikert 2009).

At an evolutionary scale, we highlight the possibility that addictive manipulation could have had important consequences in the evolution of bacterial lineages with ancient and evolutionarily stable interactions with eukaryotic hosts (e.g., *Rickettsiales*, *Legionellales*, *Holosporales*, *Chlamydiae*).

Box 2: How to test addictive manipulation

The inherent complexity of addictive manipulation hampers its proper identification in protists. Possible approaches to discern it could involve modelling bacterial-host interactions in case of addictive manipulation, for instance by analogy with models of addiction of bacterial cells on plasmids (Rankin et al. 2012), and then subject those models to experimental validation.

Herein, it seems appropriate to outline some simple general criteria as a starting ground, in particular by evaluating the effect of symbiont removal on the host. For this purpose, we assume that: i) the host is reproducing asexually, ii) host survival, reproductive success and/or well-being can be measured (here collectively termed as "fitness"), iii) a method for removing the addictive manipulator is available (e.g. antibiotics), iv) any addictive manipulation phenomenon is not 100% effective. The latter assumption seems reasonable based on the available knowledge on *Wolbachia*, *Caedibacter*/*Caedimonas*, and *L. jeonii*, for which the addictive manipulation mechanisms are conditionally regulated (e.g. by prophage inductions) according to physiological states or external factors such as temperature (Jeon 1987; Schrallhammer and Schweikert 2009; Shropshire et al. 2020). Although this may represent a confounding factor, it can also be instrumental in discriminating an addictive manipulator from a necessary mutualist (see below).

If an addictive manipulator is removed, we expect an initial reduction of host fitness, up to complete death, or followed by a subsequent recovery (by hosts escaping from non-100% effective addictive manipulation) (Figure 2). The post-recovery fitness level would depend on whether the overall effect of the addictive manipulator is mutualistic or parasitic. Notably, the end results would be indistinguishable from canonical parasites or canonical mutualists, and, if taken alone, may mislead in the classification of the interaction. This seems to be the case of *L. jeonii*, originally interpreted as a necessary mutualist (Jeon and Hah 1977). Rather, it is the temporal trajectory of the variation of fitness that matters, as the fitness "reduction-recovery" process would be distinctive for an addictive manipulator (Figure 2).

Inevitably, such an approach is prone to confounding factors and to detection limits (in particular relative to the speed of the process and the effect size). We put forward that identifying molecular determinants could complement such limits, not only demonstrating the mechanism for addiction manipulation of protists (or other asexual hosts), but also validating that it is actually taking place.

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