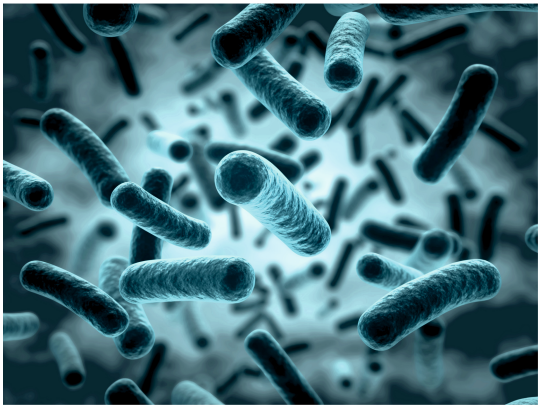
Gopala Krishna Mannala

# Dual non-coding RNA profiles of host and bacteria and their role in the regulation of innate immune response during *Listeria monocytogenes* infection



**INAUGURAL DISSERTATION** (Cumulative thesis)
submitted to the Faculty of Medicine
in partial fulfilment of the requirements
for the PhD degree
of the Faculties of Veterinary Medicine and Medicine
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by

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Giessen 2016

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# 1. List of publications

During his doctoral work, the author was involved with the following publications which are part of this thesis.

1. **microRNA response to *Listeria monocytogenes* infection in epithelial cells.**
   Izar B*, **Mannala GK***, Mraheil MA, Chakraborty T, Hain T. Int J Mol Sci. 2012; 13(1):1173-85. doi: 10.3390/ijms13011173. Epub 2012 Jan 20. **(Equal first authors)**

2. ***Listeria monocytogenes* induces a virulence-dependent microRNA signature that regulates the immune response in *Galleria mellonlella*.**
   **Mannala GK*,** Izar B*, Rupp O, Goesmann A, Chakraborty T, Hain T. (**Manuscript was submitted for publication**)

3. **Ultra-deep sequencing of *Listeria monocytogenes* sRNA transcriptome revealed new antisense RNAs.**
   Behrens S, Widder S, **Mannala GK**, Qing X, Madhugiri R, Kefer N, Abu Mraheil M, Rattei T, Hain T. PLoS One. 2014 Feb 3;9(2):e83979. doi: 10.1371/journal.pone.0083979. eCollection 2014.

4. **Detection of Very Long Antisense Transcripts by Whole Transcriptome RNA-Seq Analysis of *Listeria monocytogenes* by Semiconductor Sequencing Technology.**
   Wehner S, **Mannala GK**, Qing X, Madhugiri R, Chakraborty T, Mraheil MA, Hain T, Marz M. PLoS One. 2014 Oct 6; 9(10):e108639. doi: 10.1371/journal.pone.0108639. eCollection 2014.

5. **Current status of antisense RNA-mediated gene regulation in *Listeria monocytogenes*.**
   Schultze T, Izar B, Qing X, **Mannala GK**, Hain T. Front Cell Infect Microbiol. 2014 Sep 30;4:135. eCollection 2014. Review.

# 2. Introduction

Infectious diseases are one of the significant contributors of mortality and morbidity worldwide. These are caused by bacteria, viruses and other multicellular organisms such as fungi and are spread directly or indirectly from one person to another. Abu Ali ibn Sina (Avicenna) discovered the contagious nature of infectious diseases in the early 11$^{th}$ century. In 13$^{th}$ century, Europe had devastating pandemic plague outbreak, caused by *Yersinia pestis*, which led to a decrease of 30-60% of the total European population. During the course of any infection, the pathogens follow several strategies to evade the host defense system and, adapt to the host environment for efficient survival, which in turn is encountered by host mediated cellular process, innate and adaptive immune system. At molecular level, the pathogens deploy different strategies, mainly by modulating their gene expression profile according to intercellular and intracellular compartments of the host. In a similar way, the host also alters its gene expression profile specific to each pathogen as defense mechanism. For a long time, proteins were considered to be the predominant molecules to regulate gene expression until the discovery of regulatory non-coding RNAs (ncRNAs) both in prokaryotes and eukaryotes. The discovery of these regulatory non-coding RNAs opened a branch in further understanding of gene regulation which can be exploited for drug targets and biomarkers.

## 2.1. Discovery of non-coding RNAs

The term non-coding RNA (ncRNA) represents nucleotide sequence which does not code for any protein, but has other functional roles (*1*). The first described ncRNA was alanyl-transfer RNA (tRNA) from baker's yeast by Robert W. Holley group in 1965 (*2*). In 1974, the cloverleaf secondary structure of tRNA was elucidated using X-ray crystallography (*3*). In later years, ribosomal RNA (rRNA), which is highly abundant and the major structural component of ribosomes, was discovered. These two ncRNAs (tRNA and rRNA) are involved in protein synthesis machinery with coding mRNA in both prokaryotes and eukaryotes. Later, in the eukaryotic nucleus, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) were identified and associated with splicing machinery and modification of ribosomal RNA, respectively. Furthermore,

remarkable developments in molecular biological methods (microarray, whole genome sequencing and bioinformatics approaches) led to the discovery several novel types of ncRNAs; small non-coding RNAs (eukaryotic miRNAs, piwiRNAs, siRNAs and prokaryotic *trans*-encoded sRNAs, *cis*-acting riboswitches, CRISPR elements) and long non-coding RNAs (both in prokaryotes and eukaryotes) (*1*, *4*). microRNAs were firstly discovered by Victor Ambros in *Caenorhabditis elegans* during developmental studies (*5*). siRNAs are associated with RNAi machinery which was discovered by Andrew Fire and Craig Mello which won them the Nobel prize for Medicine in 2006. CRISPR are clustered regularly interspaced short palindromic repeats of prokaryotic DNA which confers protection against plasmids and phages. These CRISPR molecules were first described in *Escherichia coli* in 1987 however, exact functions were not known at that time (*6*). In recent years, the CRISPR/Cas system has been widely used for RNA guided genome editing in eukaryotic systems. Recently, circular RNAs were identified, a type of RNA that is closed as continuous loop by joint 5´ and 3´ ends. These are considered as non-coding RNA and potential gene regulators in eukaryotes and archaea (*7*).

## 2.2. Role of non-coding RNAs in host-pathogen interactions

During the course of infections, interplay between host and the pathogen leads to dynamic changes in their global gene expression. These infections not only alter the gene expression, but also non-coding RNAs in both, host and pathogen. Broadly, these ncRNAs act as regulators of gene expression through complementary base pairing with target mRNAs to either suppress the translation of mRNA into functional protein at post transcriptional level or stabilize the mRNA (*8*). In the past decade, the ncRNAs are under extensive investigation to reveal their functions in several cellular and physiological processes. Recently, efforts have been taken to prove the roles of ncRNAs in bacterial infection and subsequent host immune responses. So, a unique profile of ncRNAs can be associated to fine tuning of mRNA expression for immediate adaptation of cellular physiology in response to environmental changes during in host and pathogen interaction. These ncRNAs have been explored as drug targets and biomarkers for diagnostic purposes (*9*).

## 2.3. Eukaryotic non-coding RNAs

Eukaryotes express a larger and more diverse group of ncRNAs than prokaryotes because of their higher complexity at cellular levels. The functions of some of the ncRNAs, for instance, snRNAs and snoRNAs are confined to nucleus and involved in splicing of mRNA and modification of other RNA molecules. Other ncRNAs are involved in regulation of gene expression by acting as *cis*- or *trans*-regulatory elements.

### 2.3.1. Classes of eukaryotic non-coding RNAs

The eukaryotic non-coding RNAs are categorized into following types:

snRNA: small nuclear RNAs are found in nucleus of eukaryotic cells associated with proteins to form ribonucleoprotein complexes. These snRNAs (U1, U2, U4, U5, and U6) are components of major splicesome and involved in splicing of mRNAs (*10*).

snoRNA: small nucleolar RNAs are involved in guiding other RNA molecules such as tRNA and rRNA through base modifications. These snoRNAs are divided into two classes: C/D box snoRNAs and H/ACA snoRNAs (*11*).

siRNA: siRNAs are small interfering RNAs with double stranded 20-25 nt size and regulate target gene expression by complementary base pairing.

miRNA: microRNAs are small single stranded RNA molecules with 22-25 nt size and are involved in post transcriptional gene regulation. These miRNAs were well elucidated for their regulatory roles in several physiological and pathological processes (*12*).

piRNA: piRNAs are small RNA molecules, associated with piwi protein and linked to gene silencing of retrotransposons in germ cell lines (*13*).

lncRNA: lncRNAs are RNA molecules with size more than 200 nt, transcribed from intergenic and intragenic/intronic genomic regions (*14*).

Circular RNAs: These are single stranded, containing exon sequence of a gene and produced from ligation of 5´ and 3´ ends of linear mRNA (*7*).

### 2.3.2. microRNA biogenesis and functional mechanism

The biogenesis of microRNA begins with transcription of large hairpin transcripts by RNA polymerase II from the miRNA gene. These transcripts, called primary miRNA (pri-miRNA), are processed into preliminary miRNA (pre-miRNA) by RNase III endonuclease Drosha-DGCR8 complex in the nucleus. This precursor miRNA is exported out of nucleus through exportin-5, where it is processed into 21-24 nt duplex miRNA by RNase III enzyme called dicer. This duplex strand contains one functional guide strand, which is complementary to target and another passenger strand that undergoes degradation after RISC complex formation (*15*). Later duplex is loaded onto argonaute protein to form miRNA RNA-induced silencing complex (miRISC) along with other accessory proteins (TNRC6). The three dimensional structure of RISC complex revealed that mature miRNA bases from 2-8 were involved in hydrogen bond formation with target mRNA (*16*). Mature miRNA directs RISC to target mRNA with perfect base pairing and regulates its expression either by destabilization or translational repression. After binding of argonaute-miRNA complex to target mRNA, TNRC6 inhibits the translation of target mRNA with recruitment of CCR4-NOT1 deadenylase complex that initiates degradation of target mRNA(*17*). The process of miRNA biogenesis and its regulatory mechanism is depicted in (Figure 1).

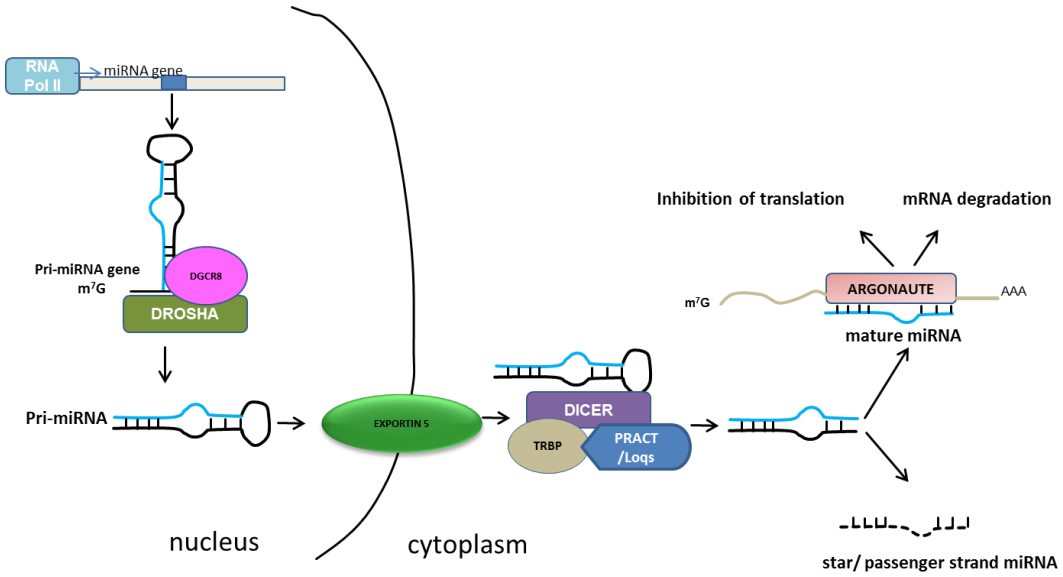


**Figure 1.** miRNA biogenesis and its regulatory mechanism. microRNAs are transcribed by RNA polymerase II from miRNA gene to give primary miRNA (pri-miRNA) transcript. This pri-miRNA is processed into precursor miRNA (pre-miRNA) by Drosha and DGCR8 complex. This hairpin pre-miRNA is transported into cytoplasm through exportin 5 where further processed by the dicer complex.

The dicer cleaves loop of the pre-miRNA resulting in 21-24 nt miRNA duplex which is loaded on to argonaute complex to form miRNA-induced silencing complex (miRISC). After formation of RISC complex the passenger strand undergoes degradation. Subsequently guide strand mediated downregulation of target mRNA through either decay of mRNA or inhibition of translation results. Adapted from reference(*18*).

### 2.3.3. microRNA response to infection of bacterial pathogens

Since the discovery of miRNAs, these molecules have been well explored for their regulatory roles in several physiological and pathological processes such as development, energy metabolism, immunity, apoptosis, cancer, and cardiovascular diseases (*12*). Additionally, from recent studies it is evident that miRNAs also play an important role during microbial infections. Several studies have demonstrated the role of miRNAs in host-pathogen interactions. In case of infection, miRNA's roles were firstly elucidated in viral and parasitic infections. From analysis of small RNA expression profiles, it is known that DNA viruses express several miRNAs to control viral and cellular mRNA thereby affecting viral replication and pathogenesis. Besides this, host miRNAs like miR-29a and miR-32 are involved in antiviral activity against HIV and primate foamy virus (PFV) by targeting viral mRNA (*19*, *20*). In case of bacterial infection, Navaro *et al.,* demonstrated the induction of miR-393a transcription in *Arabidopsis thaliana* during the infection with *Pseudomonas syringae*. Here, sensing of bacterial flagellin by FLS2 receptor leads to induction of miR-393a that represses the auxin hormone receptor and controls plant innate immune system (*21*).

Later, several studies expanded the knowledge about miRNAs concerning bacterial infections. The first proof that a bacterial pathogen could alter a broad range of miRNA profile in infected host cells was provided for the extracellular pathogen *Helicobacter pylori*. A microarray study revealed upregulation of several microRNAs, foremost miR-16, miR-146a and miR-155, in GES-1 cell upon infection with *H. pylori*. Moreover, miR-155 and miR-146a were shown to have elevated levels of expression in gastric mucosa from *H. pylori* infected patients (*22*, *23*). The authors also observed that targets of miR-155 and miR-146a were related to negative regulation *of H. pylori* induced inflammatory response. The induction of miR-155 was further observed in various cell types like primary macrophages, human T-cells along with mucosal tissue of mice and humans. By using different mutants, several studies demonstrated that the strong induction of miR-155 was dependent on the major virulence factors of *H. pylori* such as VacA, GGT and

its LPS (*24*, *25*). Later, studies using next-generation sequencing approaches in AGS cell line, a gastric epithelial cell line model to study *H. pylori* infection, could not show altered expression of miR-155 to *H. pylori* infection. Nevertheless, the authors observed the upregulation of other miRNAs, especially miR-21 and miR-371-373 cluster. The induction of miR-21 is in good agreement with other studies from human gastric mucosa samples, hypothesized for its role in gastric cancer as it targets RECK, a tumor suppressor in gastric cancer (*26*). Yet another study came up illustrating the deregulation of 31 miRNAs in gastric mucosa with *H. pylori* infection and dependent on CagA virulence factor. Among these, miR-223 has shown elevated expression level where as 30 other miRNAs (including let-7 family members) have shown reduced expression (*27*).

In addition to extracellular *H. pylori*, there are many other bacterial pathogens such as *Salmonella*, *Listeria* and *Mycobacterium* which resides in intracellular conditions and causes severe infections in different organisms. In *Salmonella* Typhimurium, the first study was conducted to show altered host microRNA response to an intracellular pathogen. Using small RNA sequencing method, Schulte *et al*., demonstrated the induction of NF-κB dependent miRNAs (miR-21, miR-146b and miR-155) in macrophages upon *Salmonella* infection. The same study revealed the downregulation of let-7 family members in macrophages and epithelial cell lines and also the role of these miRNAs in regulation of IL-6 and IL-10 cytokines (*28*). Besides this, the external stimulus of *S*. Typhimurium was shown to be enough for the induction of the major immune regulator miR-155 regardless of invasion (*28*). In case of Gram-positive bacterial infection, *L. monocytogenes* alters the host miRNA profile upon infection. In bone marrow-derived macrophages, miRNAs (miR-155, miR-146a, miR-125 and miR-149), which are known for regulation of the immune response, were induced (*29*). In another study, during systemic infection of mice with *L. monocytogenes*, miR-29 expression was found to be downregulated in NK cells. This study showed that increased secretion of IFN-γ is associated with downregulation of miR-29 and promotes host resistance to *L. monocytogenes* infection (*30*). Recently, Cossart's group has shown that probiotic strains such as *Lactobacillus casei*, *Lactobacillus paracasei* and gut microbiota were able to interfere with miRNA response of mice with orally acquired

listeriosis, subsequently influencing the pathogenicity of *L. monocytogenes* (*31*, *32*). The overview of miRNA mediated regulation of host immune response towards bacterial infection is illustrated in (Figure 2).

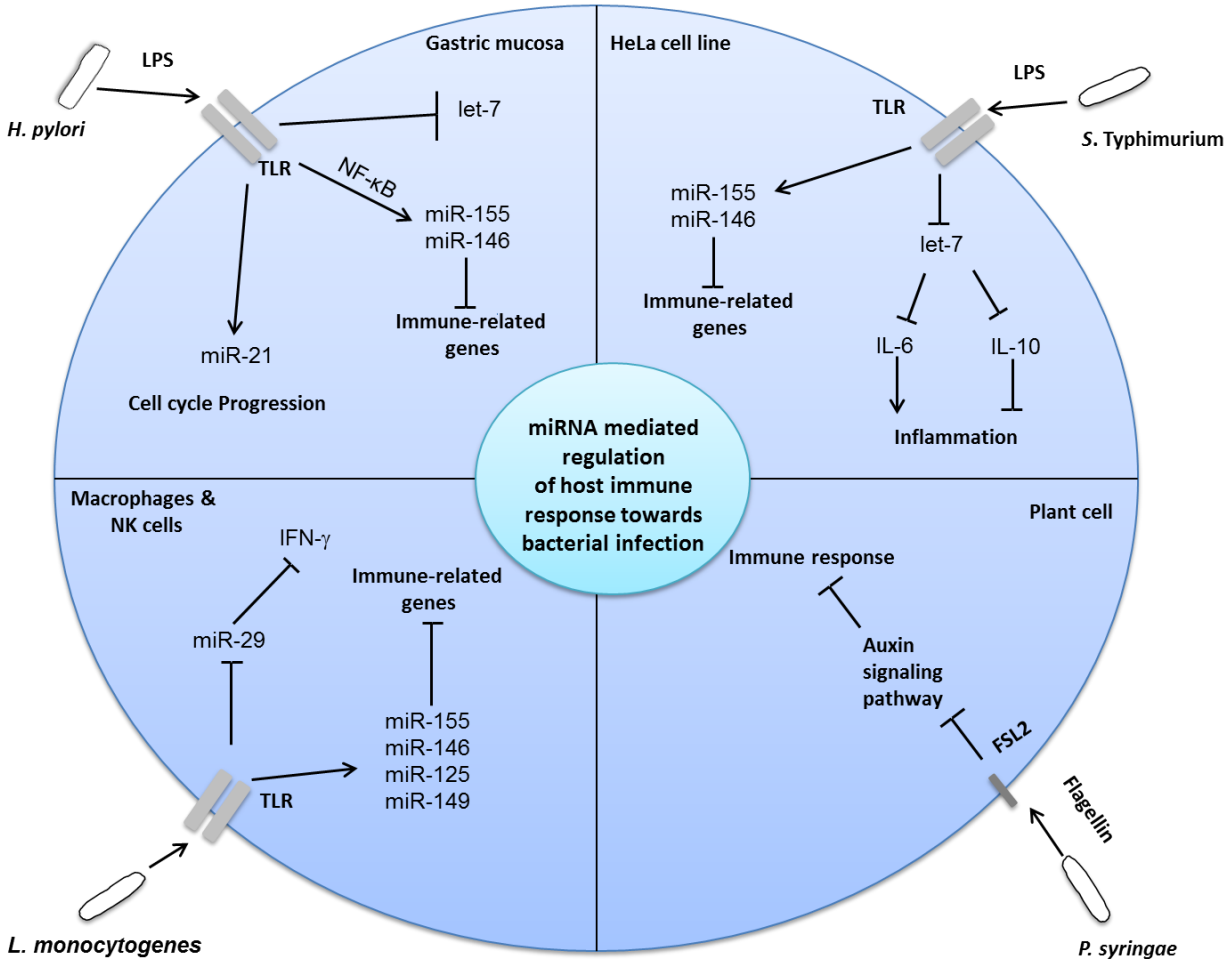


**Figure 2.** Overview of miRNA mediated regulation of host immune response to bacterial infections. Adapted from reference(*8*).

In insects, the endosymbiont bacterium *Wolbachia* has been shown to induce the expression of aae-miR-2940 which targets the metalloproteinase and cytosine methyl transferase genes and thereby plays major role in its maintenance (*33*). Freitak and co-workers have demonstrated alteration of miRNA response in *Tribolium castaneum* after infection with *Pseudomonas entomophila* and this response varies with gender specificity of the host (*34*). Recently, Mukherjee *et al.,* have investigated the role of miRNA in the developmental stages and in fungal and bacterial infections of insect

infection model *G. mellonella*. Moreover, they have also shown that, miRNAs can act as mediator for trans-generational immune priming (*35*).

### 2.3.4. microRNAs involved in immune regulation during bacterial infections

As mentioned earlier, bacterial infection lead to significant changes in the miRNA repertoire in both *in vivo* and *in vitro* infection models. The microRNA response differs based on the bacterial pathogen as well as host, but some miRNAs have exhibited similar ways of regulation and effects on host innate immune system irrespective of the type of pathogen. Some miRNAs e.g., miR-146, miR-155, miR-21 and let-7 family members have shown crucial functions in host immune responses during bacterial infection.

**miR-146**

The expression of miR-146 is found to be elevated in host cells in response to various bacterial pathogens such as *S*. Typhimurium, *H. pylori*, *Mycobacterium* species and *Francisella tularensis* (*36*). First time, the induction of miR-146 along with miR-155 and miR-132 was observed in monocytes after treatment with LPS. This phenomenon was subjected to surface TLR signaling receptors rather than cytosolic TLRs which sense nucleic acids (*37*). In parallel to activation of transcription of cytokine genes during different TLR signaling pathways, major transcriptional regulator NF-κB binds to the promoter of miR-146 gene and induces its transcription. The miR-146 targets TRAF6 and IRAK1, which are important adapter molecules in TLR/NF-κB signaling cascade, thereby regulate host innate immune response. In this way, the functions of miR-146 can lead to negative regulation of TLR signaling pathways in response to bacterial products, minimizing LPS sensitivity and protecting the host from excessive inflammation (*38*). Over expression of miR-146 along with miR-132 and miR-212 is linked with macrophages tolerance to septic shock, induced by extracellular bacterial stimuli. The tolerance is resulted from reduced MyD88 recruitment to TLR signaling pathways, subsequently diminishing NF-κB activity and TNF-α production. Similar innate immune tolerance was observed in vaginally delivered neonatal mice with downregulation of IRAK-1 and strong upregulation of miR-146. The epithelial TLR susceptibility was restored by oral administration of anti-miR-146a to neonatal mice (*39*).

**miR-155**

Another miRNA, miR-155 was known for its induction through TLR sensing of bacterial and viral components and also by TNF-α and interferons (*40*, *41*). Recently, activation of miR-155 has been also reported by bacterial peptidoglycan components that are sensed by cytosolic NOD2 receptors (*28*). Thus, it appears to be a major component of inflammatory mediators of innate immune system. Several adapters (FADD, IKKε, TAB2, SOCS1 and RIPK1) from TLR signaling pathways were identified as targets of miR-155. Thus similar to miR-146, miR-155 is also involved in negative regulation of innate immune responses. miR-155 is located within highly conserved sequence of non-coding RNA gene *bic* (B-cell receptor inducible gene) on chromosome 21. The non-coding RNA gene *bic* is reported to be highly expressed in Hodgkin and Burkitt lymphoma cells (*36*). Deletion of miR-155 portion in *bic* gene in mice influenced several aspects of adaptive immune system. Vaccination with attenuated *Salmonella* strain, miR-155 null mice were unable to mount protective immune responses against challenge with wild type strain (*42*). Mice lacking miR-155 were highly susceptible to infection with *Citrobacter rodentium* and showed impairment in humoral immune responses (*43*). Furthermore, these mice also showed deficient $CD8^+$ T cell response to facultative intracellular pathogen *L. monocytogenes* (*44*). Above examples state that miR-155 is also essential for functioning of adaptive immune system that includes both T cell and B cell activation during bacterial infections.

**let-7 family**

let-7 family members are highly conserved between vertebrates and invertebrates in both sequence and functions. These are well known to be involved in cell differentiation and development. It also seems to be involved in innate immune response. As let-7i has target region for TLR4, it is downregulated after infection with *Cryptosporidium paruvam* or LPS treatment in human cholangiocytes (*45*). From these results, It is speculated that downregulation of let-7 facilitates TLR activation to mount innate immune response. let-7 family members were downregulated in human gastric mucosa infected with *H. pylori* (*27*). Among these, let-7b is related to acute inflammation by neutrophil infiltration whereas let-7a is linked to acute and chronic inflammation mediated through

mononuclear cell infiltration. Furthermore, *Salmonella* infection also led to reduced expression of let-7 both in murine macrophages and epithelial HeLa cell line. The same pattern of let-7 regulation is sustained in endotoxin tolerated macrophages. TLR4 signaling that is activated by bacterial LPS leads to suppression of let-7 family members and subsequently expression of its target cytokine genes IL-6 and IL-10. Interestingly, IL-6 and IL-10 can have opposite effects on host immune response, IL-6 supports whereas IL-10 inhibits pro-inflammatory program. So, the downregulation of let-7 family can be attributed to fine tuning of immune response to *Salmonella* infection (*28*). The regulation of host innate immune response at various levels by miRNAs is represented in (Figure 3).

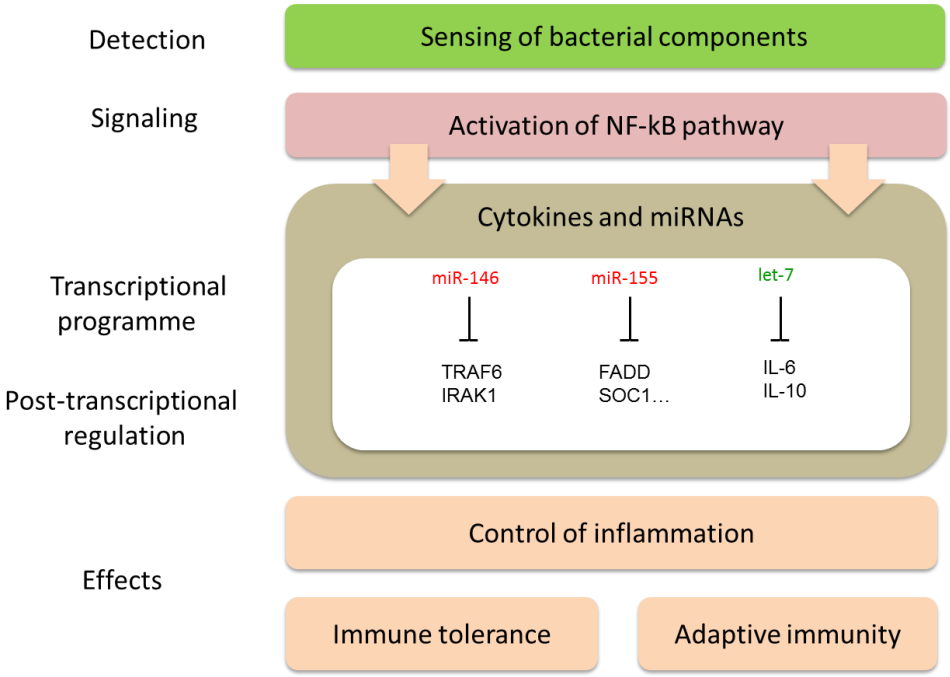


**Figure 3.** Regulation of host innate immune response by miRNAs. Adapted from reference (*36*).

### 2.3.5. Overview of insect immune system and its regulation by miRNAs

Insects are the most diverse group of organisms with around a million of described species and are present everywhere on the earth. In the course of evolution, approximately 500 million years ago insects and vertebrates diverged. Insects easily can get infected by viruses, bacteria and fungal pathogens from their surroundings and thus have evolved different defense systems to combat them. They have an effective primitive immune system called innate immune system, but lack an adaptive immune

system like higher mammals. As a lot of similarities exist between innate immune system of insects and mammals, insect innate immune system is well explored to understand the innate immune system of higher animals with avoidance of complex adaptive immune system (*46*). *Drosophila melanogaster* has been shown as a popular model to study insect immune system. It consists of humoral and cellular responses: humoral immunity consists of localized melanization and the production of AMPs in fat body, which is similar to human liver whereas cellular immunity is composed of immune cells called as hemocytes that engulf invading pathogens (*47*). The expression of anti-microbial peptides is mainly under the control of two signaling pathways. Those are the Toll and Imd (immune deficiency) signaling pathways showing similarities to Toll-like receptor/interleukin-1 and TNF-$\alpha$ pathways in higher mammals. These signaling pathways are activated based on the interactions between receptors and ligands or pathogen associated molecular patterns (PAMPs) that are released by different pathogens (*48*).

