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1926-2016: 90 years of listeriology

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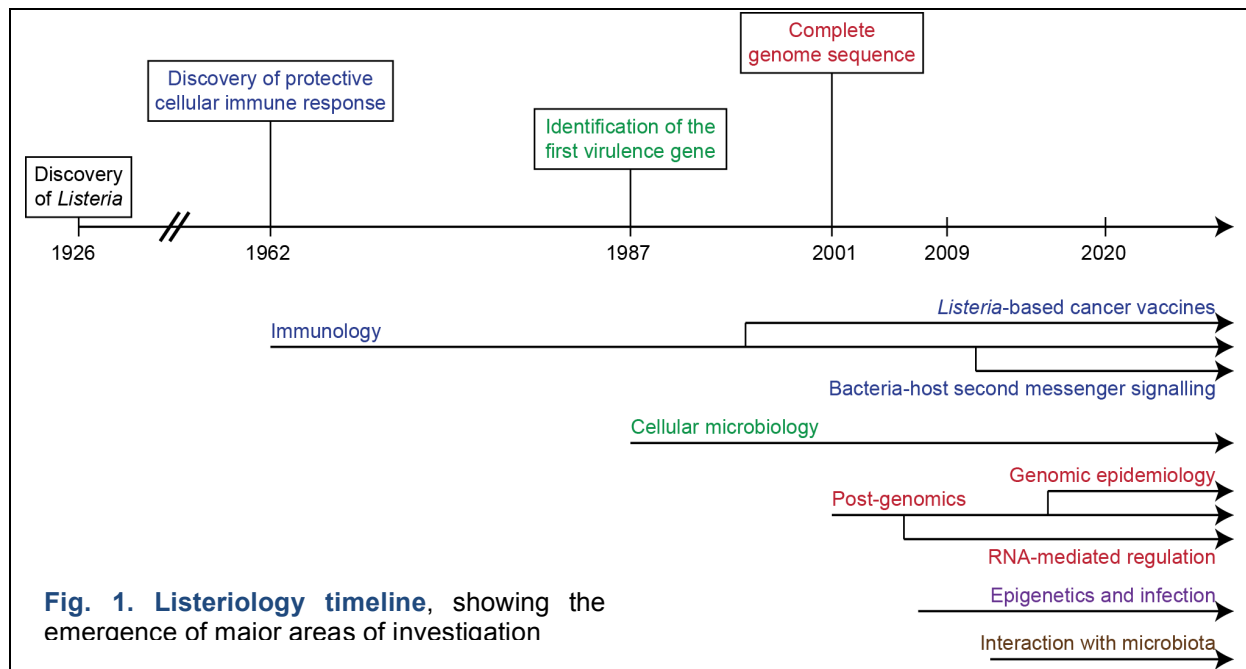
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Abstract

ISOPOL – for “International Symposium on Problems of *Listeria* and Listeriosis” – meetings gather every three years since 1957 participants from all over the world and allow exchange and update on a wide array of topics concerning *Listeria* and listeriosis, ranging from epidemiology, diagnostic, and typing methods, to genomics, post-genomics, fundamental microbiology, cell biology and pathogenesis. The XIXth ISOPOL meeting took place in Paris from June 14th to 17th, 2016 at Institut Pasteur. We provide here a report of the talks that were given during the meeting, which represents an up-to-date overview of ongoing research on this important pathogen and biological model.

Since the original article published by EGD Murray, RA Webb and MBR Swann in 1926 in the Journal of Pathology and Bacteriology [1], ninety years of research have raised *Listeria monocytogenes* among the most documented pathogenic bacteria and listeriosis among the best studied infectious diseases, at least in animal models (Fig. 1) [2-4].



Since 1957, a meeting entitled ISOPOL for “International Symposium on Problems of *Listeria* and Listeriosis” gathers every three years participants from all over the world and allows exchange and update on a wide array of topics concerning *Listeria* and listeriosis, ranging from epidemiology, diagnostic, and typing methods, to genomics, post-genomics, fundamental microbiology, cell biology and pathogenesis. Attendees include advanced scientists, students and post docs from academia, industry and public health agencies, *i.e.* fundamental microbiologists, medical microbiologists, veterinary microbiologists and public health specialists as well as researchers in cell biology and immunology.

The XIXth ISOPOL meeting organized by one of us (PC), with an international committee (S. Barbuddhe, H. Bierne, S. Brisse, C. Buchrieser, T. Chakraborty, J. Farber, P. Glaser, E. Gouin, J. Kreft, M. Lecuit, P. Martin, D. Portnoy) took place from June 2016 14th to 17th in the Institut Pasteur, Paris, one of the birth places of microbiology. It successfully gathered 360 scientists, including most of the experts in the field who were present during the whole meeting (Fig. 2).

While immunology was the first discipline to focus on *Listeria* infection, in particular the induction of adaptive T cell immunity, adaptive immunity was not extensively represented during the conference, whereas infection biology including cell biology and virulence gene regulation, as well as innate immunity were discussed in detail. A large amount of presented results were post genomic studies, with genomic approaches benefitting also to strain genotyping procedures.



Fig. 2. ISOPOL XIX group picture.

One of the highlights of the meeting was the HPR Seeliger award ceremony. This year, the award was given to Werner Goebel (Fig. 3), who has been working on *Listeria* since three decades.



Fig. 3. Seeliger Awardee Werner Goebel (centre) with H. Hof (left on the picture) and J. Kreft (right).

We provide here summaries for most of the talks delivered during the meeting by invited lecturers, or by speakers selected after abstract submission. For confidentiality reasons on unpublished results, a few talks could unfortunately not be included in this report.

Keynote speakers of the opening session

Fernando Baquero (Ramón y Cajal Institute for Health Research, Madrid, Spain) provided an overview of the resistome of *Listeria monocytogenes* (*Lm*). He first pointed out the difference between the extrinsic and intrinsic resistomes of *Lm* to antibiotics and underlined the intrinsic resistance to some antimicrobial agents such as clindamycin and the cephalosporins [reviewed in 5]. He then discussed the atypically small extrinsic resistome, *i.e.* resistance genes acquired through horizontal gene transfer, possibly from microorganisms sharing the same ecological niche. He reasoned that this is consistent with the small pangenome of *Listeria*, and the high synteny between different strains, pointing to an infrequent acquisition of foreign genes by *Lm* for its ecological diversification and evolution. Restricted access of *Lm* to foreign genes is also attested by the limited number of plasmids isolated from the bacterium to date and may also result from its impaired competence system [6] combined with the presence of CRISPR-mediated defence systems to defeat DNA invasion by phages. This low rate of integration of foreign genes in the *Lm* genome may reflect its strong adaptation to a stable environmental niche. Recent anecdotal reports of antimicrobial resistant strains of *Lm* remain to be confirmed.

