**Old war, new battle, new fighters !**

Sandra Sousa, Francisco Sarmento Mesquita and Didier Cabanes
IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal.

Correspondence:
Didier Cabanes
Institute for Molecular and Cell Biology
Group of Molecular Microbiology
Rua do Campo Alegre, 823
4150-180 Porto
Portugal
(didier@ibmc.up.pt)

Since the discovery of penicillin, antibiotics had been of critical importance in the control of infectious diseases. However, the extensive use and misuse of antibiotics during the last decades led to the wide development and spread of multiple drug resistance in bacteria. Associated to the alarming levels of antibiotic resistances and the difficulty to discover novel antibiotics, the re-emergence of infectious diseases poses new challenges. We are probably close to reach the end of the antibiotics period and researchers are now investigating alternatives to treat infectious diseases. Contrarily to traditional strategies that aim to kill bacteria or prevent their growth, these new approaches intend to block the ability of bacteria to harm the host and are thought to apply less selective pressure limiting the development of bacterial resistance. These emerging strategies that seek to directly inhibit bacterial virulence factors, benefit from the detailed knowledge of the functional and molecular mechanisms underlying key pathogenic determinants acquired during the last decades of research in host-pathogen interactions [1]. In particular, bacterial toxins are primary targets for these novel anti-virulence strategies. In this issue of *The Journal of Infectious Diseases*, Wang and colleagues show that fisetin, a natural flavonoid with negligible antimicrobial activity, has an effective anti-virulence activity against *Listeria monocytogenes* by directly interfering with a bacterial secreted toxin.

*Listeria monocytogenes* (*Lm*) is a facultative intracellular human food-borne pathogen that causes listeriosis, an infection characterized by gastroenteritis, meningitis, encephalitis and maternofetal infections. Listeriosis is the most frequent cause of death due to the consumption of contaminated food in Europe, and is the third foodborne infection in terms of cost of illness and quality life losses [2, 3]. *Lm* enters the host *via* the ingestion of contaminated foods, invades the intestine, translocates to mesenteric lymph nodes and spreads to the liver, spleen, brain and to the placenta. During infection, *Lm* has the ability to cross the intestinal, the blood-brain and the placental barriers, entering, surviving and multiplying inside phagocytic and non-phagocytic cells [4]. To establish and sustain infection *Lm* employs an arsenal of virulence factors to hijack host-signaling pathways [5, 6]. While remaining a real public health concern, *Lm* has emerged as an exceptional model to address the different facets of host-pathogen interactions and the design of new therapeutic strategies.

Listeriolysin O (LLO) is a crucial virulence factor produced by *Lm* [7]. It is a pore-forming toxin (PFT) member of the cholesterol dependent cytolysins (CDC) family [8]. LLO monomers are secreted by the bacteria and oligomerize at the surface of target cell into a ring [9]. Membrane insertion of LLO results in ion fluxes across damaged membranes and ultimately leads to cell lysis in conditions of extensive damage and/or inefficient membrane repair mechanisms [10]. Inactivation of LLO results in the inability of *Lm* to escape from the internalization vacuole thereby decreasing the *Lm* virulence potential [11, 12]. Besides membrane lysis, it has become

recently apparent that LLO acting from the intracellular or extracellular milieu exerts additional effects on the host cell [7]. Intracellular LLO affects host cell signaling [13, 14], induces autophagy [15] and suppresses reactive oxygen species [16]. LLO was also shown to deregulate host SUMOylation [17], to induce endoplasmic reticulum stress [18] and mitochondria fragmentation [19], and to promote regulatory epigenetic changes [20].