Toll pathway is a serine protease cascade, activated by fungal and Gram-positive bacteria. It contains three branches: two pattern recognition receptors (PRR) based pathways and the virulence dependent danger-signaling pathways. Peptidoglycan components of Gram-positive bacteria bind to PRRs PGRP-SA, PGRP-SD and GNBP1, whereas the $\beta$-1, 3-glucans of fungal cell wall bind to GNBP3 receptor to activate Toll pathway. The danger-signaling pathway is induced by the virulence factors that are secreted by bacterial and fungal pathogens during infection (*49*). The Imd pathway consists of a kinase cascade activated through the receptors PGRP-LE and PGRP-LC, which bind to the cell wall components of Gram-negative bacterial pathogens (*47*). Both these Toll and Imd pathways lead to activation of transcription factor NF-$\kappa$B, which in turn activates transcription of genes encoding AMPs. Additionally, other pathways like JAK-STAT and JNK cascade are also involved in counteracting viral and Gram-negative bacterial infections respectively (*50*). Figure 4 gives an overview of the immune system in insects.

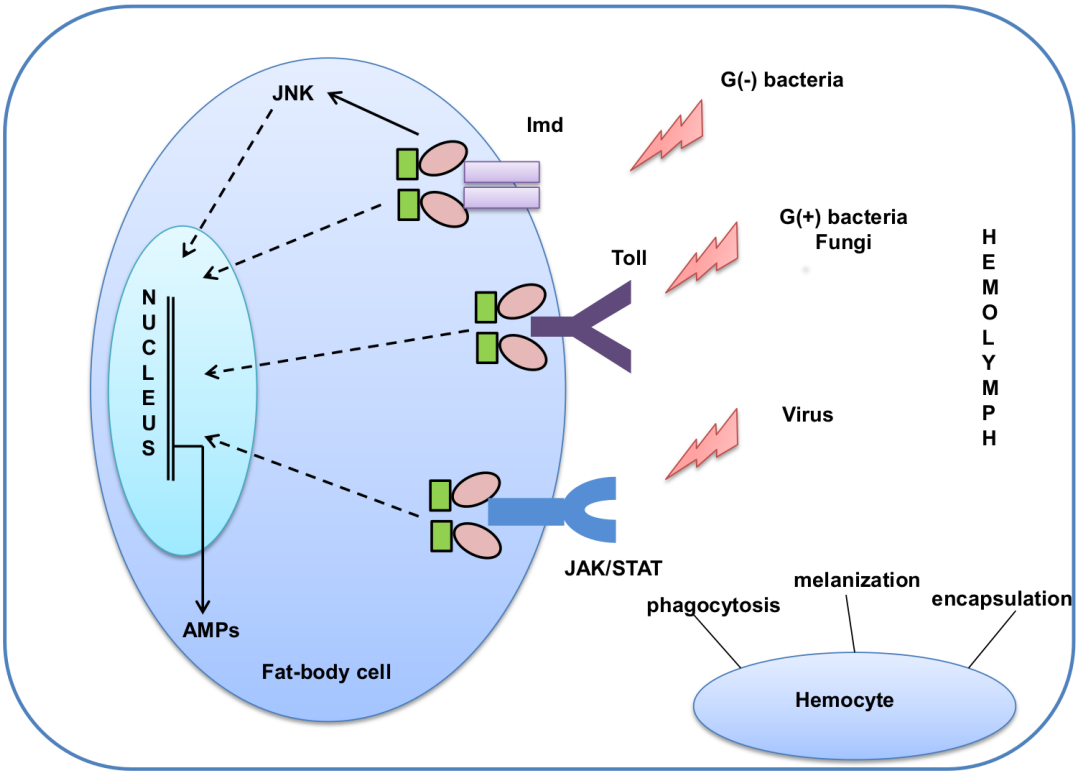


**Figure 4.** Overview of the insect immune system. Insects contain innate immune system that composed of humoral and cellular responses. The humoral response comprises of different signaling pathways that are associated with the synthesis of anti-microbial peptides (AMPs) in fat-body cells. Gram-negative bacterial infection activates Imd and JNK cascades, whereas Gram-positive bacterial and fungal infections lead to induction of Toll pathway. In addition, the JAK/STAT pathway is activated in response to viral infections. On the other hand, hemocytes are involved in cellular response with several processes as phagocytosis, melanization, encapsulation and coagulation. Adapted from reference (*51*).

In addition to regulatory roles of miRNAs in vertebrate immune system, several studies also demonstrated regulatory functions of miRNAs in insect immune system. A study based on computational target prediction for miRNA was able to identify over 60 miRNAs that are associated with immune signaling pathways such as Toll, Imd, JNK/STAT pathway and phenol oxidase pathways in *D. melanogaster* (*52*). A similar kind of study in *Anopheles gamibiae* identified two miRNAs aga-miR-2304 and aga-miR-2390 that target the genes coding supressin and prophenoloxidase respectively (*53*). One of the insect miRNAs, for which the role in insect immunity experimentally was proven, is miR-8 that negatively regulates anti-microbial peptides (AMPs) such as drosomycin and diptericin in *Drosophila*. This miR-8 is involved in keeping low

expression levels of AMPs and maintaining the homeostasis of immunity in non-infected animals. Even though, miR-8 knockout mutant showed higher levels of AMPs expression. Moreover, It was predicted that miR-8 could target transcripts of GNBP3, a receptor for Toll path way activated by fungal infection and Pvf, linked to JNK pathway (*54*). In dengue virus vector *Aedes aegypti*, blood meal induced miR-375 was found to regulate two immune related genes *cactus* and *REL1*. Interestingly, in subsequent studies, cactus, an inhibitor of Toll pathway, is positively regulated by miR-375 whereas REL1, an activator of AMPs, is suppressed by same miRNA. The cumulative effect of this miRNA regulation on *cactus* and *REL1* promotes replication of dengue virus as AMPs negatively affect virus replication (*55*, *56*).

## 2.4. Bacterial non-coding RNAs

As stated earlier, similar to eukaryotes, bacteria transcribe several types of non-coding RNA elements based on the environmental conditions. These molecules are known to control several functions in bacteria including envelop homeostasis, biofilm formation, uptake and assimilation of several nutrients, carbon metabolism and regulation of virulence gene expression.

### 2.4.1. Different classes of bacterial non-coding RNAs in *L. monocytogenes*

Bacterial ncRNAs are categorized into three major classes as *cis*-acting RNAs (riboswitches and thermosensors), *cis*-encoded antisense RNAs (asRNAs) and *trans*-encoded small RNAs (sRNAs). *cis*-acting elements are present on 3´ and 5´ UTRs of mRNA which can regulate either transcription or translation. Even though, there are evidences that these elements can also be associated with mRNA stability and turnover (*57*, *58*). The *cis*-encoded asRNAs are less explored, but are for instance thought to be involved in inhibition of translation by interfering the RNA polymerase activity through base pairing to sense strand (*59*). Finally, the ncRNAs, which are *trans*-encoded, generally function by binding to their target mRNA and modulating translational outcomes (*60*).

With the discovery of a thermosensor in *L. monocytogenes*, located on 5´ UTR of *prfA* which regulates expression of *prfA* based on temperature shifts, the organism has become a model to study RNA based regulation (*61*). In recent years, through different

approaches such as bioinformatics, tiling arrays and RNA-seq, a number of regulatory ncRNAs have been identified in *L. monocytogenes* under different experimental conditions.

### *cis*-acting RNA elements

This class of ncRNAs comprises of riboswitches and thermosensors. Riboswitches get transcribed on as part of the mRNA, bind to specific ligands like nucleotides, ions, metabolites and tRNAs and control either transcription or translation of downstream ORFs. At first, using a bioinformatics approach, 42 riboswitches were identified for *L. monocytogenes* and grouped into 13 families (*62*). Later, using different molecular methods such as tiling arrays and RNA-seq more riboswitches were identified and some of the functions were elucidated. For example, the lysine riboswitch located between the genes *lmo0798* and *lmo0799* regulates the transcription of both genes in presence of lysine as ligand. By binding to lysine, this riboswitch terminates the transcription of the downstream gene *lmo0798* and acts as terminator for upstream gene *lmo0799* (*63*). Another example of a riboswitch that can act *in trans* as a transcriptional regulator after binding to a ligand is well demonstrated in case of SAM riboswitches. Here, the two SAM riboswitches *sreA* and *sreB*, which can also act as small RNAs, are involved in regulation of major virulence gene regulator PrfA, and this phenomenon has been demonstrated through deletion mutants of these riboswitches and overexpression studies (*64*).

The majority of *cis*-acting elements are known to regulate transcription of genes, but few of them like thermometers are associated with inhibition of translation. RNA thermosensors form complex secondary structures that can respond to differences in temperature by altering their conformation and thereby masking or unmasking the Shine-Dalgarno sequence. In case of *L. monocytogenes*, a RNA thermosensor is regulating translation of PrfA protein. At 30°C, protein levels of PrfA and virulence factors is very low, despite the presence of *prfA* mRNA. This indicated that *prfA* might be regulated post-transcriptionally. The presence of a long 5´ UTR which was observed for *prfA* mRNA led to hypothesize for UTR mediated regulation. Subsequently, it has been proved that 5´ UTR adopt different structural confirmations at different temperatures. At

30°C, The 5´ UTR region forms a stable hairpin structure which prevents Shine-Dalgarno sequence interaction with 30S ribosomal subunit whereas at 37°C the stable structure of hairpin melts and allows to initiation of *prfA* mRNA translation.

**Antisense RNAs**

Traditional microarray approaches can be used only for expression analysis of annotated ORFs. But, the advent of tiling array and RNA-seq methods identified the large number of *cis*-encoded transcripts in antisense orientation to the respective ORFs. In recent years, development of transcriptomics revealed extensive antisense transcription throughout all bacteria (*59*). For example, in *E. coli* around 1005 asRNAs were detected, covering 22% of total ORFs (*65*) as well as in *H. pylori* 969 asRNAs were expressed with overlapping 46% of all ORFs (*66*) in opposite orientation. In case of *Listeria*, the tiling array studies revealed the presence of 4 antisense RNAs as well as some other mRNAs which have either long 3´ UTR or 5´ UTR and overlapping adjacent genes in antisense manner. With the help of RNA sequencing technology, various studies revealed the presence of total 86 asRNAs in *L. monocytogenes* (*67*).

So far, asRNAs are less investigated by functional studies. However, some recent work highlighted the role of asRNAs in regulation of gene expression either on transcription or translational level. The well-studied example for the function of asRNAs is the regulation of flagellar synthesis genes by their repressor *mogR*. The *mogR* gene is transcribed from two transcriptional start sites, one is located at 45 nucleotides and another one positioned far way at 1697 nucleotides of upstream of start codon, resulting in two transcripts with short and long 5´ UTRs (*63*). The long 5´ UTR of *mogR* gene overlaps the genes of flagella biosynthesis in anti-sense manner. It has been proven that the expression of these long 5´ UTR containing *mogR* transcripts resulted in decreased expression of flagellar genes, because the deletion of *sigB* consensus sequence of long transcript led to an increased expression of flagellar genes (*67*).

Recently Mellin *et al.,* described that an asRNA is regulated by vitamin $B_{12}$ binding riboswitch. *PocR* is a transcriptional regulator for *pdu* and *cob* genes, which are involved in propanediol catabolism and vitamin $B_{12}$ biosynthesis. This metabolism requires $B_{12}$

dependent diol dehydratase encoded by the *pduCDE* genes. In *L. monocytogenes pdu* genes are positioned in two cassettes with 8 and 20 genes. These two clusters are surrounded by *pocR* transcriptional regulator. Whole genome transcriptional analysis identified an asRNA opposite to *pocR*, a transcriptional regulator. It comprises of a previously identified noncoding RNAs *rli39* and *rliH* and is regulated by a $B_{12}$ riboswitch. The presence of propanediol leads to activation of *pocR* transcriptional regulator, which in turn leads to activation of *pdu* and *cob* genes and transcription of asPocR, repressor of *pocR* expression. In the presence of both propanediol and $B_{12}$, binding of $B_{12}$ to a riboswitch gives the small aspocR transcript a premature termination, increased gene expression of *pocR* regulator and consequently high expression of *pdu* genes for propanediol catabolism. Ectopically transcribed *aspocR* showed inhibitory action *in trans* on *pocR* expression, *in vitro* transcription and translation experiments showed inhibitory action of *aspocR* on *pocR* translation. This study suggested that transcription attenuation and inhibition of translation initiation as possible mechanisms of *pocR* regulation by *aspocR* (*68*).

### *trans*-encoded RNAs

*trans*-encoded RNAs (also known as small RNAs) are major class of bacterial non-coding RNAs (ncRNAs) and well recognized as important regulators in bacterial gene expression. As stated earlier, these are encoded from intergenic regions of genomes which are distantly located from their targets. Generally, *trans*-encoded RNAs act through either perfect base-pairing or discontinuous base-pairing to the target mRNA and are also known for interacting with multiple mRNAs (*69*, *70*). *trans*-encoded RNAs are involved in both suppression and stimulation of translation by binding to target mRNA. Moreover *trans*-encoded RNAs are also associated with rapid degradation of their target mRNA in interplay with RNases (*69*, *71*).

In *L. monocytogenes*, using different methodologies and experimental approaches a significant number of small RNAs were identified and some of them were validated by northern blot (*63*, *67*, *72*) . However, it has been difficult to determine the 3´ UTRs of small RNAs. Coming to regulation of small RNAs expression, very little is known and two small RNAs *sbrA* and *sbrE* are under regulation by SigB, as Δ*sigB* mutant has shown

reduced expression of these small RNAs. *L. monocytogenes* has shown expression of several small RNAs during its growth in blood, intestinal lumen as well as hypoxia conditions. Some of those small RNA deletion mutants such as *rli38* and *rliB* have sown reduced colonization in mice model, indicating their role in pathogenicity of *L. monocytogenes* (*63*). Moreover, a study through RNA-seq unraveled the important role of small RNAs *rli31*, *rli33-1* and *rli50* during intracellular growth in P388D1 macrophages as well as virulence in mice and insect models (*72*). Another set of small RNAs (*LhrA*, *LhrB* and *LhrC*) bind to Hfq protein, a RNA chaperon, shown by co-immunoprecipitation assay followed by RNA-seq. One of these, *LhrA* is known to be targeting the genes *lmo0302*, *lmo0850* and *chiA* and is dependent on Hfq protein (*73*, *74*).

### 2.4.2 Role of non-coding RNAs in other bacterial pathogens

Among the bacterial non-coding RNAs, trans-encoded RNAs are well investigated. Most of the reported bacterial small RNAs are dependent on Hfq, the global regulator, for the regulation of gene expression through complementary base pairing with their respective targets. Bacterial Hfq is a RNA chaperone first discovered as Qβ replicase, a host factor needed for RNA phage replication. This RNA chaperon Hfq is essential for virulence in several bacterial pathogens; *hfq* deletion mutants display several pleotropic changes as altered growth rates, metabolic profiles and virulence genes expression (*75*). In *S*. Typhimurium two Hfq dependent small RNAs ArcZ and SdsR control biofilm formation by targeting *csgD*, a major biofilm regulator. Similar events were observed in case of *E. coli* that several Hfq-dependent small RNAs e.g., McaS, GcvB, RprA and OmrA/B were linked to biofilm formation through regulation of *csgD* (*76*).

Horizontal gene transfer (HGT) is a process that allows transfer of genetic material between related and unrelated bacterial species and is a major contributor of antimicrobial resistance and virulence gene transfer. Virulence genes that are transferred through HGT are located as pathogenicity island on bacterial genomes. To be beneficial for recipient bacteria, the horizontally transferred virulence gene has to be well integrated into regulatory networks coded by the core genome (*77*). In *Salmonella*, Papenfort *et al*., showed that small RNA SgrS, highly conserved and Hfq dependent, regulates the expression of *sopD* virulence gene which is horizontally transferred. This

study indicate that small RNA SgrS is evolved to integrate both core and pathogenicity island through regulatory networks (*78*). Similar kind of cross regulation is reported for the sRNA invR, but in reverse direction as InvR is transcribed from pathogenicity island. *Salmonella* pathogenicity island-1 (SPI-1) of *S*. Typhimurium encodes a novel abundant sRNA, invR, whose expression is activated under SPI-I inducing conditions and regulated by SPI-I transcription factor HilD and Hfq. InvR represses the synthesis of the abundant OmpD porin encoded by the *Salmonella* core genome (*79*).

## 2.5. The facultative intracellular pathogen *Listeria monocytogenes*

*L. monocytogenes* causes severe disease called listeriosis in humans and animals. The symptoms of listeriosis include meningitis, meningoencephalitis, septicemia, abortion, prenatal infection and gastroenteritis (*80*). The occurrence of listeriosis is very low with 2-20 cases per million. But listeriosis is a deadly disease with 25-30% of mortality rate in immunocompromised patients and pregnant women (*81*).

### 2.5.1. The species *L. monocytogenes*

In 1926, E.G.D. Murray isolated for first time *Bacterium monocytogenes* from infected rabbit and guinea pigs, which had shown symptoms like an increase in the number of monocytes in their bloodstream (*82*). Later, this bacterium was named as *Listeria monocytogenes* in honor of surgeon Joseph Lister. The first method for subtyping *L. monocytogenes* was first described by Paterson (1940) and later that was improvised by Donker-Voet (1957) and Seeliger and Höhne (1979). Currently, *L. monocytogenes* can be divided into 13 serotypes (*83*). Differences in the virulence have been observed among *L. monocytogenes* serotypes using mouse, and insect model *Galleria mellonella* (*84–86*). Although there are variations in the virulence properties, the serotypes of *L. monocytogenes* are divided into four lineages based on virulence gene variation, ribo-typing, DNA arrays and multi locus sequence typing (MLST). From most studies, lineage I is known to represent most of the clinical isolates and outbreaks strains whereas lineage II strains were isolated from sporadic cases. The occurrence of lineage III and IV are rare in outbreaks (*83*, *87*). In humans, most of the listeria cases are associated with only four serotypes of *L. monocytogenes* (1/2a, 1/2b, 1/2c and 4b) (*88*).

### 2.5.2. Interactions of *L. monocytogenes* with its host

Following a major outbreak in 1986, *L. monocytogenes* has been reported as a major food-borne pathogen. Over a span of 30 years, *L. monocytogenes* has become a significant model organism to study host-pathogen interactions (*89*).

Most of the virulence genes exist in a cluster in *Listeria* genome with ~9 kb in size except internalins, which are distributed throughout the genome. This virulence gene cluster (vgc) comprises of six genes as four transcriptional units. Comparative genomics of listerial species revealed that the virulence gene cluster is absent in the genome of non-pathogenic species like *L. innocua*, *L. welshimeri* and *L. grayi* whereas it is present in the chromosome of *L. ivanovii* (*80*, *90*). The *prfA* gene is the first member of this cluster and also a member of the transcriptional activator family CRP/FnR (*91*). PrfA is the main switch to regulate the expression of this virulence gene cluster, including internalins (InlA, B and C) and acts as major virulence regulator (*92*, *93*).

**Major virulence genes**

A number of environmental, growth-phase dependent and intracellular signals affect the expression of the virulence regulon via PrfA. PrfA expression is predominantly controlled by a thermosensor, as described above. This explains the rationale behind the saprophytic *L. monocytogenes* turning into an opportunistic pathogen after entry into host with maximum expression of virulence genes (*61*). During intracellular growth, the *prfA* expression is regulated by sugar metabolism (*94*). *prfA* expression leads to synthesis of more PrfA protein by positive feedback, through a PrfA-dependent promoter, which activates synthesis of bicistronic *plcA-prfA* mRNA (*92*, *95*). In addition, PrfA activation leads to transcription of monocistronic *hly* and *mpl* genes, encoding a pore forming toxin listeriolysin O and zinc metalloprotease respectively and bicistronic *actA* and *plcB* , encoding for ActA protein and phospholipase C respectively (*96*, *97*). Internalins are the cell wall surface proteins, involved in internalization of bacteria into non-phagocytic host cells. In addition, Internalin A, B and C are best studied for their role in the infection (*98*). PrfA regulated virulence gene are represented in (Figure 5).

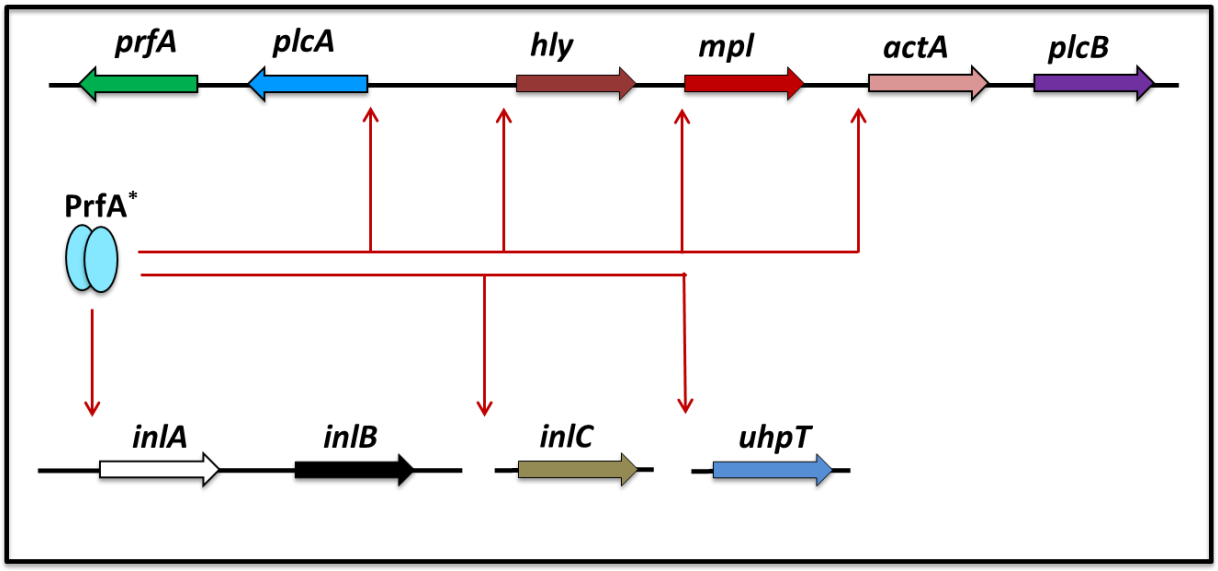


**Figure 5.** An overview of PrfA regulated genes of *L. monocytogenes* including the virulence gene cluster (vgc), internalin A, B and C and *uhpT*.

## Intracellular life cycle of *L. monocytogenes*

Entry of *L. monocytogenes* can be mediated either by phagocytosis in case of macrophages or invasion process in non-phagocytic cells. The invasion of bacteria starts by attaching to the host cell membrane by cell surface proteins; simultaneously the cell membrane engulfs the bacterium. Here, internalins A and B, surface proteins of bacteria, interact with E-cadherin and tyrosine kinase Met receptors of the host cell (*99*, *100*). After internalization, the bacteria are localize in the membrane bound vacuoles which have a mild acidic pH (pH=5.7-5.9) (*101*). After residing for about 30 min in vacuole, the bacteria rupture vacuolar membrane using their pore-forming toxin listeriolysin O (LLO), while mutants lacking this toxin are unable to escape the vacuolar compartment (*100*). Further, listeriolysin O is also shown to mediate escape of the bacterium from secondary vacuole formed during cell-to-cell spread (*102*). In addition to LLO, the bacterium utilizes two phospholipases C (*plcA* and *plcB*) to facilitate its release from vacuolar compartments into cytosol. Once released into the cytosol, it needs to adapt to the intracellular environment and replicate efficiently. To achieve this, *L. monocytogenes* utilizes glucose-6-phosphate, an intermediate component of glycolysis, present in cytoplasm in large amounts, by expressing hexose phosphate transporter

*(uhpT)* (*103*). This *uhpT* expression is also regulated by transcriptional activator PrfA (*104*).

Like other intracytosolic bacteria, *L. monocytogenes* has evolved mechanisms of actin based motility for intracellular and intercellular movements in the host. ActA, a surface protein of *L. monocytogenes*, is structurally similar to host protein WASP and thereby able to recruit host Actin-related protein 2/3 (Arp2/3) as well as actin polymerization machinery to form a comet tail posterior end of the bacterium (*105–107*). The polymerization of actin occurs at only one end of the bacterium, as the ActA protein accumulates on a specific pole of the bacterium after its division (*105*). Eventually, this makes the bacteria to propel in one direction making protrusions on host cell membrane, thereby it leading to cell-to-cell spread without host cell lysis. It is known that mutants lacking ActA are unable to spread from cell-to-cell and appear as micro colonies in cytosol (*108*). When the bacterium enters neighboring cells, it is located in double membrane vacuoles which are called as secondary vacuole. The lysis of this secondary vacuole is mediated by conjugated action of LLO and PC-PLC resulting in the release of bacterium into the cytosol. This way, once *L. monocytogenes* gets entry into cytosol, again it can disseminate from cell-to-cell, escaping from antibody mediated host humoral immune system (*109*). The invasion and intracellular life cycle of *L. monocytogenes* is depicted in (Figure 6).

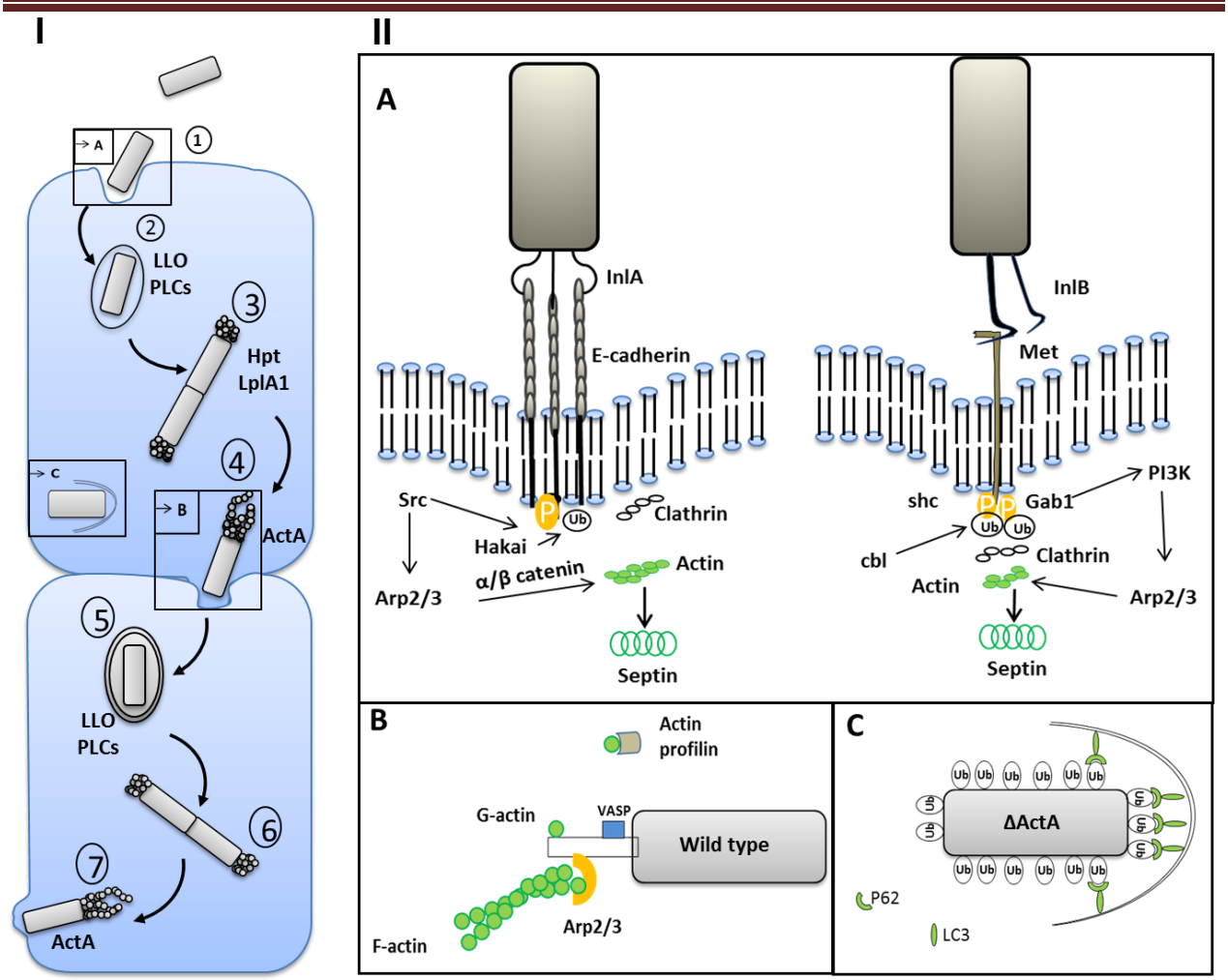


**Figure 6. Schematic representation of *Listeria monocytogenes* entry and intracellular life cycle**. **(I)** (1) *L. monocytogenes* contact with host cell and stimulates its uptake. (2) Internalized bacteria are located inside a phagosome, from which they escape due to the activity of LLO and PLCs. (3) Once the bacteria are released into cytosol, *Listeria* starts to adjust their metabolism to cytosolic environment by expression of a number of genes such as *uhpT* and *lplA1*. Further, it initiates replication and actin polymerization. (4) Polarized expression of ActA makes *L. monocytogenes* to take over the host actin polymerization machinery. With this, the bacteria propel in cytosol until it interact with host cell membrane. Upon interactions with host cell membrane, *Listeria* makes protrusions on neighboring cells in non-lytical manner, later it ends up in double-membrane vacuole which is lysed by LLO and PLCs (5). Cytosolic bacteria undergo a second round of replication and spread as mentioned earlier (6, 7).