Werner Goebel (Ludwig Maximilians University Munich, Germany) summarized the fundamentals of the intracellular *Lm* pathometabolism, which consists in a bipartite carbon metabolism where both glucose-6-phosphate and glycerol are taken up and used as carbon and energy sources essential for intracellular listerial growth. Activation of the host cell metabolism necessary for providing these compounds occurs once *Lm* reaches the intracellular stage, but the listerial factors triggering this activation are still ill-defined. Glucose-6-phosphate is mainly used by intracellular *Lm* to generate intermediates that serve anabolic purposes, such as cell wall and nucleotide biosynthesis [7]. Glycerol is preferentially utilized instead of glucose by intracellular *Lm* to ensure its ATP production, perhaps because excessive consumption of host cell glucose could result in metabolic stress of the host cell leading to its premature death. Other intracellular bacterial pathogens, such as *Legionella*, *Chlamydia* and enteroinvasive *Escherichia coli* show a bipartite metabolism as well, but these bacteria use different combinations of carbon sources, the production of which is induced in the host cell through distinct mechanisms [8].

Martin Loessner (Eidgenössische Technische Hochschule (ETH) Zürich, Switzerland) presented the world of *Listeria* phages (Fig. 4), consisting mostly of temperate phages that belong to the Siphoviridae or Myoviridae. Among the few lytic phages, the A511 phage kills *Listeria* within minutes due to its endolysin, which degrades the *Listeria* cell wall. The Loessner lab has harnessed the exquisite binding capacities of the endolysin to *Lm* teichoic acids by isolating its cell wall binding (CBD) domain [9]. This opened the way to novel methods for labelling and enriching of *Listeria*, and allowed the detection of specific serovars [10]. These findings may provide simple and affordable *Listeria* diagnosis tests, enabling the search of *Listeria* in food or clinical isolates in a broader range of countries and settings [11].

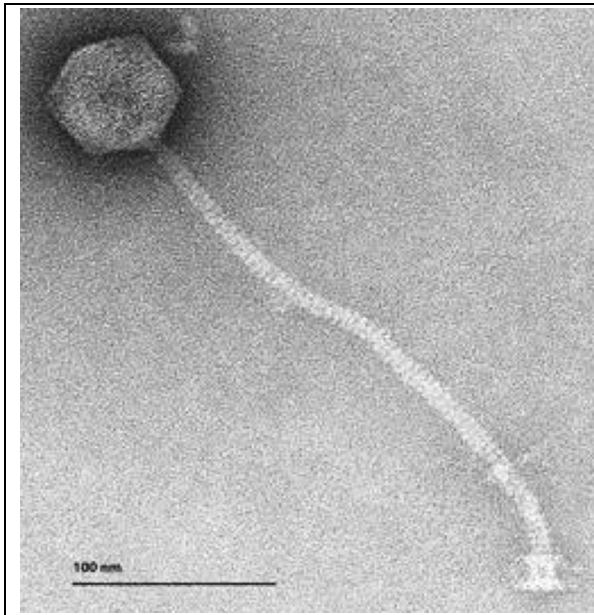


Fig. 4. Transmission electron micrograph of a *Listeria* phage [Courtesy of J. Klumpp and M.J. Loessner, ETH Zürich, unpublished]. More than 500 *Listeria* phages are known, all of which belong to *Siphoviridae* or *Myoviridae*. Among these, only a few are lytic phages.

Seeliger Prize ceremony

The Seeliger award is bestowed every three years at the ISOPOL meeting by the Heinz P.R. Seeliger Foundation to recognize an outstanding contribution to research on *Listeria*. This year, the Seeliger prize was awarded to **Werner Goebel** (Fig. 3).

Jürgen Kreft (University of Würzburg, Germany) introduced the Seeliger prize with a documented historical perspective on listeriology, from the early report of a bacterium causing liver necrosis in rabbits by G. Hülphers in 1911 [12], to the onset of molecular research on virulence determinants and cellular microbiology at the end of the 1980s. He reminded us how, from a bacterium isolated sporadically from the fluids of infected patients and animals, it became a crucial tool for immunologists enabling the identification of cell-mediated immunity by G. Mackaness [13], and was only confirmed as a food-borne pathogen in 1983 [14]. Through his discourse, it became apparent how *Lm* gradually became a model bacterial pathogen, allowing seminal findings in microbiology, infection biology, immunology and cell biology.

Epidemiology and surveillance of *Listeria* and listeriosis

The epidemiology and surveillance session of the meeting was the opportunity for a timely update about the detection and monitoring of listeriosis outbreaks, and counter-measures that need to be taken. The session underlined the ongoing generalization of whole genome sequencing (WGS) for the identification of outbreaks, and of the use of multi-locus sequence typing (MLST) as a unifying language to define *Lm* strains. Several speakers illustrated the high resolution of WGS, compared to serotyping and pulse-field gel electrophoresis (PFGE), the strain typing methods that have been widely used for many years. The session clearly illustrated how WGS improves the definition of outbreaks versus sporadic cases, and allows for a more accurate linkage between patients, contaminated food isolates and processing plants.

Peter Gerner-Smith (Center for Disease Control and Prevention (CDC), Atlanta, USA) presented the genomics-based surveillance of listeriosis by CDC and its public health and food regulatory partners in the USA, combining epidemiological data from each reported patient in the *Listeria* initiative with information about the genome sequences of the

thousands of *Lm* isolates from clinical cases and food [15]. He reported how the use of WGS of bacterial isolates allowed the identification of a larger number of outbreaks and their food source. He also pointed out that despite the extreme specificity of WGS for outbreak detection and case definition, solid epidemiological data remain critical to decipher the etiology of food contamination events.

Jeffrey M. Farber (University of Guelph, Ottawa, Canada) recapitulated the main food items that present a high risk of *Lm* contamination for humans. He illustrated the importance of high-risk food such as particular meat, dairy and vegetable products by presenting their involvement in past outbreaks [16]. He also pointed to more unexpected sources of listeriosis, including water tanks contaminated by swallows. He reminded the audience that failure of basic sanitation measures in industry can have severe consequences, and discussed the specific case of food served in hospitals, where particular care should be applied to food preparation, in light of its consumption by particularly vulnerable individuals.