A previous study showed that sub-inhibitory concentrations of plant essential oils could inhibit LLO activity and decrease *Lm* virulence, however the specific compound responsible for this effect remained unknown [21]. In this issue of *The Journal of Infectious Diseases*, Wang and colleagues report the discovery of the natural flavonoid fisetin as an effective anti-virulence agent against LLO activity. They showed that fisetin inhibits the hemolysis capacity of LLO and is able to protect mice from lethal infection by *Lm*. Molecular modeling studies revealed that fisetin directly engages LLO, causing a conformational shift of the LLO domains critical for its binding to cholesterol and oligomerization. Coupling mutational and biochemical approaches they identified LLO amino acid residues involved in the sensitivity to fisetin.
This work thus establishes fisetin as a novel anti-virulence compound that targets LLO by a unique mechanism, counteracting toxin binding to host cells and oligomerization. Interestingly, fisetin is able to decrease *Lm* virulence in tissue-cultured cells and animal infection models, without affecting the *Lm* growth or the phagocytic capacity of macrophages. Notably, while new roles have been frequently assigned to LLO, this study supports that, during *in vivo* infection, pore formation remains its main function.

Anti-virulence strategies targeting bacterial toxins to prevent their deleterious effects were previously developed. Such approaches were based in the use of soluble analogues or specific antibodies against toxins, thereby preventing their interaction with their receptors at the host cell membrane. Alternatively, they aim at blocking toxin pores using synthetic compounds [1]. In the case of LLO, neutralizing monoclonal antibodies were previously described to control *Lm* intracellular growth and virulence [22]. However, one important caveat is that these strategies are often associated to high costs of production and maintenance. By the contrary, Fisetin is present in many fruits and vegetables being associated to low costs of production, and was shown to have broad biological properties ranging from antioxidative to cancer therapeutic effects. The bioavailability and toxicity of fisetin are also well established [23]. In addition, an important advantage with dietary plant flavonoids is that they are perceived as non-toxic and have wide human acceptance [24]. This study thus pave the way for the development of broad anti-virulence approaches based on natural products. Importantly, as CDCs generally present structural homologies, it would be interesting to test the effect of fisetin on CDCs produced by other bacterial pathogens like *Streptococcus pyogenes* and *pneumoniae*, *Arcanobacterium pyogenes* or *Clostridium perfringens* [25]. Indeed, an optimal therapeutic agent would target virulence factors present in several pathogens. Results presented by Wang and colleagues associated to the recent determination of the LLO crystal structure [26] could allow the generation of fisetin derivatives with improved activity against CDCs.

Due to their rapid evolution rate, bacteria are experts in finding alternative routes to achieve growth and infection. Most virulence traits being not essential for bacterial survival, therapeutic strategies based on the inhibition of virulence should apply mild evolutionary pressure and limit the development of resistance. These promising approaches are sought to dampen pathogen progression allowing the control of infection through an effective host immune response or increasing the efficacy of classic therapies targeting bacterial growth. Indeed, in the presence of anti-virulence compounds bacteria can still grow and produce the targeted virulence determinants, being able to injure again the host in the absence of such inhibitors. It is thus probably coupled with traditional antibiotics that anti-virulence therapies will bring an undeniably advantage in the

fight against infectious diseases.

While resistance development and side effects on the host including microbiota are always possible, this work by Wang and colleagues opens new perspectives for natural compounds as effective anti-virulence strategies against human bacterial pathogens.

*Potential conflict of interest.*   Authors certify no potential conflicts of interest.

References:

1. Rasko DA, Sperandio V. Anti-virulence strategies to combat bacteria-mediated disease. Nat Rev Drug Discov **2010**; 9:117-28.
2. European Food Safety Authority ECfDPaC. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2009. EFSA Journal - Alabama Dental Association **2011**; 9:2090.
3. Allerberger F, Wagner M. Listeriosis: a resurgent foodborne infection. Clin Microbiol Infect **2010**; 16:16-23.
4. Lecuit M. Human listeriosis and animal models. Microbes Infect **2007**; 9:1216-25.
5. Pizarro-Cerda J, Kuhbacher A, Cossart P. Entry of *Listeria monocytogenes* in mammalian epithelial cells: an updated view. Cold Spring Harbor perspectives in medicine **2012**; 2.
6. Camejo A, Carvalho F, Reis O, Leitao E, Sousa S, Cabanes D. The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. Virulence **2011**; 2:379-94.
7. Hamon MA, Ribet D, Stavru F, Cossart P. Listeriolysin O: the Swiss army knife of *Listeria*. Trends Microbiol **2012**; 20:360-8.
8. Alouf JE. Pore-forming bacterial protein toxins: an overview. Current topics in microbiology and immunology **2001**; 257:1-14.
9. Shatursky O, Heuck AP, Shepard LA, et al. The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. Cell **1999**; 99:293-9.
10. Schnupf P, Portnoy DA. Listeriolysin O: a phagosome-specific lysin. Microbes and infection **2007**; 9:1176-87.
11. Lety MA, Frehel C, Berche P, Charbit A. Critical role of the N-terminal residues of listeriolysin O in phagosomal escape and virulence of *Listeria monocytogenes*. Mol Microbiol **2002**; 46:367-79.
12. Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC, Berche P. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. Infect Immun **1989**; 57:3629-36.
13. Tang P, Sutherland CL, Gold MR, Finlay BB. *Listeria monocytogenes* invasion of epithelial cells requires the MEK-1/ERK-2 mitogen-activated protein kinase pathway. Infect Immun **1998**; 66:1106-12.
14. Zwaferink H, Stockinger S, Hazemi P, Lemmens-Gruber R, Decker T. IFN-beta increases listeriolysin O-induced membrane permeabilization and death of macrophages. J Immunol **2008**; 180:4116-23.
15. Meyer-Morse N, Robbins JR, Rae CS, et al. Listeriolysin O is necessary and sufficient to induce autophagy during *Listeria monocytogenes* infection. PLoS One **2010**; 5:e8610.
16. Lam GY, Fattouh R, Muise AM, Grinstein S, Higgins DE, Brumell JH. Listeriolysin O suppresses phospholipase C-mediated activation of the microbicidal NADPH oxidase to promote *Listeria monocytogenes* infection. Cell host & microbe **2011**; 10:627-34.

17. Ribet D, Hamon M, Gouin E, et al. *Listeria monocytogenes* impairs SUMOylation for efficient infection. Nature **2010**; 464:1192-5.

18. Pillich H, Loose M, Zimmer KP, Chakraborty T. Activation of the unfolded protein response by *Listeria monocytogenes*. Cell Microbiol **2012**; 14:949-64.

19. Stavru F, Bouillaud F, Sartori A, Ricquier D, Cossart P. *Listeria monocytogenes* transiently alters mitochondrial dynamics during infection. Proc Natl Acad Sci U S A **2011**; 108:3612-7.

20. Hamon MA, Batsche E, Regnault B, et al. Histone modifications induced by a family of bacterial toxins. Proc Natl Acad Sci U S A **2007**; 104:13467-72.

21. Smith-Palmer A, Stewartt J, Fyfe L. Inhibition of listeriolysin O and phosphatidylcholine-specific production in *Listeria monocytogenes* by subinhibitory concentrations of plant essential oils. J Med Microbiol **2002**; 51:567-74.

22. Edelson BT, Unanue ER. Intracellular antibody neutralizes listeria growth. Immunity **2001**; 14:503-12.

23. Adhami VM, Syed DN, Khan N, Mukhtar H. Dietary flavonoid fisetin: a novel dual inhibitor of PI3K/Akt and mTOR for prostate cancer management. Biochem Pharmacol **2012**; 84:1277-81.

24. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer **2003**; 3:768-80.

25. Tweten RK. Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. Infection and Immunity **2005**; 73:6199-209.

26. Koster S, van Pee K, Hudel M, et al. Crystal structure of listeriolysin O reveals molecular details of oligomerization and pore formation. Nat Commun **2014**; 5:3690.