**(II)** (A) Bacteria induce its uptake into non-phagocytic cells. (A) *L. monocytogenes* attaches to E-cadherin and Met receptors with InlA and InlB respectively and induces clathrin mediated endocytic machinery and actin polymerization and endocytic machinery through several adapter molecules to internalize host cell. (B) Actin tail formation. ActA protein accumulates in a polar fashion on the *L. monocytogenes* surface. Thereby it a mimics the host zyxin–vinculin and WASP–Wave proteins, so it recruits the host cell VASP and the Arp2/3-complex. These recruited Arp2/3 complex and VASP proteins support the elongation of actin filament (C) ActA mediated autophagy escape. Bacteria that lack of ActA are subjected to autophagy. Unknown receptors on the bacterial surface are ubiquitinylated and those are recognized by the autophagy adapter p62 which recruits LC3 protein and form link to the autophagic membrane. Adapted from reference (*110*).

## 2.6. Host response to *L. monocytogenes* infection

Since long time, *L. monocytogenes* has been used as a model to study host innate and adaptive immune system which demonstrates that cellular immunity plays critical role in control of *Listeria* infection (*111*). In mouse model, the bacteria are injected intravenously into the blood stream, as oral route is not lethal. Through blood stream, bacteria reach organs such as spleen and liver and colonized them as they get internalized by macrophages (*112*). During infection, mice develop specific T cell response to eradicate *Listeria* and memory T cells to provide protection against reinfections (*113*). Though other animals, such as guinea pigs, are also used to study immune response to *Listeria*, mouse model have proven to be successful to study immune response with wide availability reagents including gene specific knockouts (*114*).

### 2.6.1 Innate immune response

The Innate immune system is host's rapid defense process against pathogenic infection by recognizing and responding to pathogens in non-specific way. Host cells identify and respond to pathogens by recognition of pathogen associated molecular patterns (PAMPs) through their pattern recognition receptors (PRRs). The identification of pathogens leads to activation of signaling pathways which results in production of proinflammatory cytokines. The activation of the innate immune system prepares the host for adaptive immune responses.

#### 2.6.1.1. Innate immune cells

At early time of infection, immune cells such as neutrophils, macrophages, natural killer (NK) cells and dendritic cells form the first line of defense against *L. monocytogenes*. After intravenous infection of *L. monocytogenes*, neutrophils migrate towards the site of infection by chemical signals such as chemokines secreted by infected hepatocytes (*115*, *116*). These neutrophils kill bacteria by phagocytosis and release of reactive nitrogen and oxygen species (NOS and ROS). Moreover, neutrophils are attracted to infection site by IL-6 and IL-8 and amplify the inflammation response by releasing inflammatory mediators and chemokines. Infection of mice that lack neutrophils showed increased susceptibility to *Listeria* and higher bacterial burden in spleen and liver (*117*,

*118*). Yin *et al.,* showed that the adaptive transfer of IFNγ producing neutrophils protects mice that are deficient in IFNγ against *L. monocytogenes* infection (*119*).

In case of macrophages, particularly resident macrophages like Kupffer cells in the liver are well described for their role in *L. monocytogenes* infection (*120*). Mice depleted of Kupffer cells with pretreatment of liposome-encapsulated dichloromethylene diphosphonate showed 75% decrease in *Listeria* burden in liver after 10 min post infection. This study suggests that initially majority of the *Listeria* recovered in the liver were bound to Kupffer cells (*120*). These Kupffer cells are involved in eradication of *Listeria* directly by phagocytosis, or indirectly by inducing the biological response of other cell populations. Indeed, Kupffer cells are able to express intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and release various soluble factors such as TNF-β, IL-1β, IL-6, nitric oxide and leukotrienes. All these molecules can induce the infiltration, localization and antimicrobial activity of natural killer (NK) cells (*121*, *122*). It is known that macrophages that are infected with *L. monocytogenes* secrete TNF-α and IL-12 to trigger NK cell activation (*123*). Dendritic cells (DCs) are major antigen presenting cells (APCs) and exist as an immature form in peripheral tissues, where they show high phagocytic activity rather than priming T cells. When they capture antigen, it leads them to mature by expressing major histocompatibility complex and costimulatory molecules. These activated DCs migrate from tissues to regional lymph nodes, where they present antigens efficiently to naive T cells. In this way, DCs play important role in bridging innate and adaptive immune systems (*124*). These DCs were classified into different subsets based on the surface markers present. In mice, the DCs are categorized into two main subsets (1) the conventional DCs (cDCs) and (2) the plasmacytoid DCs (pDCs). It has been demonstrated that cytosolic *Listeria* can induce DCs to express IFNβ that sensitizes naive T cells for antigen mediated activation (*125*).

#### 2.6.1.2. Toll like receptors, NOD-like receptors and RIG-1-like receptors

As described earlier, PRRs are involved in recognition of the pathogens based on pathogen associated molecular patterns (PAMPs) and induce different signaling pathways to counteract pathogens. To date, there is existence of three main families of

PRRs: the Toll like receptors (TLRs), the NOD-like receptors (NLRs) and the RIG-1-like receptors (RLRs). Several studies demonstrated that *L. monocytogenes* activates these PRRs during infection (*126*).

TLRs are expressed on the host cell surface as well as endocytic vesicles; their roles have been well-studied during infection of different pathogens. TLRs are able to recognize different cellular components derived from *L. monocytogenes* such as peptidoglycan, flagellin, lipoteichoic acids and nucleic acids. Activation of TLRs leads to recruitment of various adapter proteins like MyD88, TIRAP, TRIF and TRAM, which in turn activate major transcriptional factors like NF-κB to induce production of several cytokines and chemokines. During *L. monocytogenes* infection, TLR2 senses listerial lipoproteins and activates NF-κB. The process of lipidation of prelipoproteins is required for the sensing of lipoproteins by TLR2 (*127*). Macrophages which lack TLR2 expressed less TNF-α, IFNγ and IL-1β with infection of *L. monocytogenes in vitro* (*128*). Boneca *et al*., demonstrated that TLR2 has role in production of IFNβ upon infection of *L. monocytogenes pgdA* mutant. This study explains that N-deacetylation of peptidoglycan is an efficient mechanism for *L. monocytogenes* to evade host TLR2 and NOD1 mediated innate immune system (*129*). In case of TLR5, purified flagellin has been shown to activate TLR5 receptor mediated signaling pathway *in vitro* but not *in vivo*. The possible reason for the absence of flagellar induced TLR5 pathway might be the specific regulation of flagellin expression dependent on the temperature in *L. monocytogenes*. It is well-known that *L. monocytogenes* is highly flagellated and motile at low temperature but non motile at 37°C because of low expression of flagellar genes (*130*).

Nod-like receptors (NLRs) are other important microbial sensors, which are located in cytoplasm of the cell. These NLRs can be divided into three sub families: the NOD, NLRP, and IPAF. NOD2 and NALP3 of NLR family are well studied during the infection of *L. monocytogenes* infection (*131*). The effector domains of NODs activate signaling pathways by interacting with an adapter protein the, receptor-interacting protein (RIP2). RIP2 leads to activation of IKK complex, which in turn activates NF-κB, resulting in expression of several cytokines. In addition, NOD2 activation is capable of activating MAPK signaling pathways, subsequently inducing AP-1 transcription factors. Park *et al.,*

showed that RICK/RIP2 is involved in cytokines (IL-6 and TNF-β) induction mediated through NOD1 and NOD2 but not TLRs in *Listeria* infected macrophages (*132*). Some members of the NLR family are involved in formation of large caspase-1 activating complexes called inflammasomes. These inflammasomes control maturation and secretion of IL-1β and IL-18 cytokines, whose proinflammatory activities are important for host response to infections (*133*). Recent reports stated that caspase-1 activation is important for the clearance of *L. monocytogenes in vivo* and activation mediated through several NLRs such as NALP1, NALP3, IPAF and AIM2 which can assemble inflammasomes (*131*) . In addition to TLRs and NLRs, the other cytosolic receptor RIG-1 also plays considerable role against viral infections by detecting intracellular RNA and activating downstream signaling pathways including the secretion of cytokines. An Immunohistochemical study has revealed the upregulation of RIG-1 in hepatic Kupffer cells and in splenic reticular cells of infected mice with *L. monocytogenes.* This study suggests a possible role of RIG-1 in host innate immune system against *L. monocytogenes*(*134*).

### 2.6.1.3. Cytokines

Cytokines are the products of immune cells and act as mediators between the cells. These cytokines include interleukins (ILs), interferons (IFNs), colony-stimulating factors (CSFs), TNFs and chemokines. During early infection by *L. monocytogenes*, the host induces a number of cytokines (*126*). Cytokines TNFα, IFNγ and IL-12 are known to be crucial for the elimination of *L. monocytogenes* during the infection. Around 10 types of IFNs are known in higher animals. These are broadly categorized into three classes based on the type of receptor they recognize. Type 1 IFNs like IFNα and IFNβ bind to cell surface receptor known as IFNα receptor (IFNAR) and leads to activation of IFN stimulated genes (ISGs). Type II IFNs includes IFNγ, bind to IFNGR. Type III IFNs are composed of IFNλ molecules. In addition to inducing IFNγ, it is known that *L. monocytogenes* infection leads to expression of IFNα and IFNβ also. Type 1 IFNs are generally known to protect against viral infection, several reports showed that IFNβ response to *L. monocytogenes* is detrimental to host, as is the case with other pathogens such as *S.* Typhimurium and group B *Streptococci* (*135*). Several studies

demonstrated that expression of type1 interferons IFNα and IFNβ is beneficial for *L. monocytogenes*. Type 1 IFNs, IFNα and IFNβ sensitize T cells to apoptosis and reduce resistance to *L. monocytogenes* by enhancing secretion of anti-inflammatory cytokine IL-10 which shuts down innate immune response induced by *L. monocytogenes* (*136*, *137*). The major components of innate immune response towards *L. monocytogenes* infection are depicted in (Figure 7).

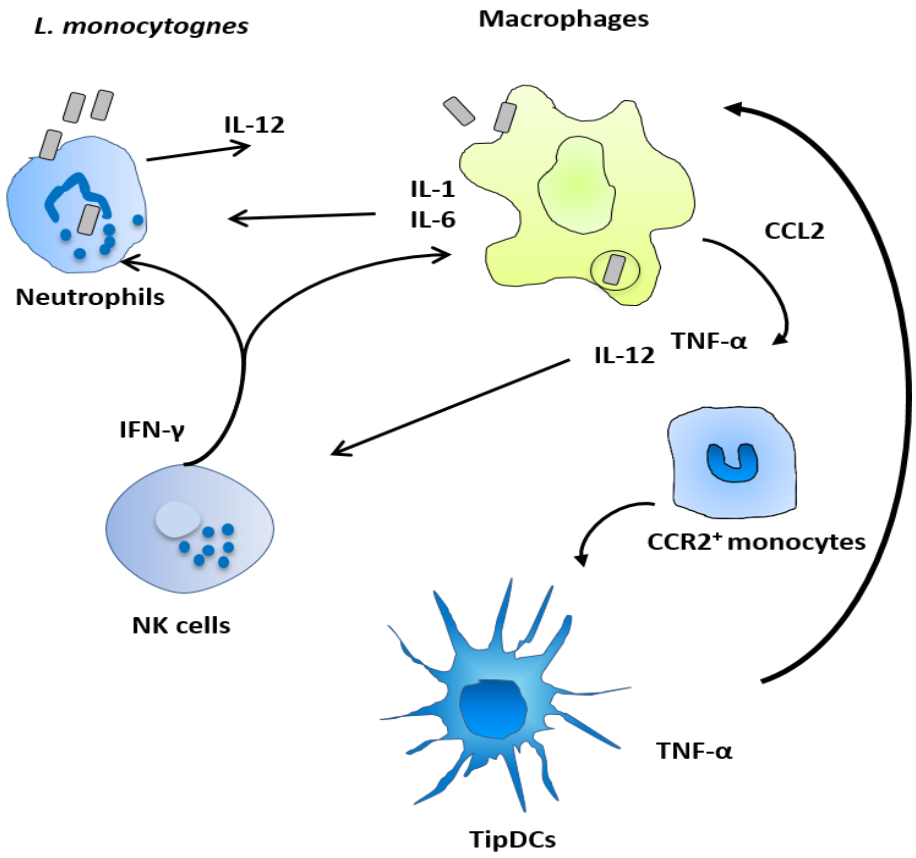


**Figure 7. Some aspects of innate immune response to *L. monocytogenes* infection:** Neutrophils are able to phagocytose *L. monocytogenes* and produce reactive nitrogen and oxygen species (NOS and ROS) to kill intracellular bacteria. Moreover, neutrophils are participated in amplification of the anti-*L. monocytogenes* inflammatory response through releasing IL-12. Macrophages are also able to phagocytose *L. monocytogenes*, release cytokines such as IL-1, TNF-α, and IL-12. TNF-α and IL-12, then stimulate natural killer (NK) cells to secrete IFNγ, which further activates macrophages. Cytokines that are released from either infected epithelial cells or macrophages able to activate DCs to produce IFNγ, which in turn stimulate macrophages and neutrophils to synthesize nitrogen and oxygen species (NOS and ROS) to kill *L. monocytogenes*. In spleen, infection of *L. monocytogenes* leads to maturation of monocytes into TipDCs which produce TNF-α and NO to clear *Listeria* infection. Adapted from reference (*110*).

## 1.7 Objectives of the study

The main objectives of the study are to investigate the role of non-coding RNAs (ncRNAs) involved in host immune response as well as host-pathogen interactions.

First part of the study involves investigation of miRNA deregulation during infection by *L. monocytogenes,* non-virulent mutant strains and purified endotoxin LLO treatment in Caco-2 cells. Further, target gene analysis of selected miRNAs was performed to find out miRNA mediated regulation in host immune responses.

The second part deals with virulence dependent microRNA signature that controls immune responses in *G. mellonella* during *L. monocytogenes* infection. Here, we obtained the miRNA profile of infected larvae using miRNA microarray analysis. Later, we created publically available transcriptome database and performed target gene prediction for selective miRNAs using miRanda. Finally, we did *in silico* estimation of minimum free energy (MFE) of miRNA-mRNA duplexes and the expression levels of selected target genes to reveal regulatory network of the host immune response to *L. monocytogenes* infection.

The third part of the study addresses whole genome transcriptomic analysis of *L. monocytogenes* that grows inside P388D1 macrophages using SOLiD and Ion Torrent sequencing technologies. RNA-seq data was analyzed by using different pipelines and showed an extensive antisense transcription. Later the asRNAs were validated by using northern blot and qRT-PCR methods. The relevant target gene expression levels were estimated to estimate the role of asRNAs in adaptations to intracellular environment and virulence.

# 3. Chapter I microRNA response to *Listeria monocytogenes* infection in epithelial cells

## 3.1 Publication

**microRNA response to *Listeria monocytogenes* infection in epithelial cells**

Izar B*, **Mannala GK***, Mraheil MA, Chakraborty T, Hain T. Int J Mol Sci. 2012; 13(1):1173-85. doi: 10.3390/ijms13011173. Epub 2012 Jan 20. **(Equal first authors)**

## 3.2 Contribution

The author (G.M) jointly contributed in designing and writing of the manuscript with other authors. He performed all cell culture infection experiments with *L. monocytogenes*, its isogenic mutants and listeriolysin O (LLO) treatment experiments. Later, the RNA isolation was performed and processed for microarray and qRT-PCR analysis. Further, the expression levels were estimated and statistical analysis was performed by G.M.

## 3.3 Abstract

microRNAs are small non coding RNAs that are well investigated for their significant roles in different physiological and pathological processes such as development, host immune response and cancer. miRNAs work by targeting the mRNAs especially at 3´ UTR region. Little is known about miRNA response to bacterial infections, so we took effort to reveal the microRNA response to *L. monocytogenes* infections in Caco-2 cell line. With infection, Caco-2 cell line exhibited an altered miRNA expression with *L. monocytogenes* and its isogenic mutants (Δ*inlAB* and Δ*hly*) as well as with treatment of purified listeriolysin O (LLO) toxin. From miRNA microarray analysis, we screened and validated five miRNAs (miR-146b, miR-145, miR-16, let-7a1 and miR-155) that were significantly deregulated with infections. The expression patterns of these miRNAs are dependent on localization of pathogen inside the host cell and interactions with bacterial effector proteins. Interestingly, miR-155, which has been well demonstrated for its role in inflammatory response, was upregulated with *L. monocytogenes*, its mutant strain Δ*hly* and following treatment with purified LLO. But miR-155 was downregulated upon infection with Δ*inlAB* strain. This indicates the novel role of internalins in miRNA

regulation and listerial pathogenicity. Further, the target gene expression analysis of selected miRNAs revealed the role these miRNAs in regulation of host response towards bacterial infections (*138*).

## 3.4 Results and discussion

### 3.4.1 *L. monocytogenes* infection alters miRNA profile dependent on its cellular localization

With microRNA microarray analysis, we chose a subset of miRNAs that showed altered expression following *L. monocytogenes* infection compared to control non infected Caco-2 cells. These microRNA microarray results were further validated by qRT-PCR and it showed robust correlation with microarray analysis. In detail, miR-146b, miR-16 and miR-155 showed upregulation whereas let-7a1 and miR-145 showed downregulation with *L. monocytogenes* infection (Figure 8).

In addition to with wild-type infection, we studied expression levels of these miRNAs with two isogenic mutant strains Δ*hly* and Δ*inlAB*. The Δ*hly* strain is unable to synthesize poreforming LLO and thereby results in entrapment of bacteria in phagocytic vacuoles. The mutant strain Δ*inlAB* lacks two major internalins A and B, and is unable to enter into host epithelial cells and remains in extracellular space. The miRNAs miR-146b and miR-16 exhibited significant deregulation with the infection of mutant strains when compared to wild type infection. But the directionality of these microRNA expressions was different; these miRNAs were downregulated with mutant strains but inversely upregulated upon wild type infection (Figure 8). Moreover, we detected significant downregulation of let-7a1 by these mutant strains, but there was no difference in expression when compared to wild type infection (Figure 8). Interestingly, in case of miR-155, it showed strong upregulation with wild type and haemolysin deficient strain but significant downregulation with Δ*inlAB* strain (Figure 8).

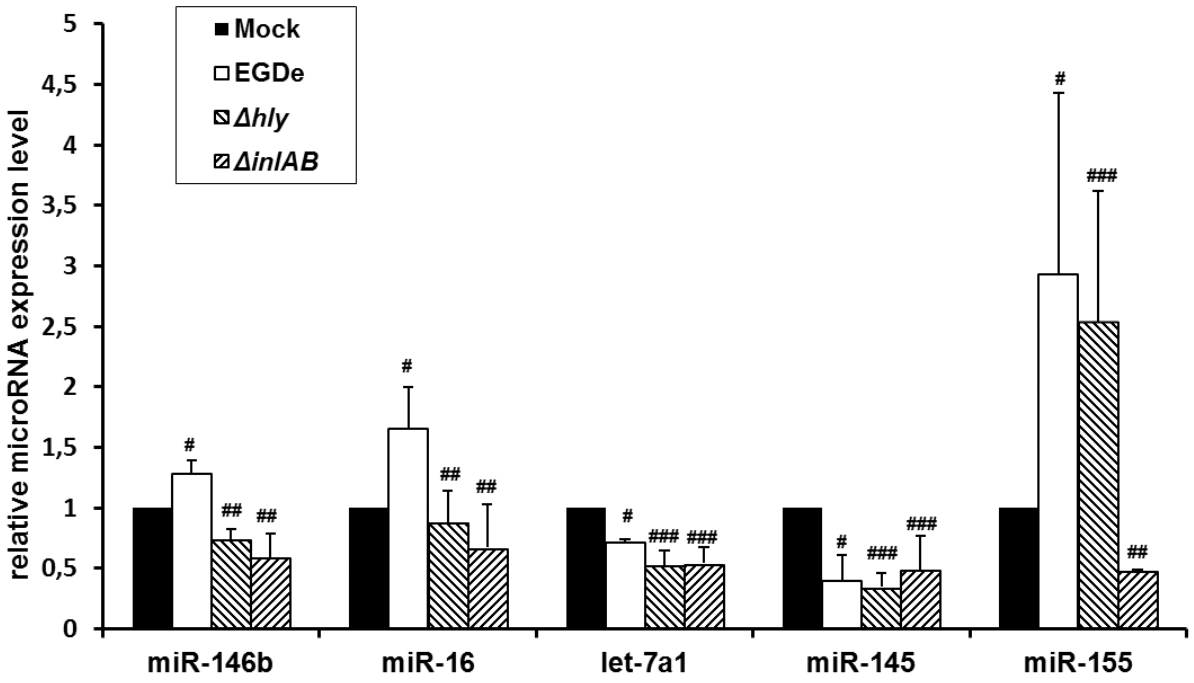


**Figure 8**. Measurement of the expression levels of miRNA candidates in Caco-2 cells that are infected with *L. monocytogenes* EGD-e wild-type, Δ*hly* or Δ*inlAB* compared to uninfected Caco-2 cells. Error bars indicate standard deviations of miRNA candidate fold expressions levels from three independent experiments. # denotes significant difference between control and infected samples (p-value < 0.05). ## denotes significant difference compared to wild-type infection (p-value < 0.05), ### denotes no significant difference compared to wild-type infection (p-value > 0.05).

As previously described for *Salmonella* infection (*28*), during our study we examined the downregulation of let-7a1, which is a member of let-7 family that is well investigated for its roles in immune response and cancer development. miR-16 mainly acts on inflammatory molecules such as TNF-$\alpha$, IL-6 and IL-8 through rapid degradation of their mRNAs which contain AU-rich sequences. Strikingly, miR-16 was reported to be induced in NIH 3T3 cells that were infected with murine gamma herpesvirus 68, similar to Kaposi's sarcoma associated herpesvirus (KSHV) and Epstein-Barr-Virus (*139*). In a similar way, induction of miR-16 was also observed with infection of *Cryptosporidium parvum,* a protozoan parasite, in cholangiocytes (*140*).

### 3.4.2 Purified listeriolysin (LLO) leads to induction of miR-146b, miR-16 and miR-155 in Caco-2 cells

Further, we investigated the effect of purified listeriolysin (LLO) toxin on regulation of the subset miRNAs. Upon treatment of LLO toxin, three miRNAs (miR-16, miR-155 and miR-146b) were significantly upregulated compared to controls (Figure 9). Interestingly, miR-146b showed opposite expression pattern to Δ*hly* strain infection suggesting a direct correlation to miR-146b regulation with treatment of LLO. In case of miR-16, this miRNA did not show any upregulation with Δ*hly* strain, whereas with treatment with LLO it was upregulated indicating the role of LLO in induction of miR-16 during *L. monocytogenes* infection (Figure 8&9). Different studies have shown that the stable expression of miR-16 in variety of cell lines. So, LLO toxin mediated induction of miR-16 and subsequent targeting of immune modulators is triggered by *L. monocytogenes* rather than a non-specific host cell response to infection (*141*). In contrast to other miRNAs expression patterns, miR-155 did not show any difference in both experimental setups as it is upregulated following infection with Δ*hly* mutant strain and LLO treatment (Figure 8&9). The miR-155 expression levels were dose independent of LLO treatment.

Several miRNA expression profiling studies in human macrophages have revealed that miR-146 and miR-155 are endotoxin responsive genes that are associated with various immune signaling pathways (*37*, *142*). Liu *et al.,* demonstrated that upregulation of miR-146b is involved in inhibition of inflammatory response induced by *H. pylori* through diminishment of IL-8 expression in gastric epithelial cells. They have proposed a possible mechanism, where miR-146b negatively regulates interleukin-1 receptor-associated kinase 1(IRAK1) and TNF receptor-associated factor 6 (TRAF6), two adaptor molecules in TLR signaling and NF-κB activation, thereby affecting cytokine production (*23*). In our study, miR-146b is induced majorly in a LLO dependent manner upon infection with *L. monocytogenes* and underlines crucial role in host miRNA regulation. Caco-2 cells carry TLR2 and TLR4 on their surface; it is well-known that these receptors are targeted by major listerial virulence factors along with LLO. So we propose that *Listeria* infection might led to induction of miR-146b and subsequent target gene interactions might be activated by LLO through a TLR dependent pathway.

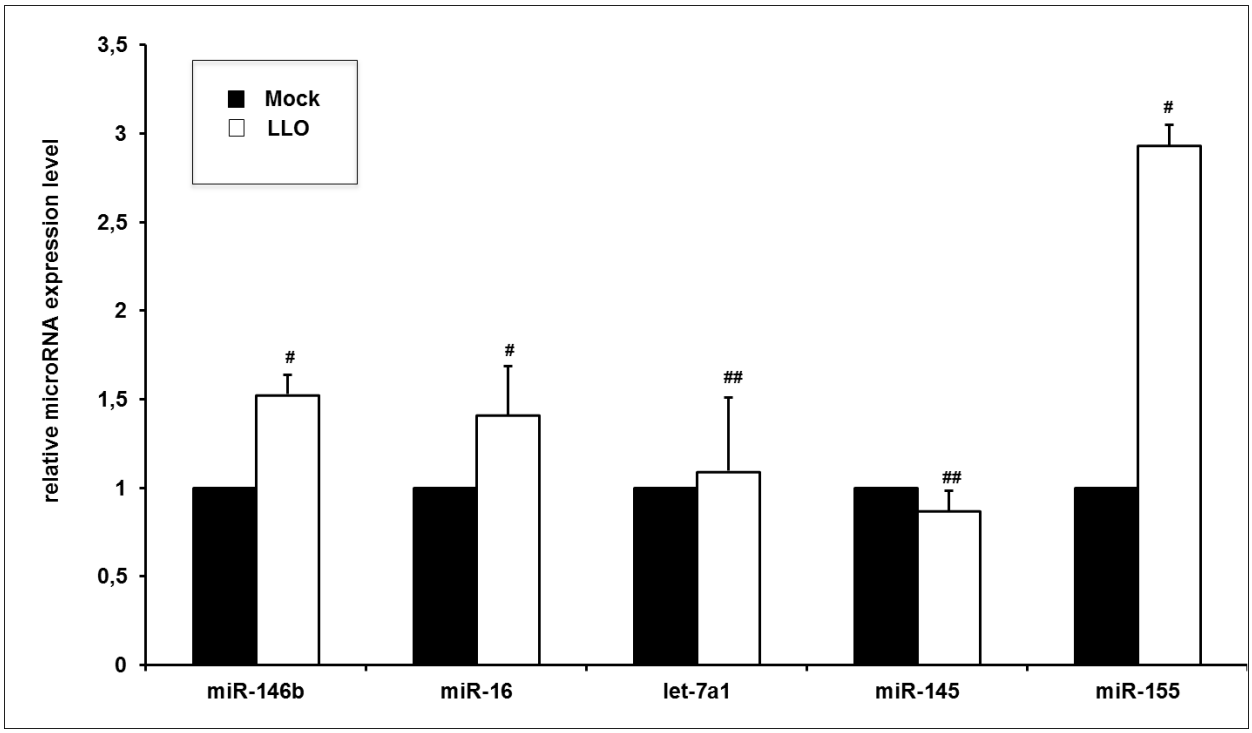


**Figure 9.** Estimation of miRNA expression levels with treatment of LLO on Caco-2 cells (A) The miRNA expression levels were measured from Caco-2 cells treated with purified listeriolysin (LLO) for 1 h. # denotes significant difference with p-value < 0.05 compared to control, ## denotes no significant difference.

As mentioned earlier, miR-155 is well investigated for its regulatory roles in several innate and adaptive immune pathways (*142*). Our results showed that miR-155 is induced to similar extent with both *L. monocytogenes* infection and LLO treatment. However, the miR-155 is also upregulated with Δ*hly* mutant, indicating the induction mediated through vacuole dependent pathway. This induction might be mediated through MyD88, as vacuolar signaling and further regulations during listerial infection are mainly dependent on this adaptor molecule (*143*). Moreover, MyD88 is also involved in integration of TLR-signaling that is induced by external stimulation such as LLO. So we conclude that miR-155 induction might be mediated through LLO dependent and LLO independent vacuolar mediated pathways, which finally may merge into a single pathway that results in a similar pattern of expression of miR-155 as observed in our present study.

### 3.4.3 Target gene expression analysis

To determine the correlation between miRNA deregulation and its effects on target mRNA of specific miRNAs, we estimated the expression levels of important targets of these miRNAs. The target genes include major cytokines such as IL-6, IL-8, TNF-α and IFN-β. In concordance to miRNA deregulation, the above selected target genes have shown significant deregulation in Caco-2 cells infected with WT and its isogenic mutants when compared to controls (Figure 10).