Eva Møller Nielsen (Statens Serum Institute, Copenhagen, Denmark) presented the recent shift of typing methods from PFGE to WGS in the Danish reference laboratory and how this shift in genotyping strategy impacted on *Lm* surveillance by improving the definition of cases during outbreaks and by providing more confidence in incriminating the food sources [17]. This strategy proved particularly useful to track back contamination during a deadly outbreak of listeriosis which arose from Danish delicatessen in 2013-2014 [18].

Henriette De Valk (Public Health France, Saint Maurice, France) presented the French organization for the surveillance of listeriosis [manuscript in preparation]. The systematic reporting of clinical cases combined with real-time genotyping of the corresponding *Lm* isolates allows the detection of phylogenetically related clusters of epidemic samples. Recent food consumption by patients is reviewed and case-case epidemiological studies are then performed by comparing cluster cases with unrelated ones, aiming at defining the statistical probability that particular food items are associated with cluster cases. Trace back investigations and environmental and food investigations are then carried out for all foods associated with the outbreak strains. H. De Valk also commented on how the whole genome sequencing approach of genotyping impacts these investigations by defining strain phylogenetic relationships in a much more precise way than the previously used PFGE method.

Kathie Grant (Public Health England (PHE), London, UK) presented the molecular genotyping strategy based on WGS deployed in Public Health England (PHE). The strategy combines MLST-based and SNP-based approaches to define the genotype of strains. Most notably, a nomenclature system known as ‘SNP address’ has been devised to provide a phylogenetic-rich naming system of genotypes, thus allowing epidemiologists to define and investigate putative clusters of cases at different depths of genetic divergence [19]. This original approach provides the flexibility that is needed to account for the varying amounts of heterogeneity of *Lm* isolates observed during single-strain outbreaks. She also illustrated how this strategy contributes to identify food products associated with disease transmission in the UK [20]. Here again, the complexity of such identification processes benefits significantly from the advent of WGS applied to all sampled isolates from contaminated food and patients.

***Listeria* genomics and post-genomics**

Sylvain Brisse (Institut Pasteur, Paris, France) presented the latest knowledge on the phylogenetic diversity of *Lm* and illustrated the strong structuration of this species into lineages and sublineages. He discussed how the data from various strain typing methods used

in research and surveillance laboratories can be compared and interpreted in light of the population structure and evolution of lineages [21]. He reported on the worldwide distribution of the major clonal groups [22], and hinted at differences of relative frequencies of these groups in food and clinical samples [23]. Finally, he illustrated how whole genome sequence data can be harnessed using publicly accessible bioinformatics tools, and how this approach provides high resolution in epidemiology and population biology studies. He argued that harmonization of WGS-based typing strategies and the use of a unified nomenclature of clonal groups will ease international surveillance and allow tracking *Lm* strains as they spread globally [24].

Martin Wiedmann (Cornell University, USA) summarized recent progress on the phylogenetic diversity and taxonomy of the genus *Listeria*. He illustrated how the study of other *Listeria* species provides evolutionary context to improve the understanding of *Lm*. Furthermore, he discussed the implications of the discovery of novel *Listeria* species, suggesting that the use of *Listeria* spp. as indicators may not be appropriate for detection or diagnostic purposes. He showed how the 17 current *Listeria* species can be classified into four groups, and discussed the pros and cons of their taxonomic reclassification as four genera (Fig. 5) [25]. He presented how the diversification of the genus was accompanied by the evolution of important characteristics such as acquisition of flagellar genes, metabolism of ethanolamine and cobalamin, and the expansion of the internalin gene family.

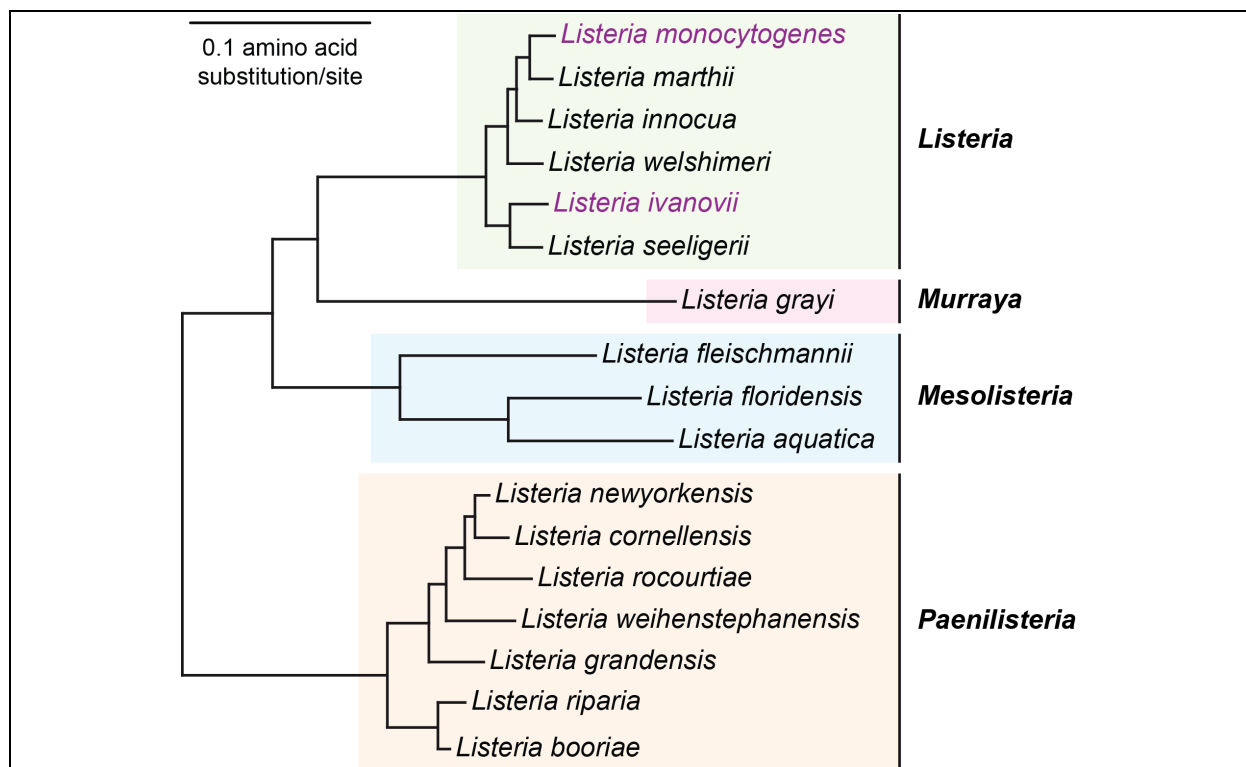


Fig. 5. Phylogenomic tree of *Listeria* species [adapted from 25]. The two pathogenic species *Listeria monocytogenes* and *Listeria ivanovii* are highlighted in purple letters. The proposed novel genera names are displayed on the right. This tree does not include yet *Listeria goensis*, the newcomer to be proposed by T. Chakraborty and S. Barbudde.