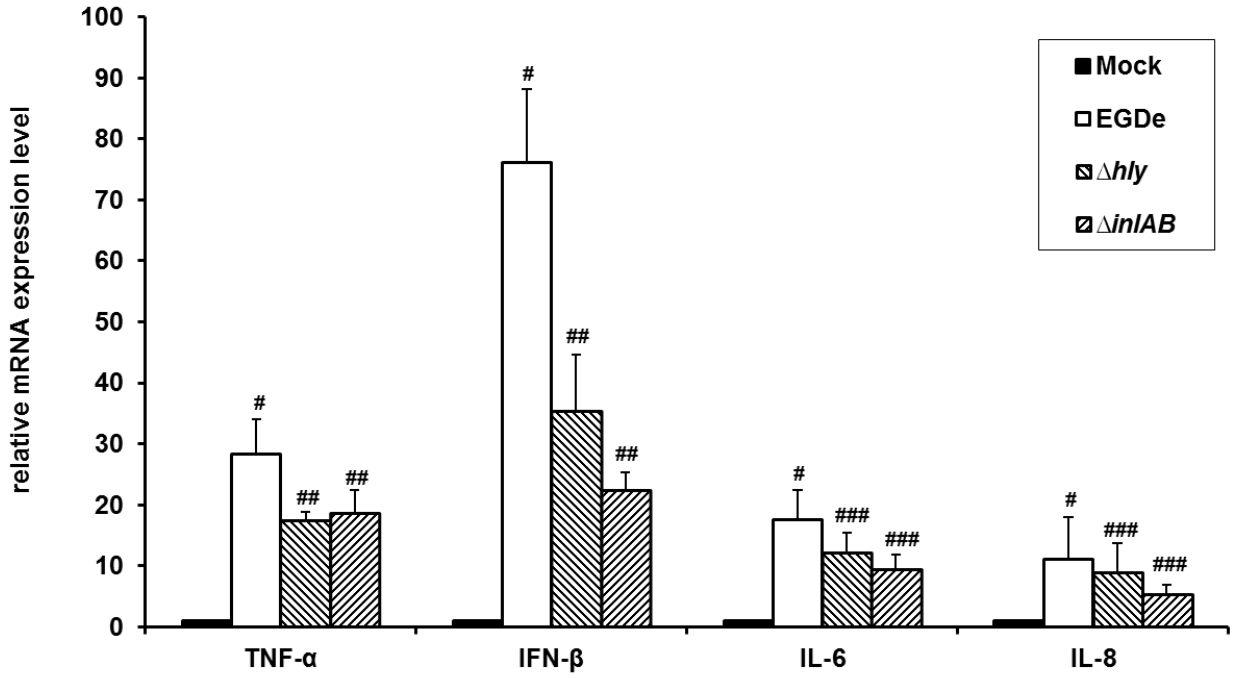


**Figure 10.** miRNA target gene analysis involved in immune response. Target genes expression analysis was performed from Caco-2 cell infected with *L. monocytogenes* EGD-e wild-type, Δ*hly* and Δ*inlAB* by using qRT-PCR method. Error bars indicate standard deviations of target gene expressions levels from three independent experiments. # refers significant difference compared to control (p-value < 0.05). ## refers significant difference compared to wild-type infection (p-value < 0.05), ### refers no significant difference compared to wild-type infection (p-value > 0.05).

A recent study demonstrated the role of miR-145 induction and its role in inflammatory response in patients suffering with ulcerative colitis (*144*). Further it was proved that blocking the miR-145 led to strong anti-inflammatory response and reduced airway hyper responsiveness (*145*). Hence, the decreased expression of miR-145b by *L. monocytogenes* infection that was observed in present study might serve as mechanism

to suppress the host immune system to support pathogen survival. Moreover, IFN-β is a predicted target of miR-145 and involved in inflammatory and anti-inflammatory effects in host during infection of *L. monocytogenes*. In present study, downregulation of miR-145 led to strong upregulation of its target IFN-β, indicating the possible role of miR-145 in host immune response (Figure 10). A recent study revealed that let-7 family members are involved in regulation of major inflammatory factors IL-6 and IL-10 during *Salmonella* infection in HeLa cells (*28*). Here, we observed similar kind of regulation with *L. monocytogenes* infection in Caco-2 cells (Figure 10) indicating the analogous roles for a host miRNAs to both Gram-positive and Gram-negative bacterial infections.

## 3.5 Conclusion

The present study demonstrated that host miRNA response induced by infection of *L. monocytogenes* in intestinal epithelial cells. The study explained that the infection with *L. monocytogenes* led to a significant deregulation of miRNA signature which is dependent on the major virulence factors such as listeriolysin, internalin and subcellular localization of *L. monocytogenes*. Moreover, it showed the possible roles of these miRNAs in post-transcriptional regulation of genes involved in immune response to bacterial pathogens. Further, miRNAs can expand the role of non-coding RNAs as major regulatory molecules in eukaryotes and also act as new drug targets.

# 4. Chapter II *Listeria monocytogenes* induces a virulence-dependent microRNA signature that regulates the immune response in *Galleria mellonella*

## 4.1 Publication

***Listeria monocytogenes* induces a virulence-dependent microRNA signature that regulates the immune response in *Galleria mellonlella*.**

**Mannala GK*,** Izar B*, Rupp O, Goesmann A, Chakraborty T, Hain T. (**Manuscript was submitted for publication**)

## 4.2 Contribution

The author (G.M.) was part of designing of the study and drafting the manuscript. He performed the experiments of infection with *G. mellonellla* and isolated RNA required for microarray and qRT-PCR analysis. Initial screening of miRNAs was jointly performed. He performed the screening of miRNA targets and estimation of minimum free energy (MFE) levels by using RNA-hybrid. miRNAs and target gene expression analysis, statistical data analysis, regulatory network between miRNA-mRNAs using cytoscape were carried out by G.M.

## 4.3 Abstract

In this study, we investigated the changes of miRNA expression levels of *G. mellonella* larvae (greater-wax moth) with infection of Gram-positive human pathogenic bacterium *L. monocytogenes*. By using insect specific miRNA microarray, we found evidence for differential expression of 97 miRNAs in response to infection *with L. monocytogenes*. Among these, 39 miRNAs were upregulated and 58 miRNAs were downregulated. These findings were validated by quantitative real time PCR. Further miRNA qRT-PCR experiments comparing *L. monocytogenes* and non-pathogenic *L. innocua* infections indicated that this miRNA deregulation in *G. mellonella* occur in a pathogen specific manner. In detail, the miRNAs dme-miR-954 and bmo-miR-3000 were upregulated whereas miR-133 and miR-998 were downregulated following a pathogenic *L.*

*monocytogenes* infection. In response to *L. innocua* infection, no significant changes of these miRNAs expression levels were measured except for bmo-miR-3000 which was even downregulated. For further detailed target analysis, from known expressed sequence tags (ESTs), we have established a novel publically available *G. mellonella* transcriptome database and performed target prediction for these selected miRNAs. Finally, minimum free energy (MFE) of miRNA-mRNA duplexes by RNA hybrid program and quantitative analysis of selected mRNA by qRT-PCR indicated the role of miRNAs in the regulation of host immune response to *L. monocytogenes* infection in *G. mellonella***.**

## 4.4 Results and discussion

### 4.4.1 miRNA microarray analysis in invertebrate *G. mellonella* duing infection

We isolated total RNA from *G. mellonella* larvae on 5$^{th}$ day post infection and studied changes in miRNA profiles by applying the RNA to insect specific miRNA microarray. As a control, RNA isolated form larvae which were injected with 0.9% NaCl was used. The RNA samples of three different experiments were tested for a total of 2064 miRNAs. Among those 919 miRNAs showed differences in signal intensity between control and infected samples. At this, it has to be mentioned that some miRNAs are conserved between insect species and therefore were measured multiple times in our study. Statistical analysis of data revealed upregulation of 39 miRNAs and downregulation of 58 miRNAs with infection of *L. monocytogenes*. A sequence homology study of the miRNAs from *C. elegans*, *D. melanogaster,* mouse and human revealed the extensive conservation of miRNAs (*146*). According to this study, the miRNAs dme-miR-133-3p and dme-miR-998-3p are conserved as miR-29 and miR-133 in higher animals respectively (*146*). Ma *et al.,* showed that infection of mouse NK cells and T cells with *L. monocytogenes* and *Mycobacterium bovis* led to downregulation of miR-29 which targets IFN-$\gamma$ and is involved in the immune response to intracellular bacteria (*30*). Similarly, our study also showed the significant downregulation of miR-998, a homolog of miR-29, following infection with *L. monocytogenes* in *G. mellonella* (Figure 11). Chronic infection with *H. pylori* led to downregulation of tissue specific miR-133 miRNA,

increased expression of its targets serum response factor and led to dysfunction of gastric emptying in mice (*147*). Here, we found similar patterns of expression of miR-133 after infection of *G. mellonella* with *L. monocytogenes* (Figure 11).

### 4.4.2 Validation of miRNA microarray results and pathogen/non-pathogen specific miRNA response

The microarray results were further validated by qRT-PCR analysis from the same RNA samples that have been used for microarray analysis. The expression levels of the miRNAs (dme-miR-133-3p, dme-miR-998-3p, dme-miR-954-5p and bmo-miR-3000) measured by quantitative PCR were in good agreement with the microarray results. In detail, miRNAs dme-miR-133-3p, dme-miR-998-3p were significantly downregulated whereas dme-miR-954-5p, bmo-miR-3000 were significantly upregulated for *L. monocytogenes* infected samples compared to uninfected control (Figure 11). Recent study in *G. mellonella* showed that the regulation of miR-263a is varied with oral infection of entomopathogen *Serratia entomophila* and non-pathogen *E. coli*, the latter caused induction of miR-263a whereas *S. entomophila* suppressed its induction (*35*). Considering this, we measured the expression values of these selected miRNAs after infection with non-pathogenic *L. innocua*. Strikingly, the results showed that there was no deregulation of those four miRNAs, except for bmo-miR-3000, which was even shown to be downregulated in *L. innocua* infected samples compared to control (Figure 11). In previous work, we investigated the role of different miRNAs during infection of *L. monocytogenes* Caco-2 cell line and showed the expression of miR-16 and miR-146b to be totally dependent on major virulence factors such as thiol activated toxin hemolysin and the adherence and invasive determinants internalins (*138*).

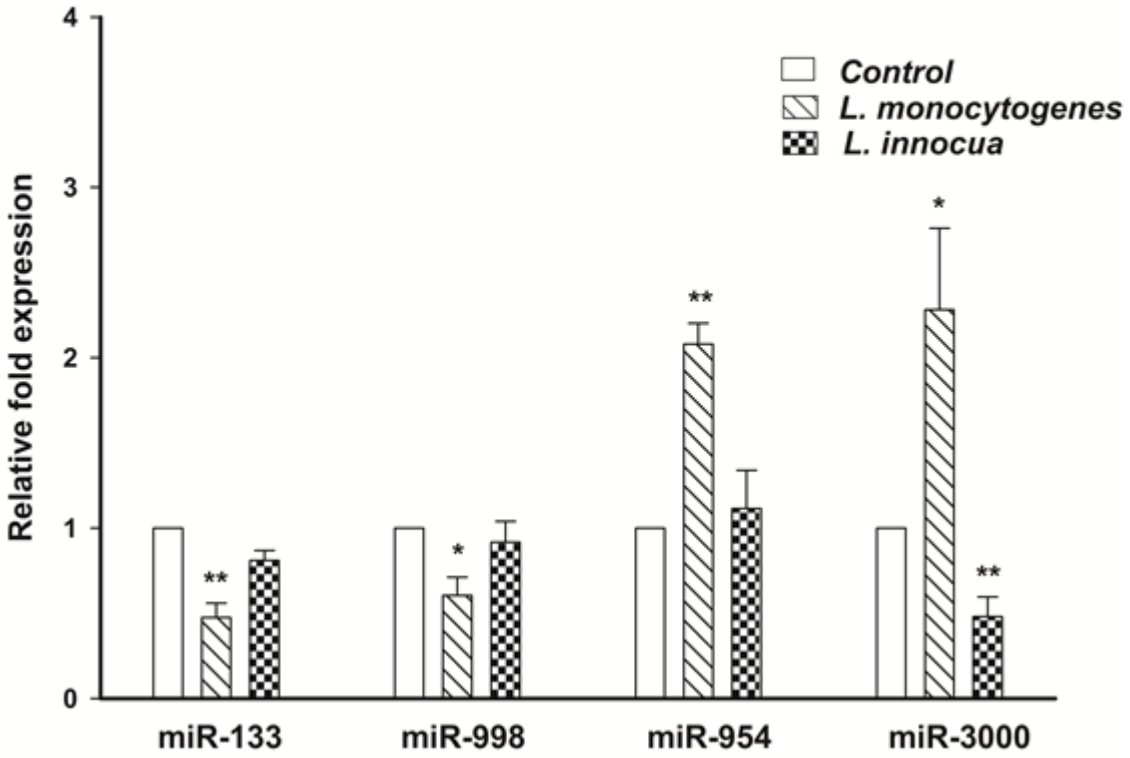


**Figure 11.** Assessment of miRNA microarray analysis results and virulence mediated miRNA response in *G. mellonella*. In support of microarray, qRT-PCR analysis revealed that infection of *L. monocytogenes* led to significant reduced expression of miR-133 and miR-998 and elevated expression of miR-954 and miR-3000. Infection with non-pathogenic *L. innocua* did not shown any significant changes in miRNA expression levels, except for bmo-miR-3000 which was downregulated with significant difference. (`*´ $P \leq 0.05$; `**´ $P \leq 0.01$).

Recently, Siddle *et al.,* investigated expression of miRNAs and their isomiRs in human monocyte derived DCs after infection of different species of *Mycobacterium* genus and other different bacterial pathogens such as *Staphylococcus epidermidis*, *S.* Typhimurium and *Yersinia pseudotuberculosis*. The same study explained the pathogen specific miRNA response and it is dependent on those mechanisms, that are employed by various pathogens to alter host immune response during infection (*148*). Moreover, this study elucidated the virulence-dependent expression of the miR-132/212 family in response to infection with *M. tuberculosis* and its isogenic attenuated mutant strains (*148*). We compared the miRNA expression levels with *L. monocytogenes* and non-pathogenic *L. innocua* infection and results indicated that this miRNA deregulation in *G.*

*mellonella* occur in a pathogen specific manner. In response to *L. innocua* infection, no significant change of these miRNA expression levels was measured, except for bmo-miR-3000 which was downregulated (Figure 11). The previous studies and our present study outlined the pathogen/non-pathogen specific and virulence mediated miRNA response to bacterial infections.

### 4.4.3 miRNA regulate gene targets that are involved in immune response against bacterial infection

Finally, to prove the results of *in silico* miRNA target predictions, we carried out qRT-PCR experiments to determine the expression levels of target genes and to correlate the miRNA and mRNA responses after infection with *L. monocytogenes* and *L. innocua* respectively. Host organisms are known to activate several immune signaling pathways and other cellular processes to counteract microbial pathogens, especially insects activate Toll, Imd, JNK-MAP kinase and prophenol oxidase pathways. Here we analyzed some of our miRNA target genes involved in immune signaling pathways such as spätzle, JNK-MAP kinase and the autophagy receptor optineurin. As we expected, the infection of *G. mellonella* with *L. monocytogenes* led to significant induction of fore mentioned genes.

For example in insects, it is known that, fungal glucans and Gram-positive peptidoglycans are recognized by endogenous ligand of the Toll pathway called spätzle. Activation of the Toll pathway finally leads to synthesis of several antimicrobial peptides to combat the pathogens (*149*). In addition, several bacterial effector proteins are able to trigger MAP kinase signaling pathway. MAP kinases are involved in innate and adaptive immune system in higher animals. *L. monocytogenes* activates MAP kinase by attaching to the cell surface of epithelial cells (*150*). In insects, MAP kinases are involved in the activation of prophenoloxidase, in turn which activates phagocytosis and melanization of hemocytes (*151*). Here, we detected upregulation of spätzle and MAP kinase, potential targets of miR-998 and miR-133 respectively, after *L. monocytogenes* infection (Figure 12). This induction in gene expression of spätzle and MAP kinase might be involved in activation of signaling pathways, synthesis of AMPs and protecting larvae against

listerial infection. Optineurin is a receptor for autophagy and plays a major role in removal of intracellular bacteria (*152*). In our study, optineurin, a predicted target for two miRNAs miR-133 and miR-998, showed reduced expression following *L. monocytogenes* infection (Figure 12). From our results, it is notable that suppression of these miRNAs by bacterial infection facilitates a strong upregulation of spätzle, MAP and optineurin and is thereby involved in clearance of pathogens in the host. Besides, infection of *L. innoucua* resulted in no changes of expression of spätzle and optineurin. MAP kinase was found to be upregulated in comparison with control, although the expression fold change was by far not as strong as compared to infection with *L. monocytogenes* (Figure 12).

Immune activation may affect regulation of cytochrome P450 enzymes, as known from direct treatment of cytokines on hepatocytes leads to downregulation of these enzymes. Analyzing the expression levels of cytochrome P450 enzymes in our case, we found downregulation after infection with *L. monocytogenes*, but not in the case of infection with *L. innocua* (Figure 12). Xenobiotic enzymes play major role in toxin and drug metabolism in multicellular organisms. Cytochrome P450 enzyme showed reduced expression upon infection with *L. monocytogenes* in mice hepatic tissue and brain, the reduction of enzymes can cause severe complication with drug metabolism (*153*, *154*). In addition, several studies have shown interactions between xenobiotic metabolism and infection and inflammation induced by bacterial pathogens and other immunostimulants as well (*155*). In juvenile carp, infection with *L. monocytogenes* 4b resulted in decreased activities of cytochrome P450 enzymes and ethoxyresorufin O-deethylase (*156*). Similarly, in *G. mellonella* infection with *L. monocytogenes* caused increased expression of miR-954, miR-3000 and subsequently downregulation of their targets genes cytochrome P450 6B4 and cytochrome P450 4g1. Strikingly, no effect on expression of these cytochrome P450 enzymes was found upon infection with *L. innocua* (Figure 12). All results suggest that infection with pathogenic *L. monocytogenes* leads to activation of immune system and impairment of xenobiotic metabolism.

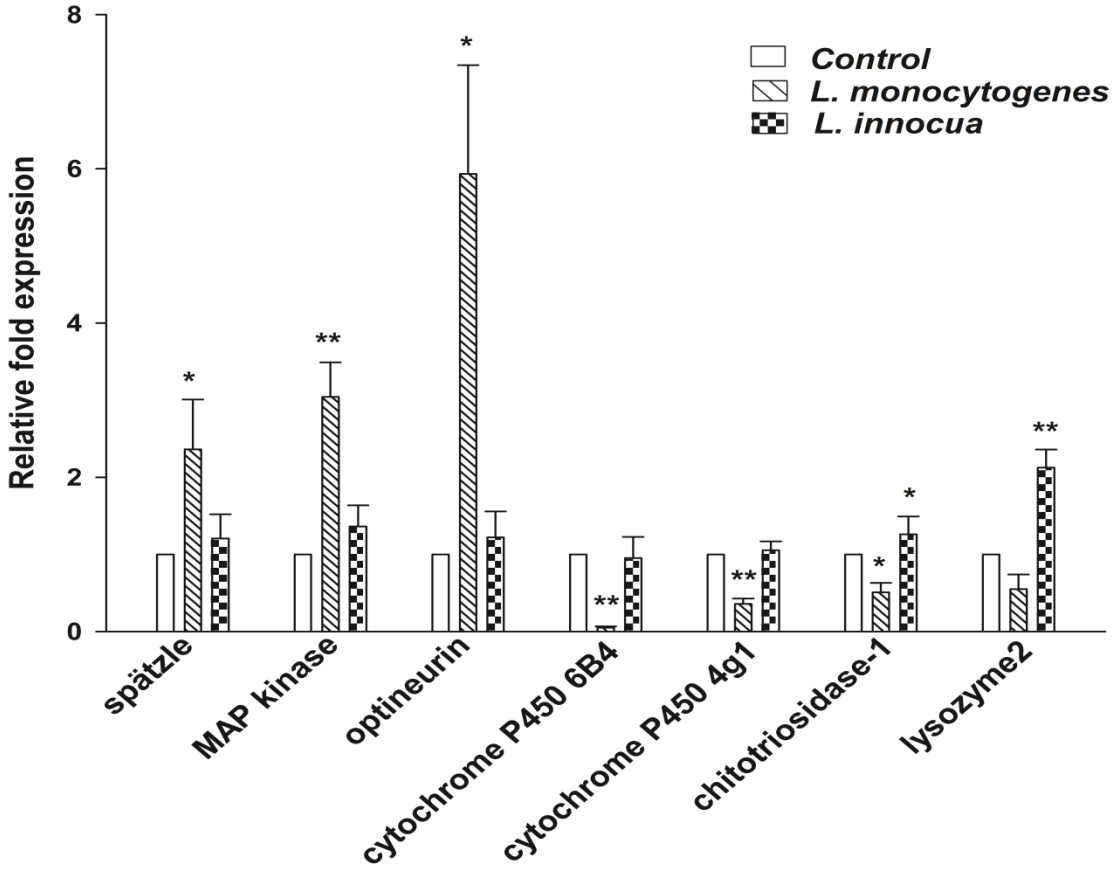


**Figure 12.** qRT-PCR analyses of predicted target genes expressions. With infection of *L. monocytogenes* target genes chitotriosidase-1, lysozyme2, cytochrome P450 6B4 and cytochrome P4504g1 are showing significant reduced expression. The factors involved in immune signaling pathways such as spätzel and MAP kinase, and autophagy receptor optineurin are significantly induced in *G. mellonella*. Expression of fore mentioned target genes were not changed with infection of *L. innocua*, except lysozyme2 and MAP kinase which were significantly upregulated (`*´ P ≤ 0.05; `**´ P ≤ 0.01).

Next, we looked for the expression levels of antimicrobial compounds among our predicted targets. Chitotriosidase-1 and lysozyme 2, which are known for degrading chitin and peptidoglycan of bacterial cell wall, were shown to exhibit reduced expression following infection with *L. monocytogenes*. Interestingly, infection with *L. innocua* led to upregulation of lysozyme 2 and there was no change in chiotriosidase-1 expression (Figure 12). The comprehensive transcriptomic analysis reveals the existence of 4

c-type lysozymes and one i-type lysozyme in *G. mellonella*. The lysozyme which was first identified in *G. mellonella* around 45 years ago is known for having antimicrobial activity against bacteria and fungi (*157*). Chitotriosidase-1 is the best characterized chitinase in mammals, known for its induction with pro inflammatory cytokines such as GM-CSF, TNF-$\alpha$ and also infection with bacterial and fungal infections (*158*). Contrary to this, we observed decreased expression of chitotriosidase-1 and another c-type lysozyme of *G. mellonella* lysozyme2 with infection of *L. monocytogenes.* Interestingly, lysozyme2 exhibit an increased expression upon infection with *L. innocua*. Here, the correlation between induced expression of miR-3000 and decreased expression of its putative target chitotriosidase-1 prompts us a negative regulation of miR-3000. No such correlation was found between the expression levels of lysozyme2 and its miRNA miR-133. The reason for reduced expression of lysozyme2 after *L. monocytogenes* might be related to different developmental stages of larvae that are induced by infection.

## 4.5 Conclusion

Here, we have shown that Gram-positive *L. monocytogenes* infection leads to modulation of miRNA expression that associated to regulation of immune response. Thereby most likely miRNAs are involved in reprogramming the immune defense and other bacterial clearance mechanisms in *G. mellonella*. Using non-pathogenic *L. innocua*, we furthermore classified these effects as pathogen/non-pathogen mediated miRNA responses. As another benefit of this work, we constructed a new publically available database, which is quite helpful to study the developmental and immune processes of the lepidopteran model *G. mellonella*. Finally, we studied the role of miRNAs regulation of some predicted targets which are involved in immune activation and its consequent processes such as impairment of xenobiotic metabolism and induction of antimicrobial compounds. As future direction of this study, miRNA inhibitors can be given orally to analyze direct link to regulation of these targets with miRNAs.

# 5. Chapter III Detection of antisense transcripts and their role during intracellular survival of *L. monocytogenes* in P388D1 macrophages.

## 5.1. Publications

**1. Ultra-deep sequencing of *Listeria monocytogenes* sRNA transcriptome revealed new antisense RNAs.**

Behrens S, Widder S, **Mannala GK**, Qing X, Madhugiri R, Kefer N, Abu Mraheil M, Rattei T, Hain T. PLoS One. 2014 Feb 3; 9(2):e83979. doi: 10.1371/journal.pone.0083979. eCollection 2014.

**2. Detection of Very Long Antisense Transcripts by Whole Transcriptome RNA-Seq Analysis of *Listeria monocytogenes* by Semiconductor Sequencing Technology.**

Wehner S, **Mannala GK**, Qing X, Madhugiri R, Chakraborty T, Mraheil MA, Hain T, Marz M. PLoS One. 2014 Oct 6; 9(10):e108639. doi: 10.1371/journal.pone.0108639. eCollection 2014.

**3. Current status of antisense RNA-mediated gene regulation in *Listeria monocytogenes*.**

Schultze T, Izar B, Qing X, **Mannala GK**, Hain T. Front Cell Infect Microbiol. 2014 Sep 30; 4:135. eCollection 2014. Review.

## 5.2 Contribution

The author (G.M.) jointly involved in performing the experiments and writing the manuscript. He performed infection experiments with P388D1 cells and isolated RNA from intracellularly grown bacteria. He was also involved in qRT-PCR analysis of asRNAs and their target genes and statistical analysis of the data.

## 5.3 Abstract

In this study we used two different technologies to detect antisense transcripts of various sizes in the genome of *L. monocytogenes* expressed during intracellular survival. In the first approach SOLiD sequencing technology and in the other Ion Torrent semi-conductor sequencing technology was utilized. Ultra-deep sequencing of RNA isolated from intracellularly grown *L. monocytogenes* revealed 9 new novel antisense RNAs. Some of these newly identified antisense RNAs are associated with regulation of housekeeping genes *purA*, *fumC* and *pgi* and underlining the significance of these antisense RNAs in metabolic adaptation of *Listeria* to intracellular environment (*159*). In a subsequent study, with semiconductor sequencing technology we identified total 611 ncRNAs candidates, among these 411 ncRNAs which were never described before. Interestingly, we detected very long antisense RNAs with up to 5400 nt size complementary to genes coding for internalins, methylases or a potassium uptake system (*kdpABC operon*). Antisense RNAs and their respective gene coding transcripts expressions were analyzed by using qRT-PCR. Comparative genomics, RNA-seq and structural conservation studies demonstrated the existence of huge ncRNA profile including novel long antisense RNAs, which might be important for intracellular survival within infected eukaryote host (*160*).

## 5.4 Results and discussion

### 5.4.1 Identification of new antisense RNAs using SOLiD sequencing technology

To identify the small RNA profile during the intracellular growth of *L. monocytogenes,* we fractionated the total RNA into three cutoffs: <40 nt, 40-150nt and >150nt length. Based on these fractions library preparation and sequencing on SOLiD platform was performed. The sequencing of 6 experimental samples gave 21 million reads. Using a special pipeline the analysis of these reads revealed the presence of 711 sRNA candidates out of which 569 are undescribed. This analysis revealed presence of a lot of antisense RNAs and we chose 9 new asRNAs for further analysis. All these nine asRNAs were expressed opposite to annotated genes and were conserved in all *Listeria* species except for anti0055 which is specific for *L. monocytogenes*.

The asRNA anti0055 is located on the opposite strand of *lmo0055 (purA)* that encodes an enzyme adenylsuccinate synthetase, important for de novo synthesis of purine nucleotides. Moreover, it has been demonstrated that *purA* plays a major role during intracellular survival of *L. monocytogenes* (*161*). The transcription start site (TSS) of anti0055 is located 365 nt downstream of the *purA* TSS in opposite direction. We found out the increased expression of asRNA anti0055 under intracellular environment by both qRT-PCR and northern blot techniques. However, there is no classical regulation pattern between the *purA* and its antisense RNA, when analyzed by both by RNA-seq and qRT-PCR methods (Figure 13 A&B).

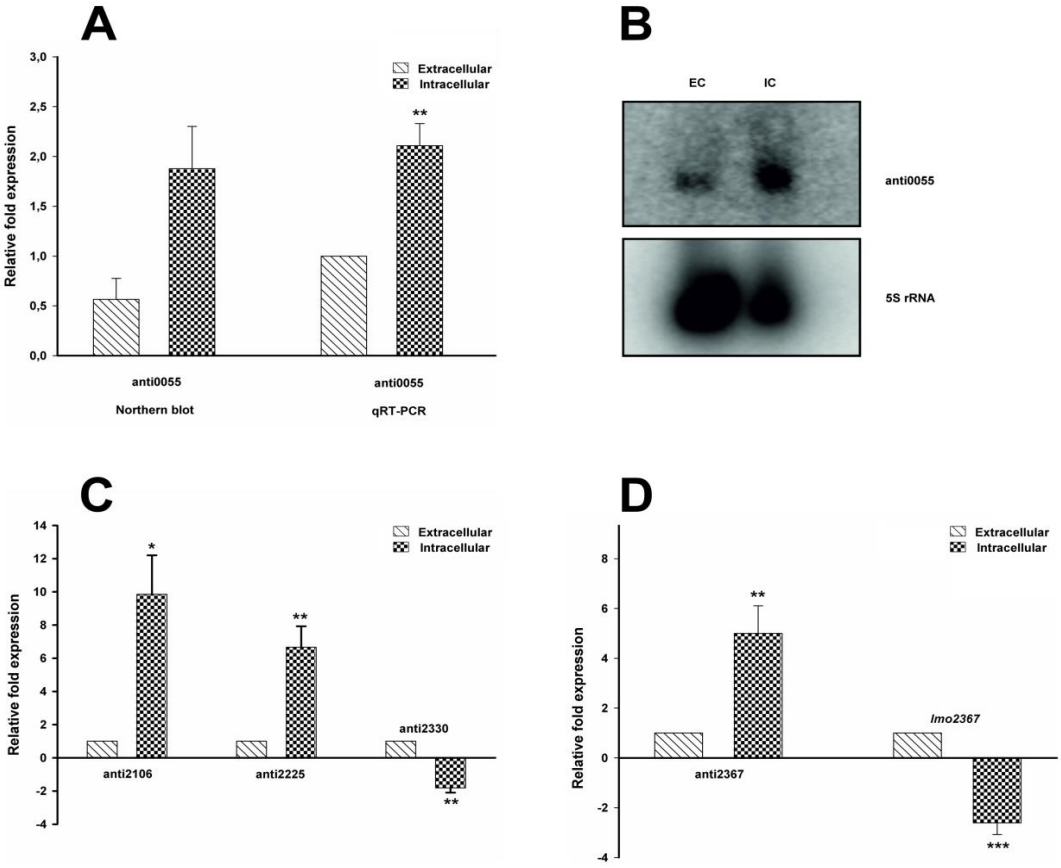


**Figure 13. Validation of asRNAs using strand specific qRT-PCR and northern blot analysis.** A) The asRNA transcript anti0055 (*purA*) is validated by both northern blot analysis and strand-specific qRT-PCR. The graph depicts the intracellular induction of anti0055. B) Northern blot images represent anti0055 and control 5S rRNA C) The induction of asRNA transcripts anti2106, anti2225, and anti2330 was detected by strand-specific qRT-PCR in intracellular conditions. D) Strand-specific qRT-PCR analysis was performed to measure antisense RNA transcript anti2367 and *pgi* (lmo2367). '*' P≤0.05; '**' P≤0.01; '***' P≤0.001. The image was taken from (*159*)

Another newly identified asRNA anti2225 is opposite to the gene *fumC* that codes for a fumarate hydratase associated with the TCA cycle. Similarly, an antisense transcript to the *fumC* homologous gene is detected in Gram-negative *H. pylori* and proven by northern blot and qRT-PCR (*162*). Some other study demonstrated in *Cyanobacterium synechocytis* that asRNA are involved in regulation of housekeeping genes at transcription level (*163*). Moreover, it is hypothesized that *L. monocytogenes* has an interrupted TCA cycle, which may act as an essential pathway to generate purines. Here we observed increased expression for both *fumC* and its antisense RNA anti2225 during intracellular growth conditions (Figure 13 C).

The sign of classical regulation of antisense RNA regulation can be observed in the asRNA anti2367 which is opposite to the gene *lmo2367/pgi.* From the analysis of sequencing the data between extra and intracellular conditions, the expression of either *pgi* or its asRNA appears to be mutually exclusive, indicating the role of antisense mediated regulation at transcription level. As shown in Figure 13.D, it is clear that *pgi* is downregulated with increased expression of its asRNA anti2367 in intracellular conditions. This *pgi* gene codes for an enzyme glucose-6-phosphate isomerase which is functions as bridge between glycolysis and pentose phosphate pathway. Previous studies stated that the transition of *L. monocytogenes* from extracellular to intracellular environments led to reduced expression of *pgi* (*164*), the changes might initiate a shift in metabolism of glucose-6-phoshpate that can be taken over by pentose phosphate pathway (*165*). As *pgi* is under the control of housekeeping promoter, it's obvious that it requires promoter independent regulation. So the finding of anti2367 shed lights on the asRNA meditated regulation on metabolic adaptions of *L. monocytogenes* in intracellular conditions (Figure 13 D).

### 5.4.2 Detection of very long antisense using Ion Torrent technology

We isolated the total RNA from extracellularly (in BHI) and intracellularly (in P388D1 macrophages) grown *L. monocytogenes*. Subsequently, cDNA libraries were prepared using Ion Total RNA-seq kit v2 and sequenced on Ion Torrents Personal Genome Machine (PGM). *In silico* studies based on characteristics such as secondary structure,

seeds, GC-content, conservation and genome wide alignment gave total 741 putative ncRNAs whereas out of them 611 ncRNAs were confirmed by RNA-seq analysis. In our set of predicted ncRNAs, we noticed some interesting long antisense RNAs (lasRNAs) with length up to 5400 nt that showed increased expression. Later these lasRNAs were validated by using qRT-PCR and expression levels of respective sense gene were measured (Figure 14).

Two long ncRNA candidates were found opposite to the genes *lmo0333* and *lmo1136* that code for similar internalin proteins harboring a typical LRR-LPXTG motif. Most of the internailn proteins and their regulation are associated with the virulence of *L. monocytogenes*. Several reports described the role of PrfA and SigB in regulation of well-studied internalins, e.g., *inlA*, *inlB*, *inlC*, *lmo0263* and *lmo0610* (*166*, *167*). Here, by using strand-specific sequencing, we revealed the regulation of internalins *lmo0333* and *lmo1136* (Figure 14). *Lmo1136* is assumed to encode an internalin, but not yet studied so far. *Lmo0333* is also known as *inlI*. Recently Sabet *et al.,* described the role of this InlI in virulence of *L. monocytogenes*, however the mutant strain of this gene was not attenuated in mouse infection model (*168*).

Another set of lasRNAs which drew our attention were antisense transcripts to several methylases like *lmo0581* (SAM methlytransferase), *lmo0935* (CspR protein, an rRNA methylase homolog) and *lmo0996* (similar to cysteine methyltransferase).The antisense transcript of *lmo0581* is of the size of 1161nt and showed increased expression during intracellular conditions. *Lmo0581* is transcribed both in extra and intracellular conditions. The other lasRNA namely las0936 (2,500 nt) runs in opposite direction to genes *lmo0936*, *lmo0935* and *lmo0934*. The expression of this lasRNA is only specific to intracellular growth of bacteria whereas the genes are expressed under both conditions (Figure 14).

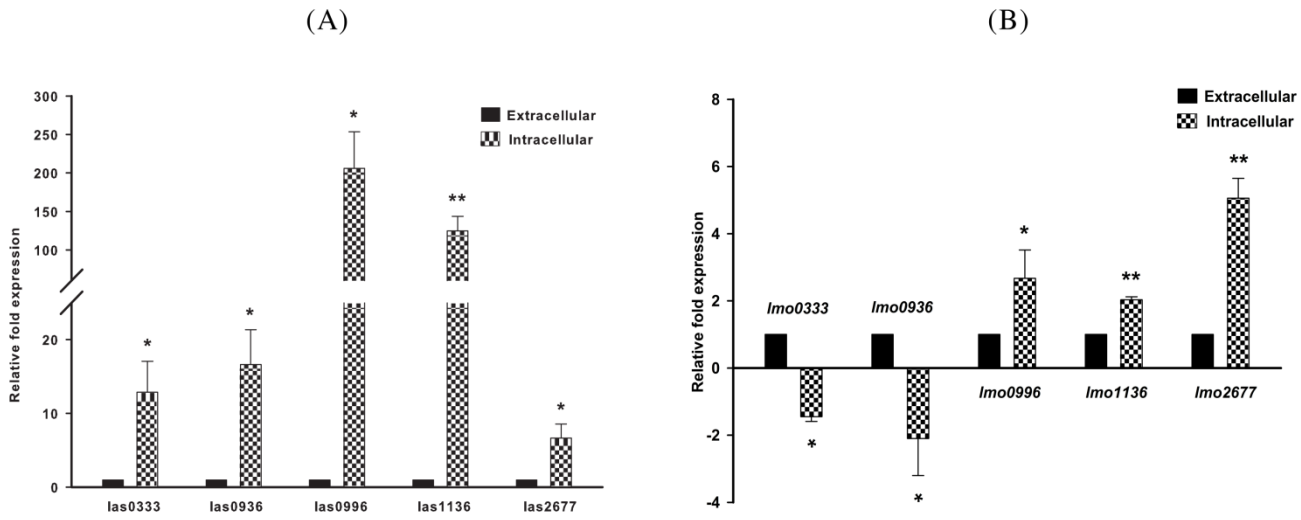


**Figure 14. Measurement of expression levels of new long antisense (las) RNAs in *L. monocytogenes* by strand specific qRT-PCR analysis.**

(A) The expression of las transcripts was measured by strand-specific qRT-PCR analysis. In support of the of RNA-seq results, the qRT-PCR analysis showed that novel lasRNA transcripts las0333, las0936, las0996, las1136 and las2677 were significantly induced in intracellular environments. '*' −P≤0.05 '**' −P≤0.01. (B) The lasRNAs respective target genes *lmo0333* (internalin) and *lmo0936* (nitroflavin reductase) exhibited significant reduced expression whereas *lmo0996* (methyltransferase), *lmo1136* (internalin) and *lmo2677* (esterase) showed increased expression under intracellular growth condtions. '*' −P≤0.05; '**' −P≤0.01. The image was taken from (*160*).

Later, we looked at lasRNA transcript to *lmo0996* that codes for cysteine methyltransferase which is transcribed as between its syntenic genes *lmo0995* and *lmo0997*. This intergenic transcript is only detected during intracellular conditions, indicating this is not attributed to 5´ or 3´ UTRs of synteny genes and a putative ncRNA (Figure 14).

Finally, we confirmed the presence of a very long antisense RNA (las2677) with size of 5400 nt that spans completely from *lmo2677* to *lmo2680* and partially *kdpB* gene by qRT-PCR method (Figure 14). Previously Wurtzel *et al.,* detected a small asRNA that covers only *lmo2678*, which is expressed under exponential growth at 37$^{o}$C and regulated by SigB (*67*). The gene of *lmo2678* produces a response regulator, part of two component system along with cognate histidine kinase encoded by *lmo2679*. In *E.coli*, the two component system *kdpED* regulates the adjacent operon *kdpABC* which is responsible for high affinity potassium uptake under high-osmolarity conditions (*169*).

For several pathogens such as *Salmonella*, *Staphylococcus* and *Yersinia*, this kdpED two component system is essential during their intracellular growth phase (*170*). But in *L. monocytogenes* it does not seem to play any major role in virulence (*171*). This phenomenon is supported by our present observation that entire locus of *kdpEDABC* is regulated by antisense transcription. This indicates the existence of some other alternative system for uptake of potassium and such a system has been already reported for *B. subtilis* (*172*).

## 5.5 Conclusion

Our studies revealed a substantial antisense transcription in *L. monocytogenes* throughout its genome. The biological significance of this extensive antisense transcription is not well understood. In intracellular conditions, many of the antisense RNA have shown increased transcription. Given the high number of asRNAs are identified along with very long antisense RNAs (lasRNAs); it is obvious that these asRNAs might have important role in regulation of *Listeria* gene expression under different environmental conditions. In our conditions, we observed extensive antisense transcripts against genes that are involved in metabolic adaptation and bacterial physiology especially in the case of potassium uptake system.

# 6. Summary

During the course of infection, the pathogens follow several strategies to evade from the host defense system and to adapt to the host environment for efficient survival. Meanwhile the host imposes several cellular processes, innate and adaptive immune systems to fight back against the pathogens. At molecular level, the pathogens deploy different strategies, mainly by modulating their gene expression profiles as per the intercellular and intracellular compartments of the host. Similarly, the host also alters its gene expression profile specific to each pathogen to protect itself. For a longtime, proteins were considered to be predominant molecules to regulate gene expression. With the discovery of regulatory non-coding RNAs (ncRNAs) in both prokaryotes and eukaryotes this assumption had to be reconsidered. These regulatory ncRNAs not only opened a new branch in the understanding of gene regulation but also furthermore represented possible biomarkers for diagnostic purposes or leverage points for drug targets. In this context, we took effort to study the functional roles of these eukaryotic non-coding RNAs (miRNAs) from the perspective of host-pathogen interactions and host immune responses during infections. In this thesis, efforts are made to reveal host miRNA response to model pathogen *L. monocytogenes* in different infection models (Caco-2 cells and *G. mellonella*) along with bacterial ncRNA (asRNAs) profilling during their intracellular survival in P388D1 macrophages.

The first part of the study revealed the altered miRNA response in Caco-2 cells that were infected with *L. monocytogenes*. Using different mutant strains (Δ*hly* and Δ*inlAB*), which are unable to invade epithelial cells and escape from phagocytic vacuoles, we demonstrated that miRNA response is dependent on the subcellular localization of *L. monocytogenes* and its virulence determinants. Even the purified endotoxin LLO from *L. monocytogenes* is able to regulate significant miRNAs in Caco-2 cells. The correlation of selected miRNAs and their predicted target gene expression levels uncovered roles of miRNAs in fine-tuning of immune related gene expression during *L. monocytogenes* infection.

In continuation of the first study, the second part involved elucidation of miRNA response to *L. monocytogenes* infection in invertebrate infection model *G. mellonella* and its role in host-microbial interactions. Insect specific miRNA microarray analysis demonstrated the deregulation of miRNA response with upregulation of 39 and downregulation of 58 miRNAs upon infection of *L. monocytogenes* in *G. mellonella*. Some of the miRNAs regulation patterns are conserved between vertebrates and invertebrates, as we observed the downregulation of conserved miRNAs miR-133 and miR-998 (homologous to miR-29) both in higher mammals and *G. mellonella* with bacterial infections. We observed pathogen/non-pathogen specific miRNA regulation in this insect model, when we compared miRNAs expression patterns with pathogenic *L. monocytogenes* and non-pathogenic *L. innocua* infections. Later, we established a public database, which would be very useful to study insect-microbial interactions that can correlate even with higher animals. qRT-PCR analysis of predicted target genes such as spätzle, MAP kinase and optineurin demonstrated the role of miRNAs in Toll pathway, MAP-kinase pathway and autophagy process with pathogenic *L. monocytogenes* infection.

In the third part of the thesis, bacterial ncRNAs especially asRNAs that are involved in metabolic adaptations and virulence of *L. monocytogenes* are unveiled using different sequencing technologies such as SOLiD and Ion torrent technologies. By using SOLiD platform, several antisense RNAs were identified and some of them were associated with regulation of housekeeping genes like *purA*, *fumC* and *pgi*, thereby involved in metabolic adaptations of *L. monocytogenes*. In another study, Ion torrent based sequencing of intracellularly grown *L. monocytogenes* RNA revealed the existence of long noncoding RNAs that overlap internalins, methylases and potassium uptake system (*kdpABCD* operon), highlighting their probable role in regulation of various group of genes.

In conclusion, the thesis provides crucial insights over the ncRNAs mediated regulation of host immune response, role in listerial adaptations to intracellular environment as well as the interplay between host and pathogens.

# 7. Zusammenfassung

Während einer mikrobiellen Infektion nutzen Krankheitserreger verschiedene Strategien um dem Abwehrsystem des Wirtes zu entgehen und sich an die Bedingungen des Wirtsmilieus für ein effizientes Wachstum anzupassen. Der infizierte Wirt wiederum reagiert mit diversen zellulären Prozessen auf die Infektion, wie z.B. mit der Aktivierung des angeborenem und/oder adaptivem Immunsystems. Die Strategien der Erreger auf molekularer Ebene beinhalten oft die Modulierung der Genexpressionsaktivität beim Wechsel zwischen inter- und intrazellulären Bedingungen. Um sich zu schützen, ändern auch die Wirtszellen in gleicher Weise ihre Expressionsaktivität in Reaktion auf eine mikrobielle Infektion. Lange Zeit ging die Wissenschaft davon aus, dass die Genexpression allein durch Proteinfaktoren bestimmt wird. Mit der Entdeckung von nicht-kodierenden RNA Transkripten (ncRNAs) in Eukaryoten wie auch in Prokaryoten musste diese Meinung jedoch revidiert werden. Damit erweitern ncRNAs nicht nur das Verständnis über die Mechanismen der Genregulation, sondern können gleichermaßen auch potentielle Biomarker für diagnostische Zwecke darstellen bzw. sich als Angriffspunkt für neue Therapeutika nutzen lassen. Vor diesem Hintergrund, haben wir uns zum Ziel gesetzt, die Rolle dieser ncRNAs für Wirt-Pathogen-Interaktionen sowie die Immunantwort des Wirts nach Infektion zu untersuchen. Die vorliegende Arbeit untersucht zunächst die miRNA-Antwort des Wirtes nach Infektion mit dem Modelkeim *L. monocytogenes* in verschiedenen Infektionsmodellen wie der humanen Darmepithelzelllinie Caco-2 und der großen Wachsmottenlarve (*Galleria mellonella*). Des Weiteren, wurden ncRNA-Profile von *L. monocytogenes* vor und nach Infektion der humanen Makrophagen-Zelllinie P388D1 analysiert, um die bakterielle Adaption zu beleuchten.

Der erste Teil der Arbeit behandelt die Identifizierung von miRNA-Profilen von Caco-2-Zellen in Antwort auf Listerien-Infektionen. Mit Hilfe direkten Vergleichs von Infektion mit *L. monocytogenes* und verschiedener chromosomaler Deletionsmutanten (Δ*inlAB* und Δ*hly*), die nicht mehr in der Lage sind in Caco-2-Epithelzellen zu invadieren oder aus dem Phagolysosom zu entkommen, konnten wir zeigen, dass die miRNA-Antwort abhängig von der Lokalisierung der Listerien ist. Ferner, war auch aufgereinigtes

Listerien-Toxin Listeriolysin (LLO) in der Lage, das miRNA-Profil von Caco-2-Zellen signifikant zu ändern. Eine Korrelation zwischen ausgewählten miRNAs und der Geneexpression ihrer vorhergesagten Zielgene zeigte die Rolle der miRNAs in der Abstimmung der Expression von Immun-zugehörigen Genen während der Infektion.

In der Fortführung des ersten Teils, befasst sich der zweite Teil der Arbeit mit der Aufklärung der miRNA-Antwort der großen Wachsmottenlarve *G. mellonella* nach *L. monocytogenes*-Infektion. Durch die Verwendung von Insekten-spezifischen DNA-Mikroarrays konnte die differentielle Regulation von 117 miRNAs der Wachsmotte nach Infektion gezeigt werden. Davon waren 39 miRNAs signifikant hoch- und 58 signifikant runterreguliert. Eine komparative bioinformatische Analyse dieser miRNAs zeigte, dass einige dieser miRNAs zwischen Vertebraten und Invertebraten konserviert sind. So wurden die von uns gefundenen Herunterregulierungen der miRNAs miR-133 und miR-998 auch in höheren Säugetieren nach bakterieller Infektion beschrieben. Darüber hinaus konnten die Deregulation in Pathogenitäts- und nicht-Pathogenitäts-abhängige Regulation unterklassifiziert werden, indem wir Referenzversuche mit den nicht-pathogenen Listerienstamm *L. innocua* durchführten. Abschließend etablierten wir basierend auf der Arbeit eine öffentlich-zugängliche Datenbank zur Vorhersage von Zielgenen von miRNAs, die hilfreich für die weitere Untersuchung von Insekten-Mikroorganismen-Interaktionen sein wird. qRT-PCR-Analysen von identifizierten Zielgenen wie z.B. spätzle und Optineurin zeigten, dass miRNAs in der Toll- bzw. MAP-Kinase-Signaltransduktionskaskade und Autophagie involviert sind.

Im dritten Teil dieser Arbeit wurden mittels verschiedener Sequenziertechnologien, wie SOLiD oder Ion Torrent, bakterielle ncRNAs untersucht, die während der intrazellulären Überlebensphase exprimiert werden. Die Studie unter Verwendung der SOLiD-Sequenzierplattform lieferte Hinweise für Genregulationen durch Antisense-Transkription z. B. für die Haushaltsgene *purA*, *fumC* und *pgi*. Dies weist auf eine Beteiligung von antisense Regulation dieser Gene bei der metabolischen Anpassung während des intrazellulären Überlebens hin. Die Ion Torrent-basierende Transkriptomstudie von intrazellulär wachsenden Listerien indentifizierte darüber hinaus auch lange ncRNAs. Diese langen ncRNAs sind gegen Internaline, Methylasen und das

Kaliumaufnahmesystem (*kdpABCD*-Operon) gerichtet und lassen somit eine vielfältige regulatorische Wirkung auf Transkriptionsebene auf eine diverse Gruppe von Gene erahnen.

Zusammenfassend erbringt diese Arbeit sowohl grundlegende Einsichten in die ncRNA-gesteuerte Anpassung des Erregers *L. monocytogenes* an intrazelluläre Bedingungen als auch die ncRNA-gesteuerte Erreger-Wirt-Interaktion.

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# 9. Abbreviations

| | |
|---|---|
| aa | Amino acid(s) |
| APS | Ammonium per sulphate |
| ARP | Actin Related Protein |
| ATP | Adenosine triphosphate |
| BHI | Brain heart infusion |
| BSA | Bovine serum albumin |
| Bp | Base pair |
| CD | Cluster of differentiation |
| °C | Centigrade |
| Cfu | Colony forming unit |
| C-terminal | Carboxy-terminal |
| DC | Dendritic cell |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Desoxynucleotide-5'-triphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine-N, N, N', N'-tetraacetate |
| FCS | Fetal calf serum |
| FITC | Fluoresceinisothiocyanate |
| G | Gravity |
| H | Hour(s) |
| HEPES | 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid |
| HGF | Hepatocyte Growth Factor |
| Hly | Haemolysin |

| | |
|---|---|
| IFN | Interferon |
| Kb | Kilobase |
| kDa | Kilodalton |
| LB | Luria-Bertani |
| LLO | Listeriolysin |
| LRR | Leucine Rich Repeat |
| LTA | Lipoteichoic Acid |
| M | Molar (mol/l) |
| mAb | Monoclonal antibody |
| Min | Minutes |
| miRNA | micro RNA |
| MOI | Multiplicity of infection |
| NEA | Non-essential amino acids |
| NGS | Next generation sequencing |
| NLRs | Nod like receptors |
| O.D | Optical density |
| ORF | Open reading frame |
| PAMPs | Pathogen associated molecular patterns |
| PBS | Phosphate-buffered saline |
| PRRs | Pattern recognition receptors |
| PCR | Polymerase chain reaction |
| qRT-PCR | quantitative Real Time PCR |
| RLRs | RIG like receptors |
| RNA | Ribo nuclec acid |
| rpm | Revolutions per minute |

| | |
|---|---|
| RT | Room temperature |
| RNA-seq | RNA sequencing |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SOLiD | Sequencing by Oligonucleotide Ligation and Detection |
| sRNA | small RNA |
| STM | Signature Tagged Mutagenesis |
| TAE | Tris acetate EDTA |
| TAT | Twin arginine transport |
| TBS | Tris-buffered saline |
| TBS-T | Tris-buffered saline - tween 20 |
| TCA | Tri chloro acetic acid |
| TCA | Tri carboxylic acid cycle |
| TE | Tris EDTA |
| TEMED | N, N, N', N'-Tetramethylethylene diamine |
| TLR | Toll Like Receptor |
| TNF | Tumor necrosis factor |
| Tris | Tris(hydroxymethyl) aminomethane |
| TSS | Transcription start site |
| U | Unit |
| UTR | Untranslated region |
| Vgc | Virulence gene cluster |
| WASP | Wiskott-Aldrich syndrome family protein |
| WT | Wild type |

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# 11. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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Place, Date Sign

**Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.**

**The curriculum vitae was removed from the electronic version of the paper.**

**List of publications:**

1. ***Salmonella enterica serovar* Typhimurium lacking hfq gene confers protective immunity against murine typhoid.** Allam US, **Krishna MG**, Lahiri A, Joy O, Chakravortty D. PLoS One. 2011 Feb 9; 6(2):e16667. doi: 10.1371/journal.pone.0016667.

2. **Needleless vaccine delivery using micro-shock waves.** Jagadeesh G, Prakash GD, Rakesh SG, Allam US, **Krishna MG**, Eswarappa SM, Chakravortty D. Clin Vaccine Immunol. 2011 Apr;18(4):539-45. doi: 10.1128/CVI.00494-10. Epub 2011 Feb 9.

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5. **Ultra-deep sequencing of *Listeria monocytogenes* sRNA transcriptome revealed new antisense RNAs.** Behrens S, Widder S, **Mannala GK**, Qing X, Madhugiri R, Kefer N, Abu Mraheil M, Rattei T, Hain T. PLoS One. 2014 Feb 3;9(2):e83979. doi: 10.1371/journal.pone.0083979. eCollection 2014.

6. **A systematic proteomic analysis of *Listeria monocytogenes* house-keeping protein secretion systems.** Halbedel S, Reiss S, Hahn B, Albrecht D, **Mannala GK**, Chakraborty T, Hain T, Engelmann S, Flieger A. Mol Cell Proteomics. 2014 Jul 23. pii: mcp.M114.041327. [Epub ahead of print].

7. **Detection of Very Long Antisense Transcripts by Whole Transcriptome RNA-Seq Analysis of *Listeria monocytogenes* by Semiconductor Sequencing Technology.** Wehner S, **Mannala GK**, Qing X, Madhugiri R,

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*Article*

# microRNA Response to *Listeria monocytogenes* Infection in Epithelial Cells

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**Abstract:** microRNAs represent a family of very small non-coding RNAs that control several physiologic and pathologic processes, including host immune response and cancer by antagonizing a number of target mRNAs. There is limited knowledge about cell expression and the regulatory role of microRNAs following bacterial infections. We investigated whether infection with a Gram-positive bacterium leads to altered expression of microRNAs involved in the host cell response in epithelial cells. Caco-2 cells were infected with *Listeria monocytogenes* EGD-e, a mutant strain (Δ*inl*AB or Δ*hly*) or incubated with purified listeriolysin (LLO). Total RNA was isolated and microRNA and target gene expression was compared to the expression in non-infected cells using microRNA microarrays and qRT-PCR. We identified and validated five microRNAs (miR-146b, miR-16, let-7a1, miR-145 and miR-155) that were significantly deregulated following listerial infection. We show that expression patterns of particular microRNAs strongly depend on pathogen localization and the presence of bacterial effector proteins. Strikingly, miR-155 which was shown to have an important role in inflammatory responses

during infection was induced by wild-type bacteria, by LLO-deficient bacteria and following incubation with purified LLO. It was downregulated following Δ*inl*AB infection indicating a new potent role for internalins in listerial pathogenicity and miRNA regulation. Concurrently, we observed differences in target transcript expression of the investigated miRNAs. We provide first evidence that *L. monocytogenes* infection leads to deregulation of a set of microRNAs with important roles in host response. Distinct microRNA expression depends on both LLO and pathogen localization.

**Keywords:** *Listeria monocytogenes*; microRNA; non-coding RNA; infection; epithelial cells; Caco-2

## 1. Introduction

microRNAs (miRNAs) represent a class of small non-coding RNAs of ~22 nucleotides in length that repress gene expression on a post-transcriptional level by targeting the 3' UTRs of cellular mRNA leading to its degradation or inhibition of translation [1]. miRNAs were implicated in a wide range of physiological as well as pathological processes, including inflammatory response, apoptosis, growth and cancer, neurodegenerative and cardiovascular diseases [2]. Increasing evidence suggests an important role of miRNAs in the immune response against infectious agents [3–5]. Previous work focused on and revealed direct anti-viral activity of miRNAs through repression of viral mRNA production [6]. Conversely, viral miRNAs were found to antagonize the host mRNA leading to a suppression of the anti-viral response [7].

Recently, a role of miRNAs in the response against bacterial pathogens has been proposed. miRNAs were shown to be effective against *Pseudomonas syringae* infection in plants [8]. Similar to viruses, *P. syringae* was found to secrete proteins that bind host miRNA and subsequently modulate immune response [8]. Furthermore, Rao and colleagues described the presence miRNAs expressed by pathogenic *Pseudomonas aeruginosa* strains which were isolated from adult patients with cystic fibrosis [9]. Xiao *et al.* uncovered a *Helicobacter pylori*-dependent induction of miR-146b and miR-155 in gastric epithelial cells with subsequent inhibition of IL-8, a central cytokine in the chemotaxis of leukocytes [10]. Further investigation revealed that miRNAs control major inflammatory pathways, such as the TLR-mediated activation of the NF-kB pathway [10]. While *P. syringae* and *H. pylori* remain extracellular during infection, a recent study showed altered immune response of mice deficient in miR-155 to the facultative intracellular pathogen *Salmonella* [5]. Schulte *et al.* uncovered the regulation of IL-6 and IL-10 by miRNAs of the let-7 family and miR-155 induction by secreted effector proteins of *Salmonella* rather than the invading pathogen [5].

In this study, we observed differential regulation of miRNAs and associated target transcripts in epithelial cells following infection with *Listeria monocytogenes*. *L. monocytogenes* is a Gram-positive, facultative intracellular bacterium that has been used widely for the elucidation of immune processes in a variety of hosts and tissues. *L. monocytogenes* facilitates its entry into non-phagocytic cells, such as epithelial Caco-2 cells, via surface bound and secreted effector proteins known as internalins. Internalized *Listeriae* are able to escape from the hostile phagocytic vacuole using the effector protein

listeriolysin (LLO), a secreted toxin that is essential for the pathophysiology and intracellular survival of *L. monocytogenes*.

Using defined mutants that variously lack individual virulence factors, this study provides evidence that the ability and extent of *Listeria* induced regulation of host miRNAs strongly depends on cellular localization, on secreted and membrane-bound proteins of the pathogen.

## 2. Materials and Methods

### *2.1. Bacterial Strains and Growth Conditions*

*L. monocytogenes* EGD-e [11] and its isogenic deletion mutants Δ*hly* [12] and Δ*inl*AB [13] were used in this study. Bacteria were grown in BHI broth overnight at 37 ℃ with shaking at 180 rpm. Overnight cultures were diluted into 1:50, grown to mid-exponential phase ($OD_{600nm}$ = 1.0) and used for further experiments.

### *2.2. Eukaryotic Cell Culture*

Human epithelial cells (Caco-2) were cultured in MEM with 10% fetal calf serum (FCS) and 5% non-essential amino acids, respectively. Cells were maintained at 37 ℃ in 5% $CO_2$.