Mario Hupfeld (ETH Zürich, Switzerland) described a novel CRISPR/Cas II subtype from the recently sequenced *Listeria ivanovii* subsp. *londoniensis* WSLC 30167 [26], which efficiently confers its bacterial host with acquired immunity against bacteriophages, and could be used as a bioengineering tool in other *Listeria* species in the future.

Trinad Chakraborty (Justus-Liebig University of Giessen, Germany) presented on the genomics and evolution of virulence in *Listeria* species. He recapitulated the phylogenetic diversity of *Listeria* species and lineages within *Lm*, and reported on the relevance of novel models such as *Galleria mellonella* larvae or *Drosophila melanogaster* cells for virulence studies [27]. Together with **Sukhadeo Barbudde** (Indian Council of Agricultural Research, Raipur, India), he highlighted the isolation of a novel *Listeria* species from a mangrove swamp environment, *Listeria goensis*. Even though phylogenetically distant from *L. monocytogenes* and *L. ivanovii*, this species displays haemolytic properties, and is more virulent than *L. innocua* in the *Galleria melonella* infection model. Its genome is highly dynamic, marked by phage invasions, and harbours two large plasmids. It does not carry the canonical *Listeria* Pathogenicity Island (LIPI), but isolated virulence genes such as lecithinases, and polymorphic toxin systems that could contribute to virulence. More broadly, **S. Barbudde** provided us with an overview of the epidemiology of listeriosis in India, and pointed out a single predominant *L. monocytogenes* serotype 4b clone persisting across the country over a longer period of time [28]. He illustrated the diversity of *Listeria* species and serovars among patients and in newly-explored habitats, such as mangroves, mosquitoes and flies.

***Listeria* in the environment, processing plants and food**

Pascal Piveteau (INRA, Université de Bourgogne-Franche-Comté, Dijon, France) discussed the ecology of *Listeria monocytogenes* in the soil environment. By assessing *Listeria* population dynamics and transcriptomics along time in a variety of soil habitats, he analysed the contribution of abiotic factors, and of resident soil bacterial communities, on the fate of the *Lm* strain EGD-e. For example, the proportion of clay in soil was found to influence the presence of *Lm*. In addition to the influence of inherent soil parameters such as pH or texture, a high diversity of microbial species in the soil microcosm can act as a biological barrier against *L. monocytogenes* [29]. Transcriptome studies in soil demonstrated the overexpression of dedicated nutrient transport and utilization systems. A prominent role of the Agr communication system for *Lm* transcriptional regulation and adaptation in soil was also reported [30,31].

Mickaël Desvaux (INRA, Clermont-Ferrand, France) reported on the role played by *Listeria* SecA2-dependent secretion pathways in its ability to colonize surfaces. He emphasized that this non-essential SecA paralogue in the Sec pathway controls the secretion of proteins important for bacterial adhesion and cell differentiation [32], with a significant impact on bacterial sedimentation, as well as biofilm architecture at ambient temperature [33].

Sophia Kathariou (North Carolina State University, Raleigh, USA) provided an overview of current knowledge on molecular mechanisms allowing *Lm* isolates to resist quaternary ammonium disinfectants and heavy metals. She underlined the strong genetic investment of *Lm* in efflux systems for these compounds and highlighted the heavy influence of horizontal gene transfer in the acquisition of resistance determinants such as the *bcrABC* and *cadAC* cassettes conferring resistance to benzalkonium chloride and cadmium, respectively, harboured on the large plasmid pLM80 [34]. The actual impact of these transfers on virulence and the ability to survive and persist in environmental reservoirs and food-processing environments remains to be evaluated.

Taran Skjerdal (Norwegian Veterinary Institute, Norway) presented the STARTEC decision support tool for food producers, which is a prototype tool developed in an EU financed project with the same name. One of the main messages was that, from the viewpoint of food producers, food safety is only one component of a complex set of process

management issues, which also include quality and cost of food production. Food contamination control must therefore be considered within this broader context, and a multidisciplinary decision support tool can help to tackle the tradeoff between safety and other criteria as well as to establish performance objectives [35].

Sophie Roussel (Anses, France) presented the genetic diversity of 1,700 *Lm* strains isolated from food samples in France. She reported that the serotypic group IIA is dominant (54% of *Lm* isolates) and discussed the association of specific genotypic groups (defined based on PFGE or MLST) with particular food matrices [36]. **Damien Michelin** from the same institute went on to discuss the future use of genomics to enhance the monitoring of food contamination by *Lm*, and presented the network of European Union Reference Laboratories for *L. monocytogenes* [37].

Haley Oliver (Purdue University, USA) presented a large study on the prevalence and modeling of *Lm* in retail deli in the USA. She reported on the detection of *Lm* in 25% of tested delis and showed that *Lm* strains can be persistent in stores [38,39]. Cold room floors were found to be the most contaminated places, which may be useful information for future surveys.

Annette Fagerlund (Nofima Ås, Norway) presented a detailed genomic analysis of *Lm* strains of clonal complex 8 (CC8), illustrating how the comparison of genomic sequences provided answers to questions regarding persistence and epidemiology of *Lm* in food processing facilities and in the food chain [40]. In one case, it was found that the relocation of a conveyor belt from one factory to another was at the source of transmission of a *Lm* strain, underlining the complex challenges posed by the control of this pathogen in food industry.