### *2.3. LLO Purification*

LLO is expressed and purified from a recombinant *L. innocua* 6a strain harboring the *hly* gene [14]. Briefly supernatant fluids were concentrated using a Millipore filtration apparatus followed by batch absorption onto Q-sepharose (Pharmacia, Freiburg, Germany) and pre-equilibrated with loading buffer (50 mM $NaH_2PO_4$, pH 6.2). The non-absorbed fraction was centrifuged and desalted by transferring through a super loop to a HiPrep 26/10 desalting column (Pharmacia, Freiburg, Germany) where loading buffer (50 mM $NaH_2PO_4$, pH 6.2) was used to elute the desalted fraction. This fraction was subsequently filtered through a Millipore filter (0.22 μm) and loaded onto a Resource-S column previously equilibrated with 50 mM $NaH_2PO_4$, pH 6.2. The pure toxin eluted reproducibly from the column at 0.21 to 0.28 M NaCl using elution buffer (50 mM $NaH_2PO_4$ 1M NaCl, pH 5.6). Fractions were collected and individually tested for hemolytic activity. Yields of the toxins range from 1 to 5 mg/L supernatant with a hemolytic activity (HU) of 20,000 HU/mg purified protein. One hemolytic unit (HU) is expressed as the amount of toxin required to lyse 50% of a 1% suspension of sheep erythrocytes. The toxin showed a high purity as seen using SDS-PAGE analysis, was efficiently recognized with LLO-specific antibodies, and exhibited hemolytic activity on sheep erythrocytes at both pH 6.0 and pH 7.4 respectively.

### *2.4. Infection Assays and LLO Treatment*

Caco-2 cells were maintained in 6-well plates following at conditions described above. Bacteria at MOI 10 were added to the monolayer of cells. One hour of post infection, followed by washing with 1 × PBS, cells were supplemented with fresh media containing 20 μg/mL gentamycin to remove

extracellular bacteria. After one hour of gentamycin treatment cells were lysed using a mixture of RLT lysis buffer and 1% β- mercaptoethanol and used for RNA isolation.

LLO at different concentrations (25 ng/mL and 50 ng/mL), was preactivated with dithiothreitol before administration to Caco-2 cells. Following incubation with LLO for one hour, cells were lysed with RLT lysis buffer and 1% β- mercaptoethanol.

### *2.5. RNA Isolation*

RNA was isolated from cell lysate samples using the Qiagen miRNeasy Kit. Briefly, cell lysate samples were transferred to the QIA Shredder column and centrifuged at 13,200 rpm. An equal amount of 70% of ethanol was added to the eluted sample and mixed thoroughly. These samples were passed through a nucleic acid binding column which is supplied by the miRNeasy Kit (Qiagen). The DNA present on the column was digested using RNase-free DNase (Qiagen) for 30 min at RT and RNA was eluted by RNase free water. The quantity of isolated RNA was measured with NanoDrop analyzer (NanoDrop Technology, Rockland, MA, USA) and quality was assessed by running the samples on Nano-chips for 2100 Bioanalyzer (Agilent, Böblingen, Germany).

### *2.6. miRNA Microarray*

For this analysis we used the biochip "Geniom Biochip MPEA homo sapiens & mus musculus" (febit, Heidelberg, Germany). The probes are designed as the reverse complements of all major mature miRNAs and the mature sequences as published in the current Sanger miRBase release (version 14.0 September 2009, see http://microrna.sanger.ac.uk/sequences/index.shtml) for homo sapiens & mus musculus. Techniqual and procedural details are described in detail in supplementary material.

### *2.7. Reverse Transcription Reaction and Quantitative Real-Time PCR Analysis*

First strand cDNA was generated for mRNA by using SuperScript II reverse transcriptase (Invitrogen) and miScript reverse transcription kit (Qiagen) for miRNAs using 1 μg of RNA for each reaction.

Quantitative real-time PCR analysis was performed by using AB Prism 7900 HT system. All forward and reverse primers used for PCR were purchased from Qiagen. We used RNUA1 as internal controls for miRNA expression normalization and HPRT for target mRNA expression normalization.

The reaction mixture volume of 25 μL for mRNA quantitative real-time PCR was applied using 100 ng cDNA for each reaction. For miRNA quantitative real-time PCR analysis 3 ng of cDNA per 50 μL reaction set-up was used. For each primer the efficiency was calculated by standard curve which was generated by using different concentrations of genomic DNA in real time PCR. The expression level of mRNA and miRNA was calculated by normalizing its quantity to the respective expression of the internal control in Caco-2 epithelial cells. Threshold cycle values (CT) of the tested transcripts were determined and normalized expression of each target gene was given as the ΔCT between the log2 transformed CT of the target gene and the log2 transformed CT of the internal control. Log2 transformed gene expression levels (ΔCT) of each target transcript were expressed as log2 differences from control (=log2 ΔΔCT method). Data was acquired and analyzed with the SDS 2.3 and RQ-Manager 1.2, respectively.

### *2.8. Statistical Data Analysis of Infection Experiments*

All infection and toxin experiments were performed for a minimum of three times. Significant differences between two values were compared with a paired Student's *t*-test. Values were considered significantly different when the $p$ value $< 0.05$.

## 3. Results

### *3.1.* L. monocytogenes *Differentially Induces miRNAs Dependent on Cellular and Subcellular Localization*

Based on miRNA expression analysis using microarrays, we selected a subset of miRNA candidates that were differentially deregulated following wild type infection of epithelial Caco-2 cells. We focused on miRNAs that have a biologically validated role *in vitro* or *in vivo*. These miRNAs were validated using qRT-PCR. Relative expression levels obtained by both techniques showed a robust correlation (Figure S1).

In addition to the wild-type infection, Caco-2 cells were infected with two isogenic mutant strains or incubated with purified listeriolysin (LLO). The *hly* mutant strain is unable to produce LLO and remains in the phagocytic vacuole after host cell infection. Δ*inl*AB remains in the extracellular space because of the inability to induce bacterial uptake into epithelial cells.

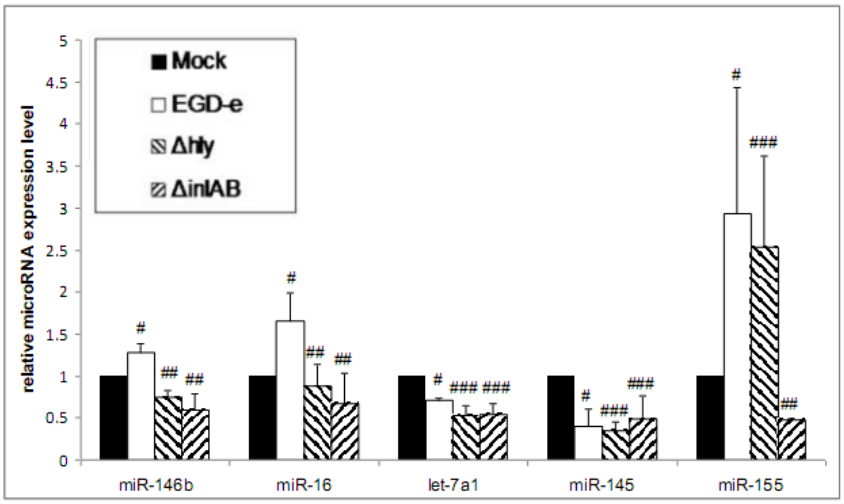
Infection with wild-type bacteria leads to significantly increased expression of miR-146b, miR-16 and miR-155 expression in Caco-2 cells compared to non-infected cells (Figure 1).

As previously described for *Salmonella* [5], we also observed a significant downregulation of let-7a1 (Figure 1), a member of the let-7 family that is implicated in immune response and cancer development. We further observed a strong downregulation of miR-145 (Figure 1). A recent study demonstrated that blocking miR-145 led to a strong anti-inflammatory and reduced airway hyper responsiveness comparable to the effects obtained following glucocorticoid treatment [15].

Compared to wild-type infection both mutant strains induced significant deregulation of miR-146b and miR-16 (Figure 1). The expression differed with respect to the directionality of regulation for these miRNAs; while upregulated following wild-type infection, the expression of both miRNAs was decreased following infection with both mutant strains. Furthermore, we observed significant downregulation of let-7a1 by both mutant strains without significant differences compared to expression following wild-type infection (Figure 1).

There was no significant difference in expression of miR-146b, miR-16, let-7a1 and miR-145 between the Δ*hly* and Δ*inl*AB strains (Figure 1).

**Figure 1.** Measurement of miRNA candidates in infected Caco-2 cells compared to uninfected Caco-2 cells at 1 h post infection with *L. monocytogenes* EGD-e wild-type, Δ*hly* or Δ*inl*AB. Error bars indicate standard deviations. # significant difference compared to control (*p*-value < 0.05). ## significant difference compared with wild-type infection (*p*-value < 0.05), ### no significant difference compared with wild-type infection (*p*-value > 0.05).




### *3.2. Wild-Type and LLO-Deficient Bacteria Induce miR-155, While the ΔinlAB Mutant Strain Suppresses miR-155 Expression*

miR-155 is one of the best characterized miRNAs and is involved in innate immune response to a variety of pathogens, including but not limited to *H. pylori*, *P. syringae* and *Salmonella*. We show that *L. monocytogenes* induces strong miR-155 expression in Caco-2 cells (Figure 1). Strikingly, infection with Δ*hly* also provoked a comparable induction of miR-155. In contrast, Δ*inl*AB not only lacked the ability to induce of miR-155, but significantly downregulated miR-155 compared to wild-type infection and control (Figure 1).

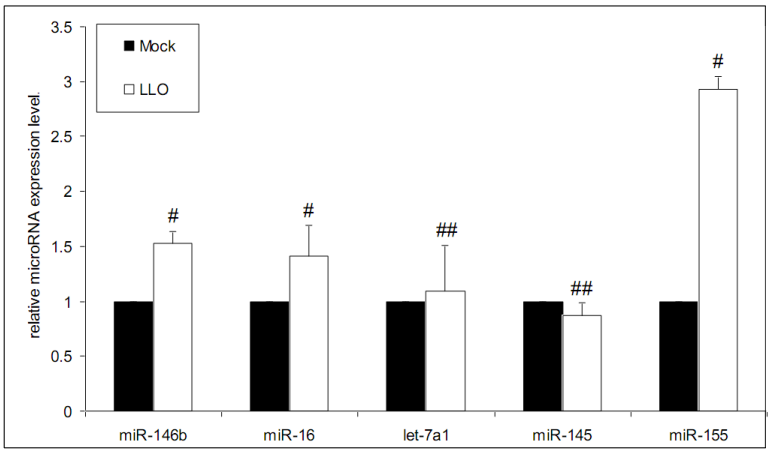
### *3.3. Purified LLO Induces the Expression of miR-146b, miR-16 and miR-155 in Caco-2 Cells*

In a further step, we sought to investigate the regulation of the above studied miRNAs after incubation with purified LLO. Expression of three miRNAs, miR-146b, miR-16 and miR-155 was significantly increased in infected cells compared to non-infected controls (Figure 2A).

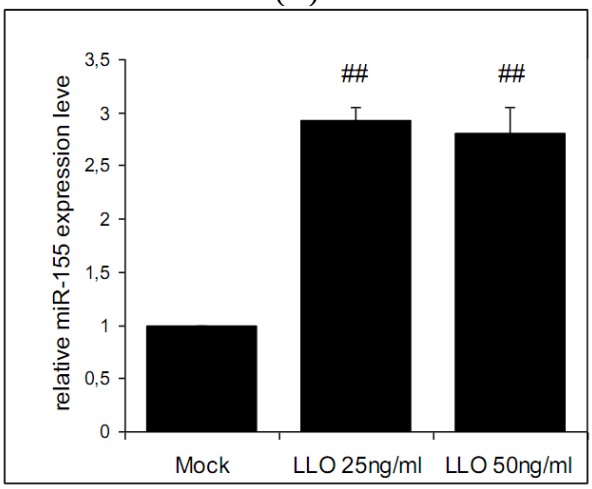
Strikingly, miR-146b displayed an inverted expression pattern compared to Δ*hly* infection indicating that expression of miR-146b is directly connected to the presence of LLO. While unchanged after Δ*hly* infection compared to control, miR-16 is seen upregulated after LLO incubation emphasizing the importance of this effector protein in miRNA regulation induced by *L. monocytogenes*. In contrast, induction of miR-155 expression was comparable in both settings, following infection with Δ*hly* strains as well as LLO incubation. To quantify the effect of higher doses of LLO on the magnitude of miR-155 induction we used a higher toxin dose. We observed no significant changes in miR-155 expression between both LLO toxin concentrations (Figure 2B).

In contrast to the changes seen in miR-155 expression, miR-145 and let-7a1 expression showed no significant deregulation in Caco-2 cells incubated with LLO (Figure 2A).

**Figure 2.** Deregulation of miRNAs following incubation with LLO. (**A**) The miRNA profile obtained from Caco-2 cells 1 h post infection for *L. monocytogenes* EGD-e wild-type was compared to Caco-2 cells treated with purified listeriolysin (LLO) for 1 h; # significant difference compared to control (*p*-value < 0.05), (**B**) miR-155 expression following incubation of Caco-2 cells with 25 ng/mL and 50 ng/mL LLO. Error bars indicate standard deviations. ## significant difference compared to control (*p*-value < 0.05), but no difference between different LLO concentrations ($p > 0.05$).




(**A**)




(**B**)

### *3.4. Deregulation of mRNAs That Are Targeted by miRNAs*

To estimate the correlation between miRNA deregulation and downstream effects on target mRNA of particular miRNAs we measured mRNA expression levels of important targets of these miRNAs. These include major inflammatory cytokines and interleukins such as IL-6, IL-8, TNF-α, IFN-β (Figure 3 and Table 1).

**Figure 3.** Conformation of immune response target genes by real time PCR analysis. Real time PCR analysis of immune response target genes was performed from uninfected Caco-2 cells compared to infected Caco-2 cells at 1 h post infection for *L. monocytogenes* EGD-e wild-type, Δ*hly* and Δ*inl*AB. Error bars indicate standard deviations. # significant difference compared to control ($p$-value < 0.05). ## significant difference compared with wild-type infection ($p$-value < 0.05), ### no significant difference compared with wild-type infection ($p$-value > 0.05).

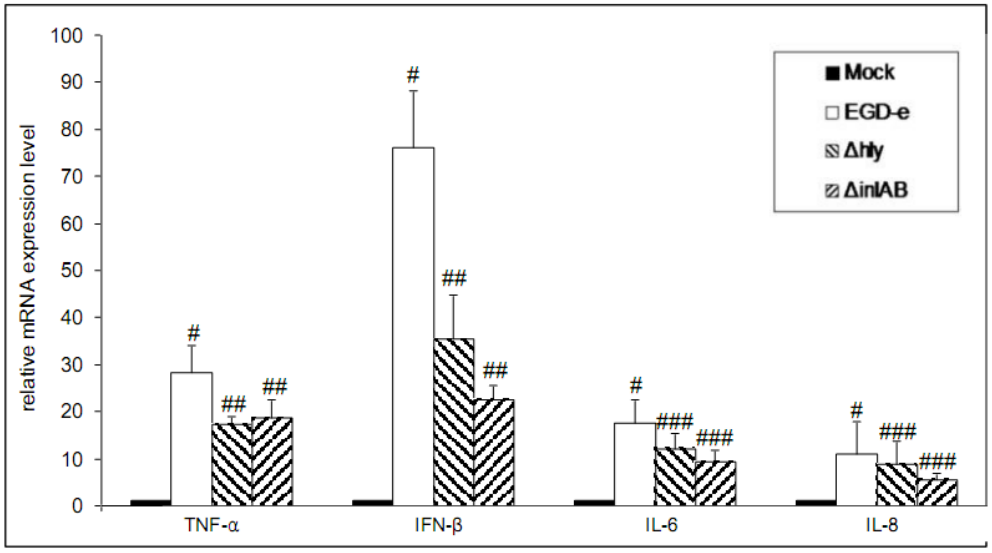



**Table 1.** Comparison of fold changes of candidate miRNAs that were identified using miRNA microarrays and validated by qRT-PCR. and target mRNAs of each miRNA. The fold changes were display the relative miRNA expression in infected Caco-2 cells 1 h following infection with *L. monocytogenes* and control cells.

| microRNA | FC microarray | FC qRT-PCR | target mRNA | Reference |
|---|---|---|---|---|
| miR-146b | 1.43 | 1.28 | IL-8, IL-6 | [16] |
| miR-16 | 0.64 | 1.65 | TNF-α, IL-6, IL-8 | [17] |
| let-7a1 | 0.63 | 0.72 | IL-10, IL-6 | [5] |
| miR-145 | 0.39 | 0.39 | IFN-β | [18] |
| miR-155 | 1.783 | 2.92 | TNF-α, IFN-β | [19,20] |

In concordance with miRNA deregulation, there are significant changes of target mRNA levels in Caco-2 cells infected with wild-type and Δ*hly* or Δ*inl*AB mutant strains compared to control cells.

## 4. Discussion

In this study we demonstrate for the first time that *L. monocytogenes* mediates differential deregulation of miRNAs in the human epithelial cell line Caco-2. Using wild-type bacteria, two isogenic mutants Δ*hly* and Δ*inl*AB, and purified toxin we show that listeriolysin and internalins are involved in miRNA expression and regulation of the putative target transcripts. miRNA microarrays were used to screen and select a subset of miRNA candidates that were significantly deregulated and

have biologically validated roles in host response to external stimuli. These miRNAs, including miR-16, miR-145, mir146, miR-155 and let-7a1 were further investigated.

miR-16 is required for the rapid degradation of inflammatory mediators that contain AU-rich sequences, such as TNF-α, IL-6 and IL-8. Interestingly, miR-16 was previously reported to be upregulated in NIH 3T3 cells infected with murine gammaherpesvirus 68, a virus closely related to Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV) [21]. Activation of miR-16 gene was also observed in cholangiocytes in a p65-independent manner by *Cryptosporidium parvum*, a protozoan parasite that infects the gastrointestinal epithelium [22]. We observed a significant upregulation of miR-16 by wild-type bacteria and purified LLO, while absence of *hly* and *inl*AB resulted in significantly decreased expression of miR-16. Other studies have shown that miR-16 expression is stable among a variety of cell lines and expression is not altered by a variety of immune modulators. The observed toxin mediated induction of miR-16 and subsequent targeting of inflammatory mediators may therefore represent a targeted miRNA mediated mechanism of immunmodulation triggered by *L. monocytogenes* rather than an unspecific host cell response to infection [17,23,24].

miRNA expression profiling in human macrophages has shown that miR-146 and miR-155 are endotoxin-responsive genes that are involved in several immune and inflammatory pathways [25,26]. A recent study revealed that miR-146b upregulation leads to inhibition of *H. pylori* induced inflammatory response in human gastric epithelial cells. miR-146b was shown to inhibit IL-8 expression, possibly through interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), two major adaptor molecules in TLR receptor signaling and NF-kB activation [27]. Thus miR-146b is a potent target to aim in order to manipulate host response. We show that miR-146b is mainly induced in a LLO-dependent manner during infection with *L. monocytogenes* and emphasize the central role of LLO the regulation of host miRNA. Caco-2 cells express TLR2 and TLR4 [28], two cell surface receptors that are targeted by listerial virulence factors including LLO. Thus, we suggest that *Listeria* induced miR-146b induction and subsequent target gene interaction may be triggered by LLO via a TLR-mediated pathway.

miR-155 has an established regulatory role in several pathways of innate and adaptive immune response [26]. Our results show that wild-type bacteria and purified LLO at two different doses induce miR-155 expression to a similar extent. However, upregulation of miR-155 also occurs following incubation with the LLO deficient mutant strain indicating that this induction is also triggered through a vacuole-dependent pathway. This process is possibly mediated by MyD88, since vacuolar signaling and subsequent expression regulation in listerial infection is entirely dependent on this adaptor molecule [29]. MyD88 also integrates TLR-signaling triggered by extracellular stimuli, such as LLO incubation. We conclude that miR-155 induction may be triggered through both LLO-dependent and an LLO-independent vacuolar mediated pathway. Both routes may merge in a common pathway that results in a comparable miR-155 induction as observed in this study.

Interestingly, the expression of miR-155 was strongly reduced following infection with Δ*inl*AB compared to wild-type bacteria or Δ*hly*. Thus, we suggest a new functional role for internalins in the regulation of miR-155 that subsequently results in increased degradation of the pro-inflammatory response mediated by TNF-α.

A recent study investigated the role of miR-145 in the inflammatory response in human colonic tissue of patients with ulcerative colitis [30]. miR-145 was strongly upregulated in inflamed colon segments of affected subjects who are at increased risk to develop colon cancer. A further study demonstrated that blocking miR-145 led to a strong anti-inflammatory response and reduced airway hyper responsiveness [15]. Thus, downregulation of miR-145 by *L. monocytogenes* as observed in this study may serve as a further mechanism of diminishing host immune response and facilitate survival of the pathogen. Furthermore, miR-145 was predicted to target IFN-β [18], a type I interferon that exhibits inflammatory and anti-inflammatory effects upon infection with *L. monocytogenes*. In line with miR-145 downregulation, IFN-β was strongly upregulated upon infection of Caco-2 cells indicating a possible contribution of miR-145 in its regulation, although it did not reach statistical significance.

Previous reports implicated miR-145 in the release of intestinal mucus components such as mucin (e.g., MUC1 or MUC2) that mediate an exocytosis mechanism leading to decreased uptake of *L. monocytogenes* into epithelial cells. *L. monocytogenes* was shown to counteract this mechanism via binding MUC2 by InlB, InlC and InlJ [31]. It is known that miR-145 controls the suppression of MUC1 causing a reduction of β-catenin, as well as the oncogenic cadherin 11 [32]. Thus, downregulation of miR-145 by the host cell results in decreased bacterial uptake. Overall miR-145 has a complex role in response to infection with *L. monocytogenes* and warrants further study.

Recently, downregulation of let-7 family members was identified as control major regulators of inflammation, including IL-6 and IL-10 in macrophages and HeLa cells upon infection with *Salmonella* [5]. We observed a similar regulation in Caco-2 cells following *Listeria* infection suggesting an analogous role of this host miRNA in Gram-positive and Gram-negative pathogens.

## 5. Conclusion

The results presented in this study contribute to our understanding of the host miRNA response induced by *L. monocytogenes* in intestinal epithelial cells. We show that (i) *L. monocytogenes* induces significant deregulation of miRNAs; (ii) major virulence determinants such as listeriolysin and internalins are involved in the regulation of a miRNA repertoire; and (iii) miRNAs interference may contribute to the post-transcriptional regulation of genes involved in the immune response to Gram-positive bacteria. Further studies are required to understand the mechanistic aspects of miRNA-mRNA interactions in the context of infections with Gram-positive pathogens. miRNAs may further expand our view on the role of non-coding RNAs as "effector-RNAs" within the eukaryotic host and represent a new target in the development of anti-microbial drugs.

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## Conflict of Interest

There is no conflict of interest.

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# *Listeria monocytogenes* induces a virulence-dependent microRNA signature that regulates the immune response in *Galleria mellonella*

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## Abstract

microRNAs (miRNAs) coordinate several physiologic and pathologic processes by regulating the fate of mRNAs. Studies conducted *in vitro* indicate a role of microRNAs in the control of host-microbe interactions. However, there is limited understanding of miRNA functions in *in vivo* models of bacterial infections. In this study, we systematically explored changes in miRNA expression levels of *Galleria mellonella* larvae (greater-wax moth), a model system that recapitulates the vertebrate innate immunity, following infection with *L. monocytogenes*. Using an insect specific miRNA microarray with more than 2000 probes, we found differential expression of 90 miRNAs (39 upregaulated and 51 downregulated) in response to infection with *L. monocytogenes*. We validated the expression of a subset of miRNAs which have mammalian homologues and known or predicted function. In contrast, non-pathogenic *L. innocua* failed to induce these miRNAs, indicating a virulence-dependent miRNA deregulation. To predict miRNA targets using established algorithms, we generated a publically available *G. mellonella* transcriptome. We identified mRNA targets of genes involved in innate immunity, signal transduction and autophagy, including spätzle, MAP kinase, and optineurin, respectively, which exhibited a virulence-specific differential expression. Finally, *in silico* estimation of minimum free energy of miRNA-mRNA duplexes of validated microRNAs and target transcripts revealed a regulatory network of the host immune response to *L. monocytogenes* . In conclusion, this study provides evidence for a role of miRNAs in the regulation of the innate immune response following bacterial infection in a simple, rapid and scalable *in vivo* model that may predict host-microbe interactions in higher vertebrates.

## Introduction

microRNAs (miRNAs) are small endogenous non-coding RNA molecules with a mature size of 22 nucleotide that regulate gene expression on a post-transcriptional level by binding the 3' UTR of their target mRNA and thereby leading to its degradation or translation inhibition[1–3]. miRNAs are involved in the control of several physiological and pathological processes, such as immunity, apoptosis, carcinogenesis, and cardiovascular diseases[4]. Recently, a number of reports described a role of miRNAs in host-pathogen interactions in models of viral and bacterial infections of a range of hosts. Various conceptual mechanisms of bacteria-mediated miRNA expression alteration in host cells were established in the last years. These include physical interaction of flagellin with a cell surface receptor[5], cell invasion[6], secretion of virulence factors and others[7,8]. Recent studies demonstrated that infection with *L. monocytogenes* alters the miRNA profile and the expression of targeted mRNAs that regulate the host immune response[9–11]. In addition, probiotic strains such as *Lactobacillus casei*, *Lactobacillus paracasei* and gut microbiota interfere with miRNA response of mice that are with orally acquired listeriosis[12,13].

The Gram-positive food-borne pathogen *L. monocytogenes* can cause listeriosis, a highly lethal systemic infection in animals and immune deficient humans[14]. The pathophysiology depends upon a variety of virulence factors that in concert result in a systemic infection of vulnerable host organisms. After consumption of contaminated food, the bacterium crosses the epithelial barrier of the gut using internalins and subsequently escapes from phagocytic vacuole by the pore forming listeriolysin and phospholipases. The greater wax moth *G. mellonella* is a powerful model system to study the pathogenesis and virulence of several microbial pathogens, including *L. monocytogenes* and for high-throughput screening of its mutants[15]. In insects, the endosymbiont bacterium *Wolbachia* has been shown to induce the expression of aae-miR-2940 in mosquitoes, which targets the metalloproteinase and cytosine methyl-transferase genes and thereby plays major role in bacterial maintenance[16]. As *G. mellonella* has been a prominent infection model organism to investigate various microbial pathogens, we took up a comprehensive study to reveal the miRNA profile and its role in immune regulation during *L. monocytogenes* infection in comparison to non-

pathogenic *L. innocua* infection. Recently, Mukherjee et al. investigated the role of miRNAs in the different developmental stages of *G. mellonella* as well as in fungal and bacterial infections, and demonstrated that miRNAs can act as mediators for trans-generational immune priming[17], highlighting the value of this *in vivo* model system.

In this study, we systematically elucidated the *in vivo* miRNA profile of *G. mellonella* larvae following infection with *L. monocytogenes* using a genome-wide insect specific miRNA microarray. Significant deregulation of a set of miRNA occurred exclusively in response to pathogenic *Listeriae* while non-pathogenic strains had little to no effect. To enable *in silico* target prediction, we generated a publically available *G. mellonella* transcriptome database. Virulence-dependent miRNAs were associated with differential expression of predicted target genes that are involved in the innate immune response and autophagy. Analysis of predicted minimum energy of miRNA-mRNA duplexes converged into a regulatory network that supports a role of miRNAs in host-microbe interactions. This study highlights the feasibility and scalability of *G. mellonella* as an *in vivo* model system to elucidate the role of miRNAs in bacterial infections.

## Materials and Methods

### Insect and bacterial growth conditions

*G. mellonella* larvae were reared on artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% bees wax, 11% honey, and 11% glycerin) at 30°C incubators before infection. Larvae in the last instar stage weighing ~150-200 mg were used for all experiments. We used 20 larvae for each experiment.

*L. monocytogenes* strain EGD-e (serotype1/2a) and *L. innocua* CLIP 11262[18] were grown aerobically in BHI broth at 37$^{o}$ C at constant shaking at 180 rpm. For infection of the larvae, the overnight bacterial culture was diluted to 1:50 and grown to mid-exponential phase ($OD_{600nm}$ =1.0) and these bacteria were washed with 0.9% NaCl twice. Each larva was injected with $1x10^{6}$ CFU bacteria and incubated at 37°C for 7 days.

## RNA isolation

On 5th day post infection, the larvae were ground well in liquid N2, dissolved in Trizol solution and centrifuged at 8000 g for 15 min at room temperature and the supernatant was collected followed by addition of 100 µl of 1-bromo-3-chloropropane (BCP reagent, Molecular Research Centre, Inc). The sample was incubated at room temperature for 5 min, 10 min on ice and centrifuged at 18000 g for 15 min at 4°C. The upper layer was transferred into fresh tube and the RNA was pelleted by adding isopropanol followed by washing with 75% ethanol. The RNA was subjected to Turbo DNase (Ambion) digestion and RNA was eluted by RNAse free water. The RNA quantity was measured with Nano Drop analyzer (NanoDrop Technology, Rockland, MA, USA) and the quality was measured by Bioanalyzer 2100 ( Agilent, Böblingen, Germany).

## miRNA microarray

To construct the insect specific miRNA microarray, we collected miRNA sequence data from the miRNA registry Database (limited to miRNAs from Arthropods) (release 18; http://www.mirbase.org/). The miRNA microarray was constructed per standard protocols by and external provider (LC Sciences, Houston USA). For each miRNA microarray, we used 2 ug of total RNA that isolated from five larvae. The assay started from 2 µg total RNA (each one consists of pooled RNA from three animals) samples which were 3'-extended with a poly (A) tail using poly adenylate polymerase. An oligonucleotide tag was then ligated to the poly (A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a Paraflo microfluidic chip using a micro-circulation pump (Atactic Technologies) [19, 20]. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide-coding segment complementary to the target miRNA (from miRBase, http://miRNA.sanger.ac.uk/sequences/) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using PGR (photo-generated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 µl 6xSSPE buffer (0.90 M

NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. After RNA hybridization, tag-conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data was analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression)[21]. For two color experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and p-values of the *t-test* were calculated; detected signals with $p<0.01$ were considered significantly differentially expressed. The total analyses of three independent experiments with log fold expression and statistical significance between control and infected larvae for each miRNA is available in Additional file1.