Pathogenesis of listeriosis in humans and animals

Caroline Charlier (Institut Pasteur, Paris, France) detailed the approach, results and conclusions of an extensive prospective survey of clinical and prognostic factors of listeriosis: the MONALISA (Multicentric Observational National Analysis on Listeriosis and *Listeria*) study. She highlighted the high morbidity and mortality of listeriosis during pregnancy and neurolisteriosis, independently of patients' underlying clinical features. She also emphasised the efficiency of amoxicillin and gentamicin against listeriosis in adults, whereas anti-inflammatory dexamethasone treatment was associated with increased mortality from neurolisteriosis.

Anna Oevermann (VetSuisse, Berne, Switzerland) reviewed the current knowledge on the pathogenesis of neurolisteriosis in farmed ruminants, which typically manifests as rhombencephalitis. She described how *L. monocytogenes* spreads from the oral cavity to the brainstem and further within the brain [41], and reported on the association of the disease with hypervirulent clones, the specific virulence factors of which still require characterization [42].

Anna Bakardjiev (University of California, San Francisco, USA) reported on identification of a secreted listerial protein named InIP that increases the bacterial burden in the placenta of mice and guinea pigs by a factor of 1000 despite only a minor role in other organs [43]. In primary human placental organ cultures InIP promotes bacterial growth and spread. Furthermore, InIP is highly conserved in virulent *L. monocytogenes* strains, and absent in environmental strains and strains that are non-pathogenic for humans. In conclusion, InIP is a novel virulence factor with strong placental tropism.

Marc Lecuit (Institut Pasteur, Paris, France) described the unexpected route employed by *Lm* to cross the intestinal epithelium through goblet cells and gain access to the lamina

propria [44]. The bacterium appears to hijack the E-cadherin recycling pathway and to remain confined in a vacuole during this transcytosis-like process. Interestingly, the process can be recapitulated in intestinal organoids, in which the cell biology of *Lm* translocation across the intestinal epithelium is currently being investigated. He also presented on the identification of hypervirulent clones of *Lm*, with increased neuro and/or placental tropisms. Comparative genomics between these clones and reference strains identified new virulence gene clusters [23].

Cellular microbiology: *Listeria* entry, vacuolar life and spread

Stéphanie Seveau (The Ohio State University, USA) discussed the role of the *L. monocytogenes* pore forming toxin listeriolysin O (LLO) in promoting bacterial internalization into epithelial cells. Indeed, LLO-mediated perforation of the host cell plasma membrane allows Ca^{2+} influx and K^{+} efflux, which both appear necessary and sufficient to promote remodelling of subcortical F-actin leading to the formation of membrane ruffles and subsequently bacterial internalization [45,46]. PKC, Rac1, Arp2/3, and dynamin act as downstream components in the entry pathway, while neither microtubules nor clathrin seem to play a role. Host cell plasma membrane perforation by LLO also activates a membrane repair pathway, which is independent of K^{+} efflux, Rac1, Arp2/3, and F-actin, but requires the influx of Ca^{2+} . *L. monocytogenes* thus appears to hijack components of the host cell membrane repair pathway for its own uptake, independently and additively to the internalin-mediated entry.

In response to membrane damage by pore-forming toxins such as LLO, plasma membrane blebbing allows host cell protection and survival. **Sandra Sousa** (Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal) introduced a role for the endoplasmic reticulum (ER) and components of the cytoskeleton in this process. By targeting the ER, LLO triggers the relocation of the ER chaperone Gp96 to cortical vacuolar structures. There, it interacts specifically with non-muscle myosin heavy chain NMHCIIA and Filamin A. The recruitment of NMHCIIA at the basis of plasma membrane blebs appears pivotal to control membrane damage and allow cell recovery [Mesquita *et al.*, in preparation].

Anat Herskovits (University of Tel-Aviv, Israel) documented the surprising mutualistic relationship adopted by *Listeria* and one of its temperate phages during the intracellular life stage [6]. An A118 prophage integrated in the *Listeria comK* gene is transiently excised during macrophage infection, and recapitulates a replicative cycle without virion production. This process, described as “active lysogeny”, relies on the excision of the phage genome followed by the expression of early phage genes concomitantly with a strong repression of the genes required for virion production and for the lytic stage. Meanwhile, the excision allows restoration of the *comK* open reading frame and activation of *Listeria* competence system, which appears to be required for phagosomal escape. The phage DNA may subsequently be reintegrated.

The spread of *Listeria monocytogenes* from cell-to-cell requires the formation of actin comet tails (Fig. 6), which propel the bacteria to form so-called membrane protrusions. In these protrusions, the laboratory of **John Brumell** (Hospital for Sick Children, University of Toronto, Canada) has recently shown that LLO induces plasma membrane damage, which promotes cell-to-cell spread by the bacteria [47]. Repair proteins such as Caspase 7 and Annexins counter LLO-mediated damage, thus maintaining the integrity of the cell. However, local LLO concentration in the protrusion overcomes these repair processes, leading to exposure of phosphatidylserine (a well-known “eat-me” signal) on the surface of the protrusion, which is then recognized by the macrophage surface protein TIM-4. Thereby,

TIM-4 promotes protrusion association with neighbouring cells and cell-to-cell spread. Brumell and colleagues also investigated the effect of type-I interferon (IFN) signalling on cell-to-cell spread. Strikingly, they found that bacterial spread and motility were impaired in bone marrow macrophages from *Ifnar1^{-/-}* mice, correlating with a defect in ActA polarisation, possibly due to a general effect of type-I IFN response on actin dynamics [48]. This very intriguing finding awaits further study, as its implications may reach well beyond *Listeria* infection.