**Reverse transcription and quantitative real-time PCR**

First strand cDNA synthesis was done for mRNA by using Super Script II reverse transcriptase (Invitrogen) and for miRNA miScript reverse transcription kit (Qiagen) was used. For both reactions 1 µg of total RNA was used as template. Quantitative real time PCR analysis was done using the Step OnePlus Real-Time PCR System (Life Technologies). All the primers for real-time PCR were purchased from Qiagen, 18sRNA was used as endogenous control for mRNA real-time PCR and endogenous controls for miRNA real-time PCR were selected based on expression stability in both infected and non-infected larvae (dme-miR-307a-3p used as endogenous control). For mRNA quantitative real-time PCR, 100 ng of cDNA and for quantification of miRNA 5 ng per reaction was used, respectively. The list of primers used for mRNA quantification and sequences used for miRNA quantification are listed in Additional file 2. Expression levels of miRNA and their target genes were determined by normalizing its quantity to the respective expression of internal controls in *G. mellonella*. The relative expression of these target genes were measured by using mathematical model for relative quantification of real-time PCR as described previously[22].

## *G. mellonella* transcriptome database generation and target prediction

Publically available Illumina and 454 RNA-seq reads and ESTs from *G. mellonella* were retrieved from NCBI (SRR1021612, SRR1272440, ERR031115, ERR031116, ERR031117, ERR031118, ERR031119, ERR031120, ERR031121, and ERR031122). Additionally 18,690 pre-assembled contigs from an additional study[23] were included. The read quality was checked using FastQC[24] and trimmed accordingly (parameter used for Illumina reads: HEADCROP:15 ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 MAXINFO:30:0.5 MINLEN:50, parameter used for 454 reads: HEADCROP:40 SLIDINGWINDOW:10:21 MINLEN:50 CROP:200 TOPHRED33) using Trimmomatic[25]. All reads were pooled and digitally normalized using the k-mer coverage approach implemented in Trinity[26]. Multiple *de novo* assemblies were performed and the reads were assembled using the Trinity[27] assembler. The Velvet/Oases[28] assembler was applied to assemble reads including the ESTs and pre-assembled contigs using the *--conserve Long* option to preserve the EST and pre-assembled contigs. To take into account the heterogeneity of the data, multiple Velvet/Oases assemblies were computed with varying k-mer parameters ranging from 19 to 75. The sequences from all *de novo* assemblies, the ESTs and pre-assembled contigs were screened for potential coding regions with Trans Decoder. The predicted amino acid sequences were clustered using cd-hit[29] with 98% global identity. For each cluster, the sequence with the longest 3' UTR and a CDS length of at least 75% of the longest CDS in the cluster were selected as final transcripts. The transcripts were uploaded into SAMS[30] and an automatic functional annotation was performed.

For miRNA target prediction only the 3' UTR parts of the transcripts were used from the above prepared database. Target sites were predicted using miRanda[31] with *-strict* option to get only exact matching seed sequences. Using cytoscape, we created miRNA-mRNA network including target genes with known function. In addition, the minimum free energy level of miRNA-mRNA duplex structure was determined by RNAhybrid tool provided by Bielefeld Bioinformatics server[32].

## Results

### Comprehensive miRNA expression profiling *G. mellonella* during infection with *L. monocytogenes*

We used *G. mellnoella* to systematically study the *in vivo* effect of infection with *L. monocytgenes* on the miRNA expression profile and downstream effect on their corresponding targets (Figure 1A). Infection with *L. monocytogenes* resulted in illness, decreased motility and weight loss of larvae (Figure 1B), whereas non-pathogenic *L. innocua* had no effect on these parameters. The median survival of larvae infected with *L. monocytogenes,* while those infected with *L. innocua* survived at least seven days ($p<0.001$; Figure 1C). Together, these results indicated that infection of *G. mellonella* with either pathogenic (*L. monocytogenes*) or non-pathogenic (*L. innocua*) bacteria adequately reflected pathogenicity as observed in vertebrate *in vivo* models. To comprehensively examine the *in vivo* effect on the transcriptional profile of miRNAs induced by *L. monocytogens*, we isolated total RNA from *G. mellonella* larvae on the 5$^{th}$ day post infection and used a miRNA microarray. The microarray chip contains 2064 unique probes from different model insects such as *Bombyx mori* (559), *Drosophila melanogaster* (1539), *Tribolium castaneum* (394), *Apis melifera* (168) and *Acrythosiphon pisum* (103). The RNA samples of three different experiments were tested for a total of 2064 miRNAs. Compared to controls (saline injection) infection with *L. monocytogenes* resulted in alterations of signal intensities of 919 miRNAs, of which 90(39 upregulated, 51 downregulated) were significantly deregulated ($p<0.01$; Figure 2; Additional files 3&4). It is notable, that a subset of miRNAs represented on the microarray is conserved between insect species and was therefore measured multiple times.

### *In vivo* deregulation of miRNAs occurs in a virulence-dependent fashion

We next used q-RT-PCR to validate a subset of miRNAs that were significantly deregulated, have homologues in human and/or mouse[33] and a known function *in vivo*[9,34]. The expression levels of miRNAs dme-miR-133-3p, dme-miR-998-3p, dme-miR-954-5p and bmo-miR-3000 measured by quantitative PCR were in excellent agreement

with the microarray results ($R^2$>0.99) (Additional file 6). miRNAs dme-miR-133-3p, dme-miR-998-3p were significantly downregulated, whereas dme-miR-954-5p and bmo-miR-3000 were significantly upregulated upon infection with *L. monocytogenes*. We recently showed that in an *in vitro* model, miRNA deregulation depends on virulence-defining factors of *L. monocytogenes*[10]. Another report showed that in *G. mellonella* that expression of miR-263a was reciprocally regulated comparing entomopathogen *Serratia entomophila* and non-pathogenic *E. coli*[15]. We therefore wished to investigate differential regulation of miRNAs after infection with pathogenic *L. monocytogens* and non-pathogenic *L. innocua,* which lack *Listeria* pathogenicity island 1 (LIPI-1)[35]. Indeed, we found that *L. innocua* failed to deregulate dme-miR-133-3p, dme-miR-998-3p, dme-miR-954-5p and induced reciprocal regulation of bmo-miR-3000, which was downregulated when compared to *L. monocytogens* infection (Figure 3).

## Generation of a publically available annotated *G. mellonella* transcriptome database for miRNA target mRNA prediction

We next sought to predict putative targets of the significantly deregulated and validated miRNAs. We therefore generated a *G. mellonella* transcriptome by collecting the ESTs of *G. mellonella* RNA-seq (454 and Illumina sequencing) from NCBI along with 18,690 pre-assembled contigs from Vogel *et al.,*[23]. After quality trimming and normalization, a total of 25,196,088 RNA-seq reads, 12,057 ESTs and 18,690 contigs were used for the assembly (see Methods). The Trinity assembler produced 60,288 sequences. The number of assembled sequences of the Velvet/Oases assembly ranged from 125,562 (k-mer = 19) to 33,860 (k-mer = 75). In total 1,909,841 sequences were screened for coding regions. About 36 % (692,004 transcripts) of the sequences contained potential CDS. Clustering of the protein sequences produced 34,404 clusters. With the automatic functional annotation of the filtered cluster sequences, using different databases 60% (20,926) of the sequences could be annotated. For this purpose, we performed blastp searches in KEGG[36], COG[37], Swissprot[38], InterProScan[39], HMMER[40] and searched against Pfam[41]. The transcriptome database is available as a public SAMS project under

the following URL: https://www.uni-giessen.de/fbz/fb08/bioinformatik/Research/Supplements/galleria.

## *In silico* prediction of miRNA targets and stability of miRNA/mRNA duplexes indicate a virulence-dependent regulation of gene transcripts of the innate immunity

A histogram of the predicted 3' UTR lengths is shown in Additional file 7. About 64.7% (22,265) of the sequences could be assembled with potential 3' UTR. *In silico* miRNA target prediction for 4 miRNAs (dme-miR-954-5p, bmo-miR-3000, dme-miR-998-3p and dme-miR-133-3p) with those 3' UTR sequences provided 1,822 potential targets. The total list of target genes along with their corresponding gene ontology is summarized in the Additional file 5. From the list of targets we selected those that have known or predicted functions in host defense system against bacterial infections and visualized them in a miRNA-mRNA regulatory network using cytoscape (Figure 4). For example, bmo-miR-3000 was predicted to target chitotriosidase-1 and cytochrome P450 6B4 and cytochrome P450 4g1 was identified as a putative target of dme-miR-954-5p. Another remarkable putative target, optineurin was predicted hybridizing with both dme-miR-133-3p and dme-miR-998-3p. dme-miR-133-3p is a putatively targets MAP kinase transcripts and spätzle was found to be a target of dme-miR-998-3p. To assess the stability of predicted miRNA-mRNA interactions, we estimated minimum free energy level of miRNA-mRNA duplexes using RNAhybrid[32]. This tool provides energetically favorable sites for miRNA and its target transcript and takes into account potential intra-molecular hybridization within the target mRNA[32]. The optimal duplexes of selected miRNAs and predicted targets are shown in Figure 5.

## Validation of target transcripts supports a virulence-dependent miRNA-mediated regulation of the innate immunity in response to listerial infections

To validate the results of the *in silico* miRNA target predictions, we performed q-RT-PCR to determine the expression levels of target genes and to correlate the miRNA with corresponding mRNA responses after infection with *L. monocytogenes* and *L. innocua,*

respectively (Figure 6). Overall, downregulation of miRNAs was correlated with increased levels of the corresponding target transcripts (Figure 6). For example, we found upregulation of optineurin, spätzle and MAP-kinase was correlated with downregulation of their regulating miRNAs dme-miR-133-3p and dme-miR-998-3p. Inversely, upregulation of miRNAs was correlated with decreased levels of predicted mRNA targets. We found that increased levels of bmo-miR-3000 and dme-miR-954-3p were associated with decreased mRNA levels of Chitotriosidase-1, CYTP-450-6B4 and CYTP-450-4G1, respectively. A subset of mRNAs was predicted to be targeted by two of the examined miRNAs, such as optineurin, which is regulated by dme-miR-133-3p and dme-miR-998-3p, resulting in strongly elevated mRNA levels of this gene product. Chitotriosidase-1 is targeted by inversely transcribed dme-miR-998-3p and bmo-miR-3000. Interestingly, the level of the Chitotriosidase-1 mRNA appeared to be regulated in an integrated fashion, indicating that multiple miRNAs may be involved in the fine tuning of the same target transcript. We also found discordant regulation of one miRNA (dme-miR-133-3p) and its target transcript (lysozyme2). It is possible, that an unmeasured miRNA may regulate this target transcript in an integrated fashion as observed for Chitotriosidase-1. We next examined the transcriptional output of these target genes following infection with *L. innocua*. Consistent with the absence of significant deregulation of corresponding miRNAs, we did not find alterations in expression levels of spätzle or optineurin. Furthermore, lysozyme2 was upregulated despite the absence of direct regulation by miRNAs, further supporting the previous observation that this gene product may be regulated by multiple miRNAs that were unmeasured in this experiment. Overall, we find good concordance between predicted miRNA/mRNA interactions and supporting evidence for a virulence-dependent miRNA-mediated mRNA regulation in bacterial infections.

## Discussion

In this study, we used an insect wide microarray containing 2064 probes to systematically examine the *in vivo* miRNAs expression profiles in the greater wax moth *G. mellonella* following infection with Gram-positive bacteria. We measured and

validated significant deregulation of several miRNAs that occurred upon infection with pathogenic *L. monocytogenes,* but not with non-pathogenic *L. innocua*. To predict putative targets of these miRNAs, we compiled a *G. mellonella* transcriptome. We estimated the energetic miRNA/mRNA duplexes and validated target transcripts derived from the prediction analysis. Consistent with previous studies, our results indicate a specific virulence-dependent induction of miRNAs that occurred upon infection with *L. monocytogenes* but not in response to non-pathogenic *L. innocua*.

Infection of *G. mellonella* with *L. monocytogenes* induced upregulation of 39 and downregulation of 51 miRNAs. These findings have been validated for four selected miRNA (dme-miR-133-3p, dme-miR-998-3p, dme-miR-954-5p and bmo-miR-3000) using quantitative real-time PCR. A sequence homology study of known miRNA between *C. elegans*, *D. melanogaster* and human showed significant conservation of miRNAs indicating that miRNAs dme-miR-998-3p and dme-miR-133-3p are conserved as miR-29 and miR-133 in higher animals, respectively[33]. Both miRNAs and their respective homologues have been implicated in the response to infection and inflammation. Ma et al. showed that infection of NK cells and T cells with *L. monocytogenes and Mycobacterium bovis* led to downregulation of miR-29 which targets IFN-$\gamma$[9]. Chronic infection with *Helicobacter pylori* led to downregulation of tissue specific miR-133 miRNA, increased expression of acute phase proteins[34]. In line with these studies, we observed significant downregulation of miR-998 and increased levels of its targets spätzle and optineurin, indicating a conserved role of this miRNA and its homologs in the response to bacterial infections.

In previous work, we investigated the role of different miRNAs during infection of *L. monocytogenes* in the epithelial Caco-2 cell line and showed that expression of miR-16 and miR-146b depends on major virulence factors such as thiol activated toxin hemolysin (listerolysin) and internalins, a family of proteins that detemrine the ability to adhere and invade specific target cells[10]. Subsequently, induction of miRNA deregulation by several pathogenic bacteria via virulence-factor dependent mechanisms has been shown in studies investigating infections with *Staphylococcus epidermidis*, *Salmonella typhimurium* and *Yersinia pseudotuberculosis*[42]*.* Concordantly, our current study further supports the concept that miRNA deregulation is specific to the virulence of

*L. monocytogens* rather than a non-specific response to bacteria, including non-pathogenic *L. innocua*. In detail, miRNAs dme-miR-954 and bmo-miR-3000 were upregulated whereas miR-133 and miR-998 were downregulated following a pathogenic *L. monocytogenes* infection. In response to *L. innocua* infection, no significant change of these miRNA expression levels was measured, except for bmo-miR-3000 which indeed exhibited an inverse expression profile. Together, these findings and previous observations strongly support a concept of virulence-dependent miRNA regulation during host-microbe interactions.

We next sought to investigate potential biological implications of differentially deregulated miRNAs. To predict the target genes of aforementioned miRNAs, we have established a publically available database from all ESTs published in NCBI that were expressed under different stress responses in *G. mellonella*. Using miRanda[31], we predicted putative targets for above mentioned miRNAs, validated these by q-RT-PCR and calculated the minimum free energy levels between mRNA-miRNA duplexes using the RNAhybrid tool[32].

Host invasion by pathogens leads to activation of a number of signaling pathways of the innate immune response. In insects, for example, Gram-positive peptidoglycans and fungal glucans are recognized by an endogenous ligand of the toll pathway known as spätzle[43]. Activation of the toll pathway results in the synthesis of antimicrobial peptides to battle pathogens[43]. In addition, several bacterial effector proteins are able to trigger the MAP kinase signaling pathway, which is pivotal in the innate and adaptive immunity of higher animals. *L. monocytogenes* activates MAP kinase by attaching to the cell surface of epithelial cells[44]. In insects, MAP kinases are involved in the activation of prophenoloxidase, in turn which induces phagocytosis and melanization of hemocytes[45]. Here, we detected upregulation of spätzle and MAP kinase, putative targets of downregulated miR-998 and miR-133, respectively, after *L. monocytogenes* infection. The concordance of miRNA/mRNA regulation indicates a role of this circuit in the activation of signaling pathways, synthesis of AMPs and the defense response of larvae against listerial infection. Optineurin is a receptor for autophagy and plays a major role in removal of intracellular bacteria[46]. In agreement with previously described roles, we observed strong induction of optineurin, which is correlated with decreased expression

of two regulatory miRNAs (miR-133 and miR-998). Together, this interaction is predicted to facilitate increased clearance of intracellularly localized pathogens by autophagy. The induction of these pathways again appears to be virulence-dependent, since we observed no deregulation of these miRNAs and their targets transcripts upon infection with *L. innocua,* with the exception MAP kinase which was upregulated to lesser extent. In contrast, clearance of *L. innocua* might be correlated with increased expression of lysozyme2, which was exclusively upregulated in this setting and downregulated in *L. monocytogens* infection. Similarly, chitotriosidase-1, the best characterized chitinase in mammals, is induced by pro-inflammatory cytokines, such as TNF-$\alpha$ and GM-CSF in in bacterial and fungal pathogens[47]. It is possible that repression of lysozyme2 and chitotriosidase-1 represents mechanisms of evading the host-response by pathogenic bacteria, while non-pathogenic pathogens, such as *L. innoc*ua are efficiently cleared via lysozyme2 acticvity. Indeed, we observed evidence that bmo-miR-3000 may actively be involved in this process, as we observed upregulation during infection with *L. monocytogenes* and downregulation upon infection with *L. innocu*a.

Infection with *L. monocytogenes* induced increased expression of miR-954 and miR-3000 and corresponding reduced expression levels of cytochrome P450 6B4 and cytochrome P450 4g1, respectively, while non-pathogenic *L. innocua* had no effects on these miRNA/mRNA pairs. Xenobiotic enzymes play major role in toxin and drug metabolism in multicellular organisms. Cytochrome P450 enzyme showed reduced expression upon infection with *L. monocytogenes* in mice hepatic tissue and brain, the reduction of enzymes can cause severe complication with drug metabolism[48,49]. Previous reports uncovered interactions between the xenobiotic metabolism and infection and inflammation processes induced by bacterial pathogens and other immunostimulants[50]. In juvenile carp, for example, *L. monocytogenes* reduces activities of cytochrome P450 enzymes and ethoxyresorufin O-deethylase[51]. While the precise effect of cytochrome P450 enzymes on this infection model requires further investigation, it seems plausible that downregulation of the enzymes may improve pathogen survival. This regulation may be an actively induced process by *L. monocytogenes*, but not *L. innocua*, via miRNA-mediated degradation of the corresponding target transcripts.

## Conclusion

In conclusion, this study demonstrates the feasibility of leveraging *G. mellonella* as an *in vivo* model to examine miRNA expression following infection with pathogenic and non-pathogenic bacteria. We used orthogonal approaches to determine miRNA expression, *in silico* algorithms to predict the occurence and energetic stability of miRNA-mRNA and direct validation of predicted targets. We uncover miRNA/mRNA expression patterns specific to pathogenic *L. monocytogenes* compared to non-pathogenic *L. innocua,* which revealed the role of miRNAs in regulation of immune response. Homologues of miRNAs described in this study were shown to have important roles in mammalian infection models. Thus, *G. mellonella* represents a simple and valuable *in vivo* model capable of recapitulating the roles of miRNAs in host-microbe interactions in higher animals.

## Acknowledgements

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## Additional files

**Additional file 1:** Table S1. Total microarray analysis of miRNAs with infection of *L. monocytogenes.*

**Additional file 2:** Table S2. List of primers used in this study.

**Additional file 3:** Text S1. Microarray signal intensities analysis

**Additional file 4:** Text S2. Microarray statistical analysis.

**Additional file 5:** Table S3. miRanda target prediction and transcript annotation.

**Additional file 6:** Figure S1. Correlation between the fold changes measured by qRT-PCR and microarray methods.

**Additional file 7:** Figure S2. Length distribution of the predicted 3'−UTRs.

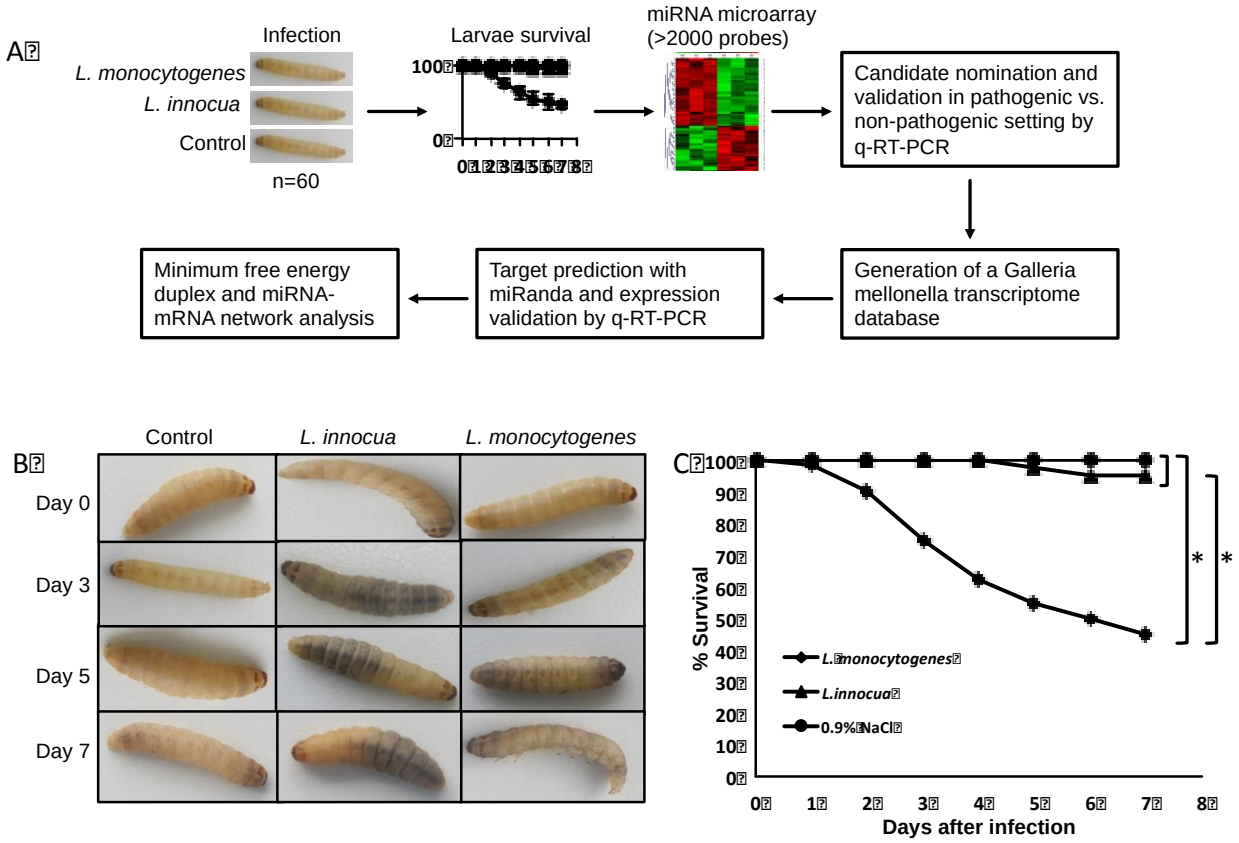



**Figure 1:** (A) Depicts the workflow taken in this study to comprehensively examine the miRNA response following infection of *G. mellonella* with pathogenic and non-pathogenic Gram-positive bacteria. (B) Macroscopic changes of *G. mellonella* larvae of infected larvae over time highlight the reduced viability and weight of parasites infected with pathogenic *L. monocytogens.* (C) Survival curves of larvae (n=60) infected with *L monocytogens*, *L. innocua* or control confirms macroscopic observations. This resulted in a median survival of six days post infection in in the *L. monocytogens* group ($p<0.001$), while *L. inncoua* did not induce significant moratlity ($p>0.05$).

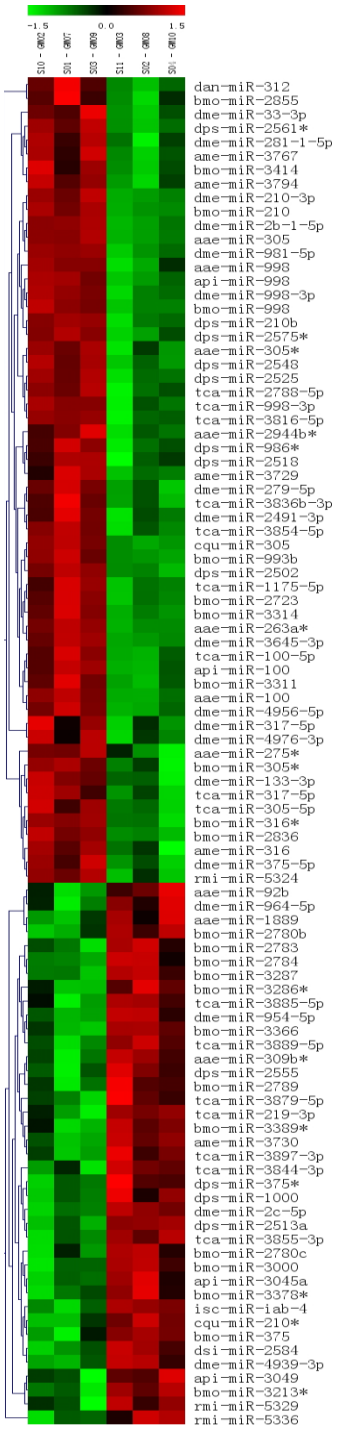



**Figure 2:** Heat map of miRNA microarray was generated between control and infected *G. mellonella*. This figure shows a set of statistically significant deregulated miRNAs upon infection with *L. monocytogenes* (p≤ 0.01). Red= increased expression; Green= reduced expression. The lines in heat map: GM02, GM07 and GM09 represent control samples; GM03, GM08 and GM10 represent infected samples.

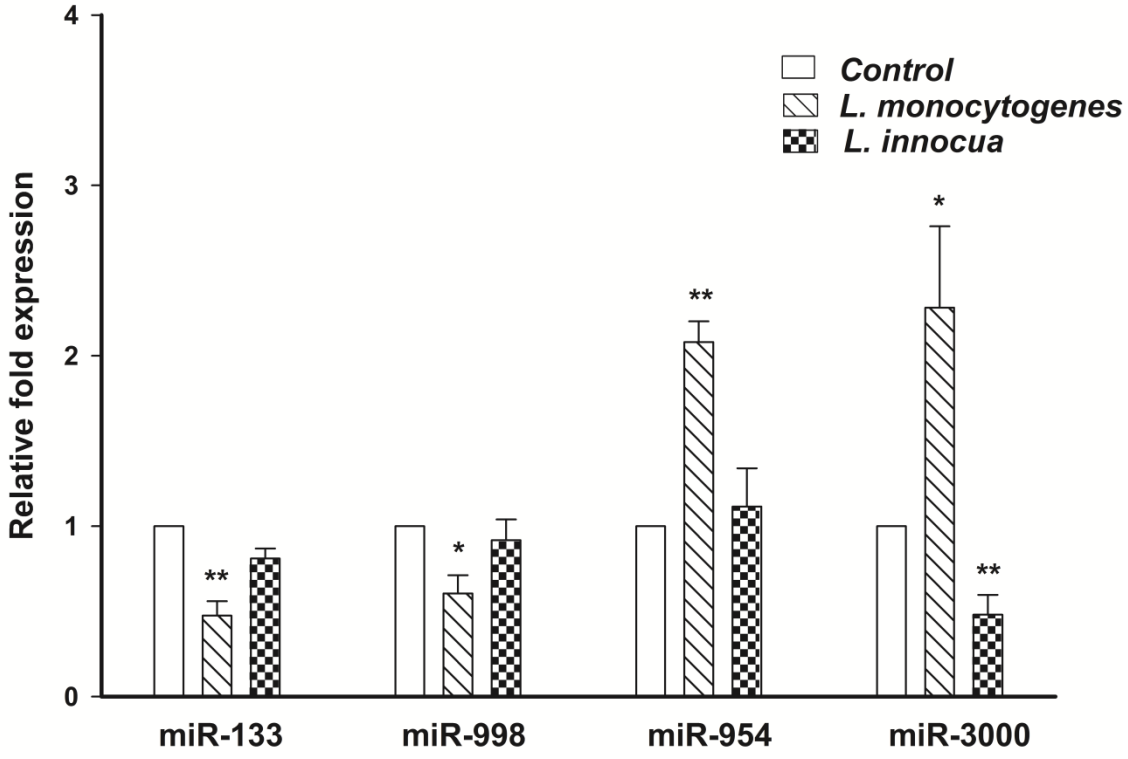
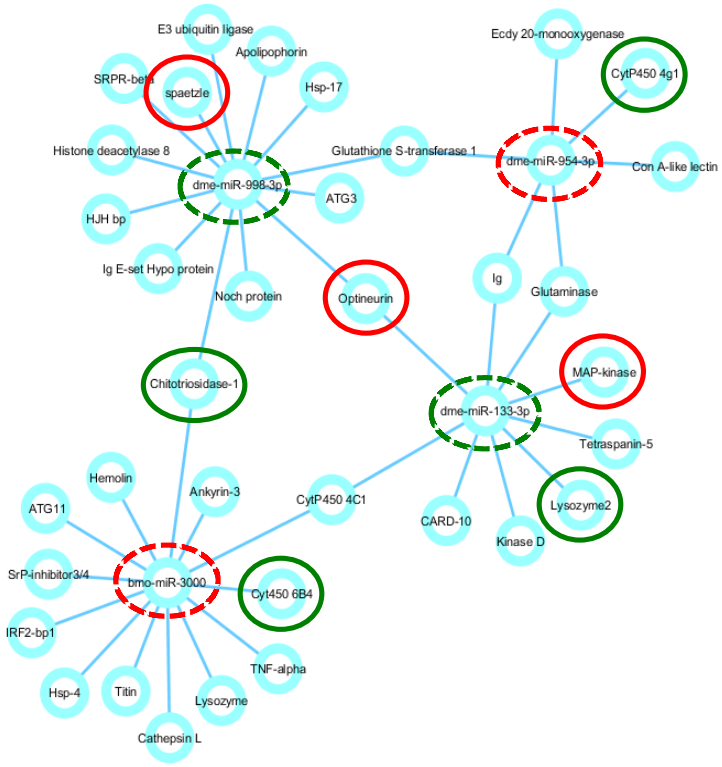



**Figure 3:** Validation of miRNA microarray analysis and patho/non-pathogenic mediated miRNA response in *G. mellonella*. In support of microarray, qRT-PCR analysis of miRNA with infection of *L. monocytogenes* showed significant reduced expression of miR-133 and miR-998 and increased expression of miR-954 and miR-3000. Upon infection with non-pathogenic *L. innocua* there is no significant changes in alteration of miRNA expression, except bmo-miR-3000 which is significantly downregulated. (`*´ p≤ 0.05; `**´ p≤ 0.01).