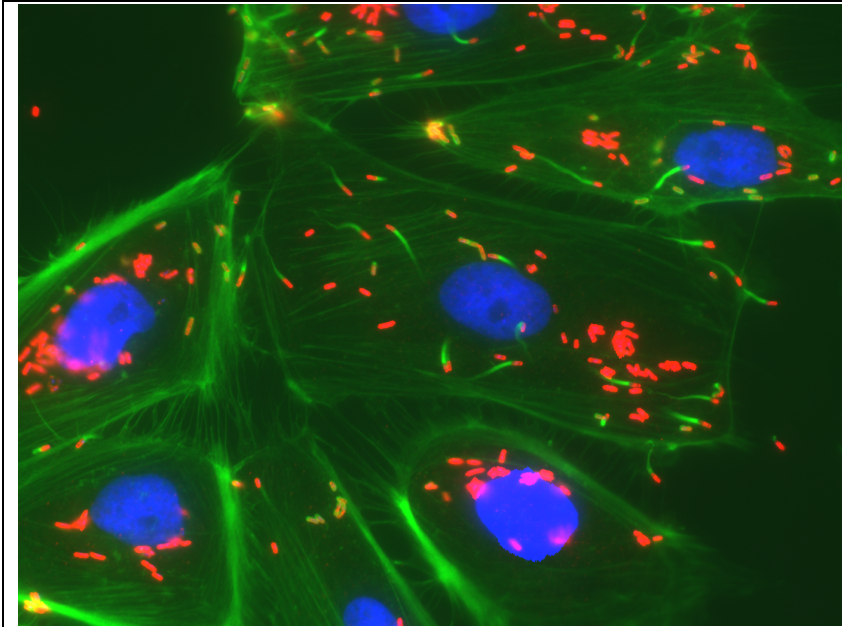


Fig. 6. *Listeria* (red) forming actin comet tails (green) in an infected HeLa cell. The nucleus is stained in blue with DAPI (courtesy of E. Gouin and P. Cossart).

Keith Ireton (University of Otago, New Zealand) further detailed the molecular interplay between bacterial and host factors allowing *Listeria* to form protrusions and spread from cell to cell in polarised epithelial cells. He highlighted how the secreted internalin InlC binds the Src Homology 3 (SH3) domain in the human scaffolding protein Tuba, thereby preventing the formation of Tuba-N-WASP and Tuba-COPII complexes. This in turn loosens cortical actin tension [49,50]. In addition, the DH domain of Tuba can activate the GTPase Cdc42, which plays a role in restricting protrusion formation and *Listeria* spread, probably by activating N-WASP [51]. Via a so-far unknown mechanism, *Listeria* infection causes a drop in Cdc42 activity, which reinforces the inhibition of N-WASP activity and potentiates the disruption of cortical tension, thus favouring cell-to-cell spread.

It has been shown by the Brumell group that *Listeria* is targeted by a non-canonical autophagy pathway referred to as LC3-associated phagocytosis (LAP) prior to escaping the entry vacuole [52]. Here, **Gabriel Mitchell** (University of California, Berkeley, USA) showed that the growth of mutants lacking ActA and the phospholipases PlcA and PlcB is restricted in macrophages [53] through a process that resembles LAP and involves the recognition of the LLO-perforated vacuole. These results suggest that *Listeria* uses ActA and phospholipases to escape a non-canonical autophagy pathway that targets the entry vacuole.

Hélène Bierne (Micalis, INRA Jouy-en-Josas, France) reported on the existence of non-motile forms of *Listeria*, which arise after long-term infections in some epithelial cells lines. These bacteria are devoid of actin and captured in LAMP1-positive single membrane vacuoles. This phenomenon coincides with the loss of the actin-polymerizing factor ActA from the bacterial surface. In addition, ActA-deficient bacteria persist in vacuoles as viable but non-cultivable forms. These results open the possibility that epithelial cells may silently carry persistent *L. monocytogenes*.

It thus seems that *Listeria* can survive in distinct types of vacuoles, depending on the cell type and on the stage of infection. The relative contribution of each of these vacuole types to the *Listeria* life cycle remains to be determined.

***Listeria monocytogenes* effectors: Virulence or niche adaptation?**

Colin Hill (University College Cork, Ireland) analysed the impact of bile produced by the host on *Lm* in the gut and identified “niche factor” genes important for survival in the presence of bile, and which are absent in *Listeria innocua* [54]. Two of these factors, the bile salt hydrolase BSH and BilE, are regulated by σ^B and PrfA, and are essential for virulence in oral route infection of mice [55,56]. Strikingly, exposure of bacteria to bile induced the expression of the flagellar apparatus gene *flaA*. In line with this observation, Hill proposed that bile acts as an environmental cue, acting as a chemorepellent for *Listeria* and thereby directing the bacterium towards the host cell epithelium.

Javier Pizarro-Cerda (Institut Pasteur, Paris, France) reported on the first *bona fide* bacteriocin found in *Listeria*, listeriolysin S (LLS) [57]. Previously described as a haemolytic factor encoded by LIPI-3, this 2.3-kDa toxin is present in a subset of strains that are often associated with human listeriosis outbreaks [58]. Upon oral infection of mice, LLS is specifically expressed in the intestine, where it promotes survival, colonisation and infection of deeper organs by challenging the resident microbiota. This feature may contribute to the higher pathogenicity of *Listeria* strains that produce LLS.

Didier Cabanes (Instituto de Investigação e Inovação em Saúde, IBMC, Porto, Portugal) illustrated how *Listeria* modifies its cell wall teichoic acids (WTA) through L-rhamnosylation, resulting in resistance to antimicrobial peptides (AMPs) and thereby enhancing virulence [59]. Rhamnosylation of WTA depends on the *rml* operon, which is overexpressed during spleen infection. By decreasing the cell wall permeability to AMPs, WTA rhamnosylation would protect bacteria *in vivo*. This might represent one of the evolutionary pressures that drive *Listeria* to maintain WTA rhamnosylation, despite the fact that the rhamnosylated WTA can serve as a phage receptor.

John-Demian Sauer (University of Wisconsin, Madison, USA) reported on novel bacterial determinants supporting bacterial survival in the host cytosol. In a screen for mutants defective for cytosolic survival, they identified that menaquinone biosynthesis was critical [manuscript in preparation]. Menaquinone is a lipid electron carrier involved in bacterial oxidative respiration. Strikingly, bacterial survival in the cytosol requires menaquinone function to maintain a membrane potential and proton motive force, but the final production of ATP is dispensable. This suggests a protective role of the energy potential maintained by the respiratory chain independently of energy production, for instance by buffering the bacterial redox balance upon exposure to reactive oxygen species (ROS).

Patrick Studer (ETH Zürich, Switzerland) described the proliferation mechanism of *Lm* cell-wall deficient variants named L-forms, by formation of intra- and extracellular vesicles, interconnected by elastic lipid tubules allowing cytoplasmic exchange [60]. *Listeria* L-forms were so far reported as non-pathogenic in phagocytes [61]. However, due to the lack of a major antibiotic target and stimulator of the immune response, L-forms could possibly give rise to persisters, an antibiotic-resistant subpopulation that is increasingly in the spotlight with respect to many different bacterial infections [62,63].

The bacterial signalling product cyclic di-AMP (c-di-AMP) is produced by several intracellular pathogens, among which *L. monocytogenes*, *Mycobacterium tuberculosis* and *Chlamydia trachomatis* [64-66]. **Fabian Commichau** (Göttingen University, Germany)

reported on the role of c-di-AMP produced by the diadenylate cyclase CdaA in *Listeria* [67,68]. CdaA is located in the bacterial cell envelope, where it interacts directly with its negative regulator, CdaR [69]. Via an as yet-unknown mechanism, the maintenance of c-di-AMP levels is required for bacterial cell envelope integrity.

In addition, c-di-AMP sensing in host cells results in the activation of type I IFNs through the innate immune protein STING [70], but it has been unclear whether STING is the only sensor of c-di-AMP in eukaryotic cells. **Joshua Woodward** (University of Washington, USA) introduced RECON as a novel eukaryotic protein with high affinity for c-di-AMP [71]. The RECON gene is highly expressed in the intestinal epithelium and encodes a promiscuous alkylreductase that reduces oxidized lipids upon infection. Binding of c-di-AMP inhibits RECON activity, leading to increased nitric oxide production, oxidative damage and inflammation in tissues, and enhanced bacterial cell-to-cell spread.

Virulence gene expression and regulation

Upon invasion of its host, *Listeria* switches from a saprophytic to a pathogenic lifestyle. Its adaptation to new conditions is allowed by a thorough reprogramming of its gene expression, the extent of which has been comprehensively characterized in the last decade using transcriptomic approaches with increasing sensitivity and resolution [reviewed in 72]. **Torsten Hain** (Institute of Medical Microbiology, Justus Liebig University Giessen, Germany) provided updated conclusions on their transcriptional studies of the intracellular response of several *L. monocytogenes* strains [73,74]. Intracellular up-regulated mRNA genes include universal stress regulons and virulence factors, while genes implicated in environmental life are down-regulated. He emphasized that small RNAs (sRNA) represent a significant part of the regulated transcriptome [75]. More recent data from his group indicate that significant transcriptional differences of certain genes, such as flagellar motility genes, occur in clinical isolates, which could contribute to their virulence potential.

Birgitte Kallipolitis (University of Southern Denmark, Odense, Denmark) discussed the role of LhrC “sibling” sRNAs in the regulation of *Listeria* genes associated with cell envelope proteins [76]. Members of this family were named Lhr (for *Listeria* Hfq-binding RNAs) due to their ability to bind the *Listeria* homologue of Hfq [77]; however, there is no evidence for a role of the RNA chaperone in their function, stability or target recognition. In addition to the five previously-described LhrC1-5, which are activated in response to envelope stress via the LisRK two component system [78], she reported the identification of two novel siblings, Rli22 and Rli33-1 [79]. Whereas all seven sRNAs share redundant targets, their expression patterns differ, suggesting that their fine-tuned control participates in the bacterial ability to cope with distinct stimuli.

Francisco Garcia del Portillo (Centro Nacional de Biotecnología, Madrid, Spain) further documented the extensive remodelling affecting the *Listeria* cell surface proteome and peptidoglycan (PG) structure in response to environmental changes. In epithelial cells and in the blood stage, the bacterial cell surface is decorated with proteins required for intracellular life, such as the actin assembly-inducing protein ActA [80,81]. Surprisingly, proteins that are required for entry in cells such as InlA are still found anchored to the PG as late as 6 hours post-infection (p.i.) of epithelial cells. The intracellular PG also harbours Lmo0514, which provides resistance to low pH stress. *lmo0514* expression is tightly regulated, and restricted to intracellular stages, via an interaction with the intracellular-specific sRNA Rli27 in its 5'-UTR, which allows its translation [82]. In contrast, the PG of bacteria grown at refrigeration temperatures displays typical features of cold adaptation. In addition to an increased abundance of specific surface proteins, the cell wall architecture and chemical modifications

of its PG backbone are drastically modified, part of which is linked to the activity of cold-shock proteins.

Pascale Cossart (Institut Pasteur, Paris, France) presented an animation (*Listeria monocytogenes*, a unique model in infection biology https://youtu.be/dlAPOa_QXAo) that visually summarizes the infection process of the whole organism and at the cellular level, highlighting the role of LLO in preparing the cell for infection [83]. She then took one step beyond the integrated -omics of *Listeria monocytogenes*, by presenting the results of a proteogenomic study performed in her group [Impens *et al.*, under revision]. High-throughput N-terminal proteomics of *Lm* strain EGD-e grown in different conditions revealed a map of translation initiation sites (TIS) covering 62% of *Listeria* known open reading frames (ORF). This analysis also revealed internal TIS in 19 ORFs, and allowed the discovery of 6 miniproteins encoded by genes previously annotated as sRNAs. One of them, *prli42*, is conserved among firmicutes and consists of a conserved helical peptide that inserts into the bacterial membrane. Its N-terminus interacts with the main component of the stressosome RsbR, anchors it at the membrane, and is required for H₂O₂ sensing by the stressosome complex, with in addition significant effects on the induction of PrfA-dependent genes.

Conor O’Byrne (National University of Ireland, Galway, Ireland) investigated the regulatory mechanisms of σ^B activity in response to stress exposure. His group has shown that the putative anti-sigma factor RsbW can interact with σ^B , or with RsbV, as previously shown in *B. subtilis* [84]. Sequestration of RsbW by RsbV exacerbates σ^B activity. He also emphasized the fitness cost of stress protection for *Listeria*, as σ^B activity appears to be detrimental to growth in the absence of stress. A selection pressure would thus ensure the maintenance of *sigB* and its tight regulation, which provides an advantage in environmental stress conditions. One such condition appears to be blue light, as addressed by **Kerrie NicAogáin** from the same group [85]. *Lmo0799*, the homologue of the stressosome component YtvA in *B. subtilis* [86,87], can sense blue light, activate σ^B and consequently inhibit bacterial growth and motility upon illumination. This response appears to be protective against damage caused by ROS production upon exposure to sunshine or to high intensities of blue light.

Claudia Guldimann (University of Zürich, Switzerland) documented the intercellular variability existing within a bacterial population in their ability to activate σ^B or PrfA in response to environmental stress [reviewed in 88]. She reported that activation appeared to be a stochastic process, that the proportion of cells activating PrfA was higher than that activating σ^B in all conditions tested, and that heat stress activated the PrfA-dependent *hly* promoter with high efficiency. These differences within a bacterial population could have consequences on the capacity of individual bacteria to cross barriers, and perhaps imply collective behaviours that remain to be characterized.

José Vazquez-Boland (University of Edinburgh, UK) explained the *raison d’être* of the PrfA virulence switch [89]. Indeed, even though PrfA activation is critical for the *in vivo* lifestyle, this activation (as determined using the constitutively activated mutant *prfA** allele) significantly impairs *Lm* fitness outside the host, *i.e.* *in vitro* or in soil microcosms. This is not due to pleiotropic effects of the activated PrfA on housekeeping determinants, but specifically to the cost associated with the up-regulation of the unneeded PrfA-regulated genes, in particular those located in the pathogenicity islands LIPI-1. The fitness cost of PrfA activity for extracellular *Listeria* is further highlighted by the fact that its On/Off switch function is rapidly lost during experimental evolution *in vitro*. In contrast, bacteria grown in alternate conditions of broth and cell cultures mostly maintain PrfA On/Off switchability. These results underline that a fine regulation of virulence gene expression is needed in order to balance

virulence with other fitness components of the pathogen.

Despite 26 years of research on PrfA, the modes of activation of this key *Lm* virulence regulator are only beginning to be uncovered [90]. **Nancy Freitag** (University of Illinois, Chicago, USA) and colleagues identified a peptide pheromone that promotes *Lm* escape from the vacuole in epithelial cells [91]. The pheromone pPplA derives from the proteolytic processing of the secretion signal of the dispensable lipoprotein PplA, and is thought to accumulate in the vacuole until it reaches a critical concentration. It contributes to the activation of PrfA through a currently unknown mechanism. This finding is reminiscent of the multipurpose secretion signals that have been identified for eukaryotic and viral proteins [92], and points to the processing of a secretion signal as a largely conserved signalling mode.

Jörgen Johansson (University of Umeå, Sweden) presented “anti-virulence drugs” as a new concept for fighting bacterial pathogens. Based on the screening of a library of ring-conjugated 2-pyridones on *Lm* infection in cultured eukaryotic cells, Johansson and colleagues identified compounds that act as potent PrfA inhibitors at micromolar concentrations, and are effective against a broad range of *Listeria* strains [93]. These molecules directly bind PrfA and thereby prevent the expression of PrfA-regulated virulence genes, such as *actA* and *hly*. The identified drugs also provide valuable tools for further probing PrfA structure/function relationships and mechanisms of action. In a broader perspective, anti-virulence drugs may emerge as valuable alternatives to antibiotics in a time of ever-rising antibiotic resistance.

Immune responses to *Listeria* infection

Mobarak Abu Mraheil (Institute for Medical Microbiology, Justus-Liebig University Giessen, Germany) reported on the nature of sec-RNAs, consisting of 60-90 bp-long RNA molecules that are secreted by *Lm* into the cytoplasm of its host cell, where they are recognized by cytosolic sensors and trigger a potent type-I IFN response [94]. Sec-RNAs are essentially small non-coding RNAs. Some of these species have the ability to activate RIG-I when transfected into bone marrow-derived macrophages (BMDM), allowing the identification of candidate structural motifs required for launching the host response. The mechanism of this active secretion of specific RNA molecules, which appears to depend on SecA2, still requires further investigation.

The group of **Thomas Decker** (University of Vienna, Austria) had previously identified the host DEAD-box RNA helicase DDX3X as a key component involved in the transcriptional induction of a type I IFN response to *Lm* infection, downstream of bacterial sensing and TBK1 activation [95]. He reported here that mice deficient for DDX3X display a complex immune imbalance and an enhanced susceptibility to listeriosis, far beyond the expected outcomes of a defect in type-I IFN signalling. Their production of cytokines and IFN- γ in response to infection is impaired, and the maturation of the lymphoid lineage is compromised, with a dramatic reduction in NK cell populations. The transcriptional role of DDX3X thus appears prominent in launching an efficient innate immune response against *Listeria*.

Dan Portnoy (University of California, Berkeley, USA) asked why *Lm* is such a potent inducer of cell-mediated immunity (CMI). The main requirements for this observation appear to be LLO-dependent access to the host cytosol and low levels of cytotoxicity. Upon entry into the cytosol, *Lm* secretes c-di-AMP which activates the STING signalling pathway and subsequent type-I IFN response, but in contrast to their original hypothesis, appears to suppress CMI [96], reminiscent of earlier reports showing that type-I IFN signalling enhances

susceptibility to *Lm* infections [97]. Taking these observations into account appears critical for the design of future *Listeria*-based cancer vaccines, to which patients' response might differ depending on their genetic background and physiological condition.

Thomas Dubensky (Aduro Biotech, USA) updated on recombinant live attenuated *Listeria* (LADD, *i.e.* *Listeria* lacking *actA* and *inlB*) expressing tumor antigens in order to induce a strong CD8⁺ T-cell response directed against the tumor [98]. Trials in mice as well as a phase 1b human mesothelioma trial, where LADD have been used in synergy with anti-PD-1 (Programmed Death-1) therapy, have been very promising [99]. We are eagerly awaiting further developments.

Concluding Remarks

The “*Listeria* book” has dramatically thickened recently (Fig. 1). In many areas, amazing achievements have been obtained including, to name a few: Phylogeny, with now at least 18 species described, and multiple sublineages within *L. monocytogenes*; *Listeria* phage biology, as lytic phages provide novel tools for diagnosis and biocontrol [reviewed in 100], while lysogenic phages appear as also involved in infection regulation [6]; genomics, post genomics, transcriptomics and now also translomics; RNA-mediated regulation by 5'-UTRs, small RNAs, riboswitches, excludons, antisense RNAs [reviewed in 72]; gene regulation by allosteric regulators, *e.g.* PrfA that was recently shown to be regulated by glutathione [90]; signalling to the host via bacterial second messengers such as c-di-AMP; and use of *Listeria* as a vaccine strain [reviewed in 101].

These are only a few highlights, which illustrate how we are reaching a level of tremendous complexity in the description of the bacterial physiology, and of the infection process. In order to mimic more closely the human disease, future research will require a better apprehension of the various cells and tissues which are infected *in vivo*, based on animal models. Clearly, understanding how *Listeria* interacts with the gut flora will also be a major challenge.

The next meeting will take place in Toronto, Canada and promises to be as interesting as the Paris meeting.

Conflict of interest

The authors have no conflict of interest to declare.

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