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587 **Figure 4:** miRNA-mRNA network shown by cytoscape: After generating a *G. mellonella*
588 reference transcriptome, we used miRanda to predict targets for validated miRNAs
589 (miR-998, miR-133, miR-954 and bmo-3000) which were implemented in this network.
590 The figure shows a network of selected targets for each miRNA. miRNAs are highlighted
591 with dashed circles and target genes, which were subsequently tested for minimum free
592 energy duplexes and validated by q-RT-PCR, are highlighted with fully closed circles.
593 Red color indicates increased expression and green color indicates reduced expression
594 of the particular miRNA or mRNA.

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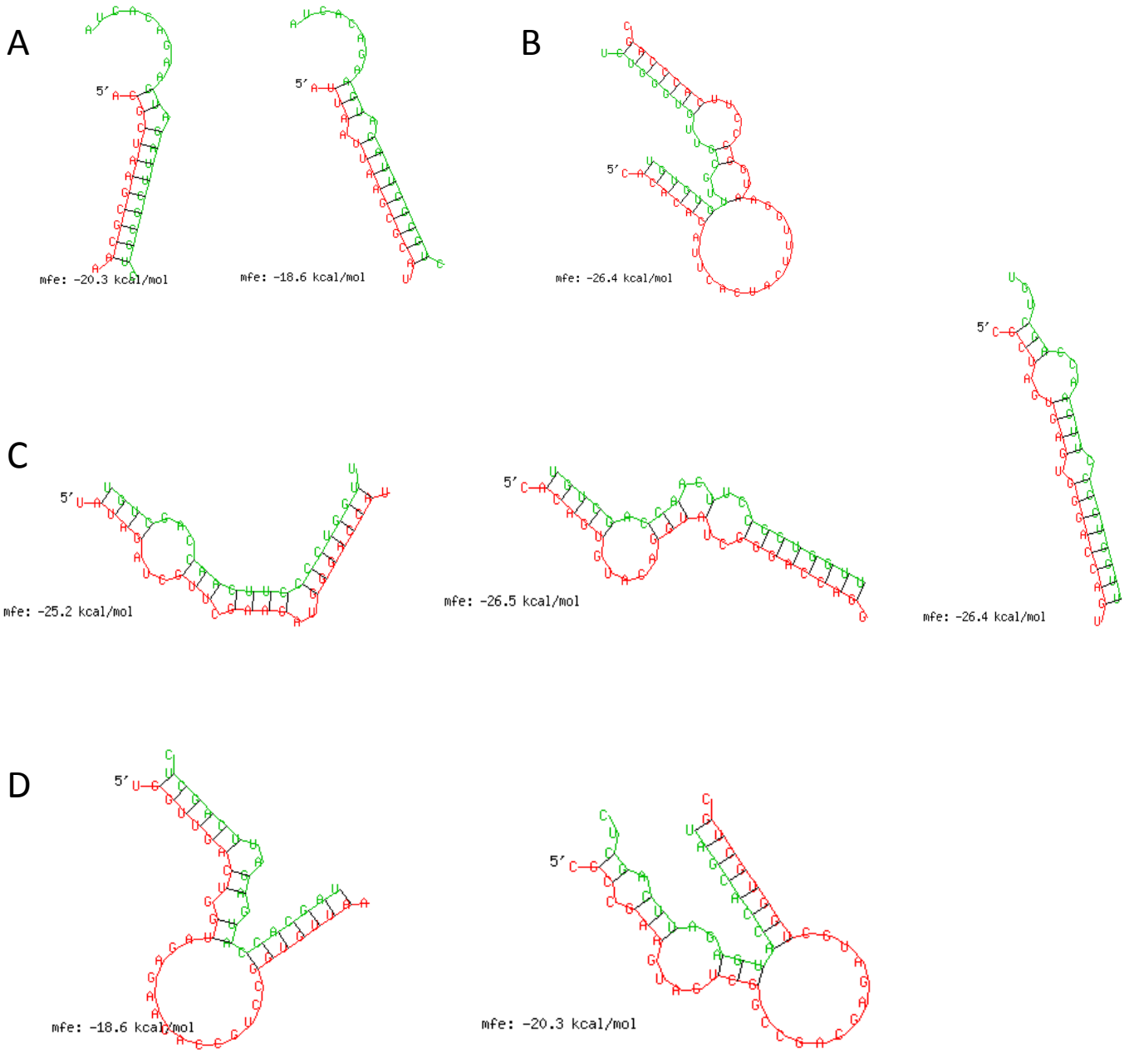


**Figure 5:** Depicts the minimum free energy duplexes of four validated microRNAs. Each figure shows the duplex of miRNAs and the 3'-UTR of target mRNAs (marked as 5') of *G. mellonella*. The alignment shows the total miRNA sequence and the seed region it hybridizes to in the target 3'-UTR. (A) Duplex of bmo-miRNA-3000 and chitotriosidase-1 (left) and cytochrome P450 6B4 (right); (B) dme-miR-954-5p cytochrome P450 4g1; (C) dme-miR-133-3p and optineurin (left), MAP Kinase (middle) and lysozyme2 (right); and (D) dme-miR-998-3p and optineurin (left) and Spätzle (right).

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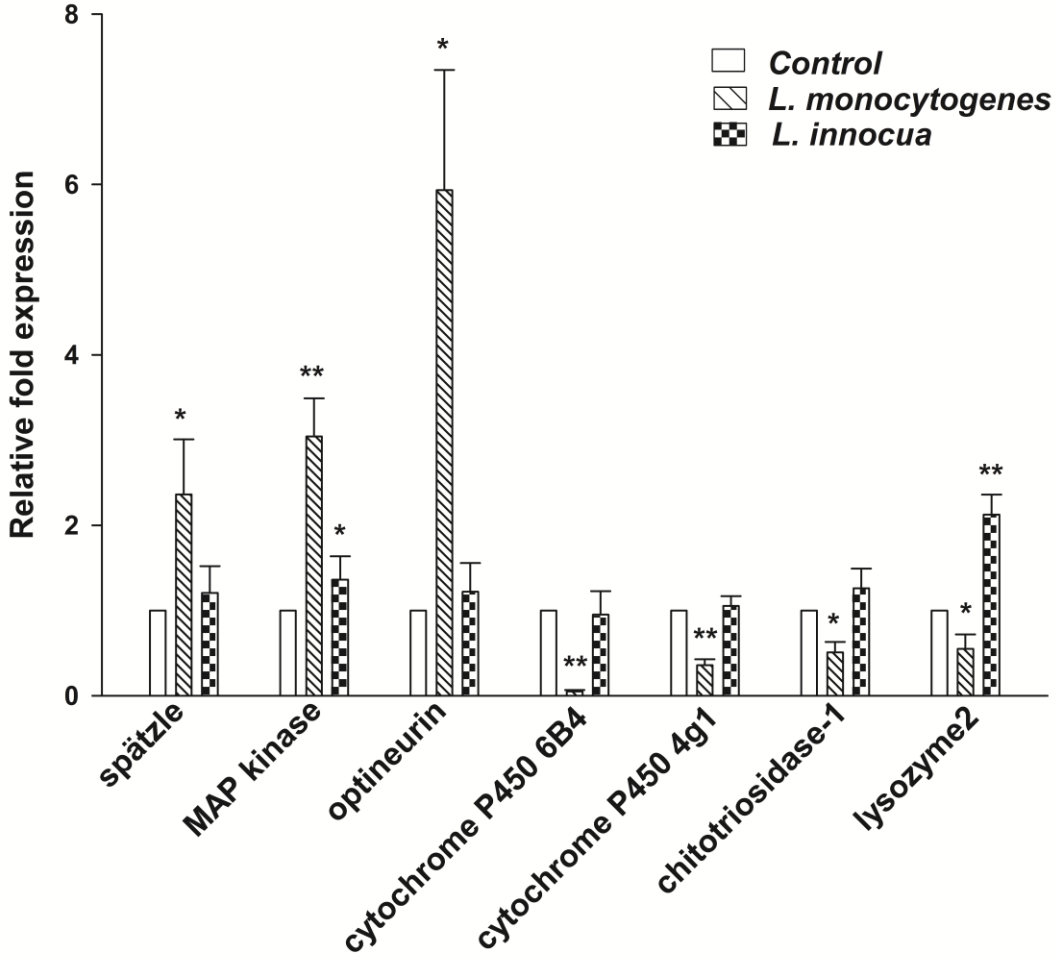


607

608 **Figure 6:** qRT-PCR analysis of predicted target genes expression upon infection with *L.*
609 *monocytogenes* and *L. innocua*. With infection of *L. monocytogenes* target genes
610 chitotriosidase-1 and lysozyme2 are showing significant reduced expression and also
611 cytochrome P450 6B4 and cytochrome P4504g1 are affected in expression. The factors
612 involved in immune signaling pathways such as spätzel and MAP kinase, and autophagy
613 receptor optineurin are significantly upregulated in *G. mellonella*. All these target genes
614 are not affected with infection of *L. innocua*, except lysozyme2 and MAP kinase which
615 are significantly upregulated (`*´ $p \leq 0.05$; `**´ $p \leq 0.01$).

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# Ultra Deep Sequencing of *Listeria monocytogenes* sRNA Transcriptome Revealed New Antisense RNAs

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## Abstract

*Listeria monocytogenes*, a gram-positive pathogen, and causative agent of listeriosis, has become a widely used model organism for intracellular infections. Recent studies have identified small non-coding RNAs (sRNAs) as important factors for regulating gene expression and pathogenicity of *L. monocytogenes*. Increased speed and reduced costs of high throughput sequencing (HTS) techniques have made RNA sequencing (RNA-Seq) the state-of-the-art method to study bacterial transcriptomes. We created a large transcriptome dataset of *L. monocytogenes* containing a total of 21 million reads, using the SOLiD sequencing technology. The dataset contained cDNA sequences generated from *L. monocytogenes* RNA collected under intracellular and extracellular condition and additionally was size fractioned into three different size ranges from <40 nt, 40–150 nt and >150 nt. We report here, the identification of nine new sRNAs candidates of *L. monocytogenes* and a reevaluation of known sRNAs of *L. monocytogenes* EGD-e. Automatic comparison to known sRNAs revealed a high recovery rate of 55%, which was increased to 90% by manual revision of the data. Moreover, thorough classification of known sRNAs shed further light on their possible biological functions. Interestingly among the newly identified sRNA candidates are antisense RNAs (asRNAs) associated to the housekeeping genes *purA*, *fumC* and *pgi* and potentially their regulation, emphasizing the significance of sRNAs for metabolic adaptation in *L. monocytogenes*.

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**Competing Interests:** Biomarker Center GmbH) which declares (formerly known as febit biomed GmbH) that CBC, has no competing interest with the manuscript entitled "Ultra deep Sequencing of Listeria monocytogenes sRNA transcriptome revealed new antisense RNAs (see also the attached letter from CBC). N. Kefer present working address is now Life Technologies as stated in the manuscript. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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## Introduction

*Listeria monocytogenes* is a Gram-positive, facultative intracellular pathogen, which is responsible for a foodborne infection, listeriosis, a rare but serious disease. It has become the prime model organism for intracellular pathogens [1]. Small non coding RNAs (sRNAs) have been proposed to play an important role in the pathogenicity of *L. monocytogenes* and some lead to attenuated infections when disabled [2,3]. These studies also showed that antisense transcription is common in *L. monocytogenes* [2,3]. Beside short antisense RNAs (asRNAs), a new type of long antisense RNAs (lasRNAs) functioning as an mRNA as well as antisense RNA that regulate adjacent genes at the level of transcription, was proposed [4].

Over the last decade reduced costs for high throughput sequencing (HTS) technologies facilitate the thorough and unbiased research of bacterial transcriptomes at an ever increasing rate [5–7]. As a result, identification of small non coding RNAs in all bacterial species have been reported [8–11]. Large numbers of small non coding RNAs have been found in both Gram-negative [12,13] and Gram-positive [14,15] bacteria. In particular *L. monocytogenes* has been subject to an extensive number of transcriptome studies using macro-/microarrays, Illumina GAIIx or Roche GS FLX sequencing platforms [2–4,16–20]. The SOLiD sequencing platform used in this study, provides a very high throughput sequencing method with increased base calling accuracy due to its unique 'color coded' di-base sequencing technique [21].

Here we report the thorough reevaluation of the small RNA transcriptome of *L. monocytogenes* with increased coverage. A large HTS transcriptome dataset containing transcriptomic data of *L. monocytogenes* grown under intracellular and extracellular conditions was the basis of this study. The transcriptomic data was generated using the SOLiD HTS platform and consists of a total of 21 million reads. In this study a newly developed computational pipeline was used to identify and classify sRNAs. Furthermore, this computational pipeline leads to the discovery of nine yet unknown small non coding RNA candidates of *L. monocytogenes*.

## Materials and Methods

### Bacterial and cell culture and RNA extraction

The strain of *L. monocytogenes* EGD-e [22] and the murine P388D1 macrophages were used for cell infection and RNA

extraction as reported recently for this study [2]. The strain *L. monocytogenes* EGD-e used in this study was grown in brain heart infusion (BHI) broth (VWR) overnight at 37°C with shaking at 180 rpm (Unitron, Infors). Overnight cultures were diluted 1:50 in 20 ml fresh BHI broth using a 100 ml Erlenmeyer flask and were incubated at the same conditions mentioned above until mid-exponential phase ($OD_{600\ nm}$ 1.0). Bacteria were added to P388D1 murine macrophage cells monolayer at a multiplicity of infection (MOI) of ten bacteria per eukaryotic cell.

For RNA extraction from extracellularly grown *L. monocytogenes*, we used aliquots of 0.5 ml from the same bacterial culture used to infect P388D1 macrophages. The bacterial cells were treated with 1.0 ml RNA protect (Qiagen) for 5 min and were collected by centrifugation for 10 min (8000×g) and subsequently stored at −80°C until use. RNA extraction from intracellularly grown *L. monocytogenes* in macrophages, 4 h post infection, was performed as described previously [33][23]. Briefly, infected host cells were lysed using cold mix of 0.1% (wt/vol) sodium dodecyl sulfate, 1.0% (vol/vol) acidic phenol and 19% (vol/vol) ethanol in water. The bacterial pellets were collected by centrifugation for 3 min (16000×g).

Total RNA was extracted using miRNeasy kit (Qiagen) with some modifications. The collected pellets were washed with SET buffer [50 mM NaCl, 5 mM EDTA and 30 mM Tris-HCl (pH 7.0)]. After centrifugation at 16000×g for 3 min pellets were resuspended in 0.1 ml Tris-HCl (pH 6.5) containing 50 mg/ml lysozyme (Sigma), 25 U of mutanolysin (Sigma), 40 U of SUPERase (Ambion), 0.2 mg of proteinase K (Ambion) and incubated at 37°C for 30 min at 350 rpm. QIAzol (Qiagen) was added, mixed gently and incubated for 3 min at room temperature. An additional incubation at room temperature was done after adding 0.2 volume chloroform followed by centrifugation at 16000×g at 4°C for 15 min. The aqueous phase, containing RNA, was transferred to a new collection tube and 1.5 volumes of 100% ethanol was added and mixed thoroughly. The probes containing RNA were transferred into columns supplied with the miRNeasy Kit (Qiagen) and treated according to the manual including an on-column DNase digestion (RNase-Free DNase, Qiagen). RNA was eluted by RNase-free water and stored at −80°C until needed. The quantity of the isolated total RNA was determined by absorbance at 260 nm and 280 nm, and the quality was assessed using Nano-chips for Agilent's 2100 Bioanalyzer. For detection and estimation of the small RNA fraction within the isolated total RNA, a small RNA-chip (Agilent) was used, which visualizes RNAs with sizes ranging from 20 to 150 nucleotides.

## RNA sequencing

6 µg of total RNA of the intracellular and the extracellular sample was used as starting material. The quality was checked by Nanodrop and Agilent Pico RNA Chip. Both samples were prepared in parallel for all three different size ranges from <40 nt, 40–150 nt and >150 nt.

>150 nt size fractionation library preparation. 2.5 µg of total RNA of the sample was rRNA depleted using the Ribo Minus Bacteria Module (Invitrogen Corporation) and purified with the RiboMinus Concentration Module (Invitrogen Corporation) with a modified protocol to keep all RNA transcripts <200 nt. After the rRNA depletion the samples were checked on the Pico RNA Chip from Agilent showing remaining rRNA in the sample. However, due to the small starting amount the rRNA depletion couldn't be repeated. Subsequently, the RNA was treated with Tobaco-Acid-Pyrophosphatase (TAP) from epicenter ® for 1.5 h at 37°C and purified with the RiboMinus Concentration Module. Fragmentation of the RNA was done with RNaseIII (LifeTechnologies, RNA-Seq Kit) (37°C, 10 min) and again purified with the RiboMinus Concentration Module. The samples were dried with a Speed Vacuum Pump, resuspended in 3 µl of nuclease-free water and the SOLiD Adapters were ligated (65°C, 10 min; 16°C, 5 min). After ligation, mRNAs were reversely transcribed into cDNA with Array Script ™ Reverse Transcriptase (Life Technologies, RNA-Seq Kit) and purification was done with Qiagen's MinElute PCR Purification Kit, eluting in 20 µl nuclease-free water. cDNA fragments between 150 nt and 250 nt (fragmented transcripts + adaptor sequences) were isolated from a 6% TBE Urea Gel (Novex-System, Invitrogen). cDNA from gel slices was amplified with 16 PCR cycles using the same 5′-Primer for each sample and two different 3′-Primers including the barcode sequences (SOLiD Multiplexing Barcoding Kit 01-16). Purification was done with the Micro PCR Purification Kit (Invitrogen Corporation).

<40 nt and 40–150 nt size fractionation library preparation. 3.5 µg of total RNA of the sample was enriched with the flashPAGE Fractionator (Ambion) with a modified protocol (runtime 40 min) in order to enrich RNA molecules up to 150 nt. Purification was done with the flashPAGE Clean up Kit (Ambion). The samples were dried with a Speed Vacuum Pump, resuspended in 3 µl of nuclease-free water and the SOLiD Adapters were ligated (65°C, 10 min; 16°C, 5 min). After ligation, small RNAs were reverse transcribed into cDNA with Array Script™ Reverse Transcriptase, (Life Technologies, RNA-Seq Kit) and purification was done with Qiagen's MinElute PCR Purification Kit, eluting in 20 µl. Afterwards, the small RNAs (cDNA) were size-selected on a 10% TBE Urea Gel (Novex-System, Invitrogen). Different size ranges were collected from the gel (60–80 nt, 80–120 nt, 120–150 nt) and amplified with 16 PCR cycles using the same 5′-Primer for each sample and four different 3′-Primers including the barcode sequences (SOLiD Multiplexing Barcoding Kit 01-16). PCR purification was done with the Micro PCR Purification Kit (Invitrogen Corporation). A total of six purified and barcoded DNA libraries were analyzed on a HS-DNA Chip on the Agilent Bioanalyzer 2100 and subsequently pooled in equimolar amounts.

Next generation sequencing. The pooled libraries were diluted to a concentration of 60 pg/µl. DNA was amplified monoclonally on magnetic beads in an emulsion PCR. Emulsions were broken with butanol and the remaining oil was washed off the double-stranded beads. DNA molecules on the bead surface were denatured to allow hybridization to polystyrene enrichment beads. Using a glycerol cushion null beads can be separated from the templated beads. In an additional denaturation step, the templated beads were separated from the enrichment beads. The 3′-ends of the DNA molecules on the bead's surface were enzymatically modified for deposition on the sequencing slide. The beads were loaded onto a slide and sequenced on a SOLiD 3 Plus analyzer producing reads of 50 nt length.

## Data processing

To identify and characterize new candidates as well as to compare known sRNAs to our transcriptome data set we implemented a novel computational pipeline. See Fig. 1 for an overview of all processing steps. We made use of the specific data set properties including the SOLiD sequencing technique, producing short and "color coded" sequencing data and data, split into two growth conditions and three RNA size fractions. The two growth conditions representing extracellular and intracellular lifestyle of *L. monocytogenes* and the size fractions containing extracted RNA of different lengths, namely <40 nt, 40–150 nt and >150 nt. The fragmentation will allow for a fine-grained

differentiation between degradation products of large RNA molecules and independently expresses sRNAs.

Fig. 1 gives an overview over this pipeline, for a detailed description of the pipeline and the used parameters see supplementary file S1. In brief, the pipeline first maps reads onto a reference genome using a short read mapper. We compared different mapping programs for this purpose, including SHRiMP, Bfast and BWA, and performed a parameter evaluation to achieve an optimal mapping. Based on this evaluation we chose BWA as mapper with a maximum mismatch rate per read of 2. Our pipeline then utilizes annotation data as well as coverage information from different size fractions to filter the dataset and identify large RNA molecules expressed on the genome. The *L. monocytogenes* genome annotation was obtained on 28/09/2011 from NCBI RefSeq: (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Listeria_monocytogenes_EGD_e_uid61583/). Our pipeline considers reads of smaller fractions that were aligned to a region in which a larger fraction indicated a transcript as degradation products originating from the larger transcript. After masking of all known transcripts as well as degradation products, an expanding window algorithm identified putative novel sRNA candidates within the remaining transcriptome.

The pipeline also implements a number of downstream analysis tools. These include an automatic comparison tool to identify equivalent sRNAs between different size fractions, samples, or studies, enabling us to quickly compare other studies of the same organism or differential expression between experimental conditions. An automated classification system is also part of the pipeline to classify transcription start sites, asRNAs, and classical sRNAs. A last tool enables a more fine-grained statistical analysis of differential expression between two given datasets. It visualizes the data in an MA-plot and lets the user select custom thresholds depending on average expression, to fine-tune the significance of the differential expression.

The pipeline as well as the corresponding java program ncFinder are accessible at http://fileshare.csb.univie.ac.at/ncFinder_associated_files/pipeline.tgz and http://fileshare.csb.univie.ac.at/ncFinder_associated_files/ncFinder.zip respectively.

## Differential expression analysis

We used NOIseq [37] to perform a differential expression analysis. The method based on the assumption, that on average, the expression is similar between case and control. We used RPKM to normalize the data and required a p-value of $<0.1$ for a locus to be considered differentially expressed. We summarized the results in supplemental table S2.

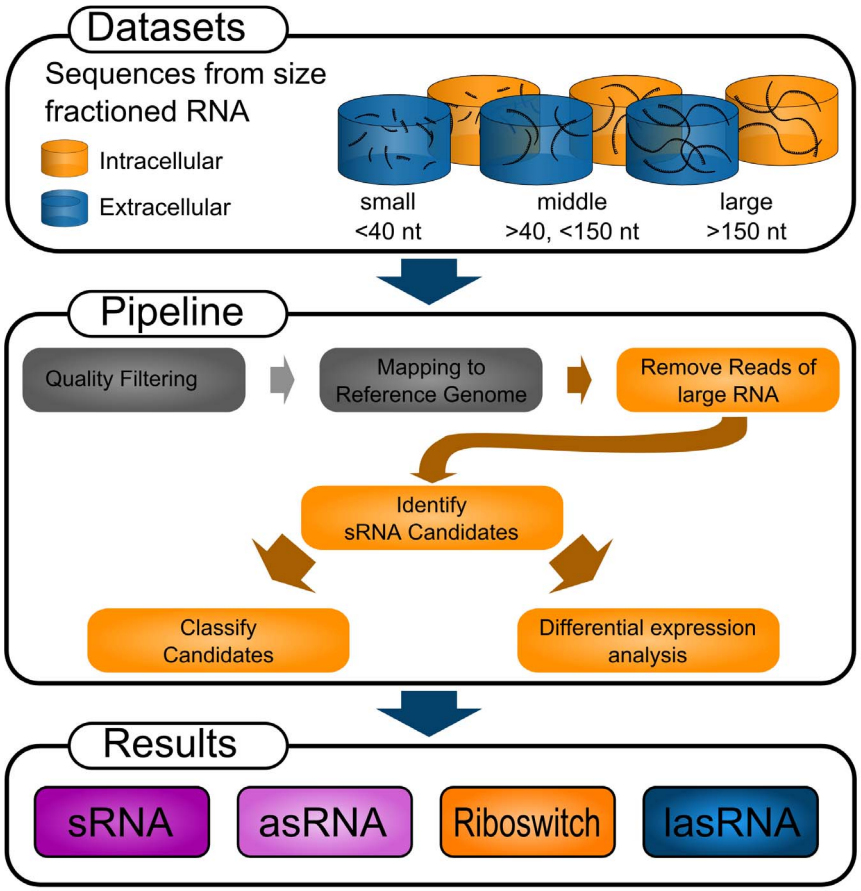


**Figure 1. Schematic representation of the main computational pipeline used in this study and its input and output.** The pipeline is optimized to work with sequence data from fractionated RNA samples containing RNA fragments of different lengths. Data gathered under various conditions can also be used for differential expression analysis. For this study we used data from the SOLiD High Throughput Sequencing (HTS) platform, but the pipeline will also process data from all major HTS platforms. The individual steps within the pipeline are colored either gray or orange representing steps for which existing software was used and newly implemented features respectively. The result of the pipeline will be lists of pre-classified sRNA candidates.
doi:10.1371/journal.pone.0083979.g001

### Conservation analysis

Mauve was used to check the conservation status of the nine sRNAs. Multiple genome alignments were calculated using default parameters for the following *Listeria* species: *Listeria monocytogenes* serovar 1/2a EGD-e (NC_003210), *Listeria innocua* CLIP11262 (NC_003212.1), *Listeria welshimeri* serovar 6b str. SLCC5334 (NC_008555.1), *Listeria seeligeri* serovar 1/2b str. SLCC3954 (NC_013891.1), *Listeria ivanovii* subsp. *ivanovii* PAM 55 (NC_016011.1) and *Listeria marthii* FSL S4-120 (NZ_CM 001047.1).

### Oligonucleotides

Oligonucleotides that were used for northern blot hybridization and qRT-PCR are listed in supplementary table S3.

### Northern blot analysis

RNA samples (~30 µg were normalized to 5S rRNA hybridization signals) were denatured for five minutes at 65°C in loading buffer containing 50% deionized formamide, separated on urea-polyacrylamide (10%) gels, and transferred to nylon membrane by electroblotting in a semi dry blotter according to the manufacturer's recommendations. Membranes were hybridized with gene-specific [$\gamma$-$^{32}$P]-end-labeled oligodeoxy-ribonucleotides [24].

### 5′end labeling of primers with [$\gamma$-$^{32}$P]ATP

DNA probes were generated by 5′-end-labelling of RNA – specific oligonucleotides with [$\gamma$-$^{32}$P] ATP which is described elsewhere [24]. All probes were purified on G25 Microspin columns (GE healthcare) and probes were further used for hybridization.

### Quantitative real-time PCR analysis

Total RNA was isolated from the *L. monocytogenes* EGD-e grown in BHI medium and macrophages as described above. RNA isolation was followed by production of strand-specific cDNA from 1 µg total RNA and SuperScript II Reverse Transcriptase (Invitrogen) by using primers designated _a (see supplementary table S3) which is complementary to the asRNA or the *lmo2673*. The generated cDNA probes were subjected to quantitative real-time PCR in a final volume of 25 µl using primers designated _b (see supplementary table S3) and QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instruction. A standard curve was generated for the used primer pairs using different copy numbers of genomic DNA from EGD-e (see supplementary table S3). For each primer pair a negative control (water), RNA sample without reverse transcriptase (to determine genomic DNA contamination) and a sample with known amount of copy numbers (to test the efficiency of the reaction) were included as controls during cDNA quantification. All samples after real-time PCR were run on a 1.5% agarose gel to verify that only a single band was produced.

### Statistical data analysis

All infection experiments for qRT-PCR and northern blots analysis were performed in a minimum of three biological experiments. Significant differences between two values were compared with a paired Student's t-test. Values were considered significantly different when the $p$ value was less than 0.05 ($p<0.05$).

### Accession number

RNA sequencing data have been deposited to EBI (http://www.ebi.ac.uk/), accession number PRJEB4644.

## Results

To investigate the transcriptome of *L. monocytogenes* RNA was extracted from bacteria grown either in BHI (extracellular growth) or in murine macrophages (intracellular growth). The RNA was then fractionated into 3 fractions with cutoffs <40 nt, 40–150 nt and >150 nt respectively to aid unambiguous differentiation between sRNA and degradation products of larger RNA molecules. Subsequently RNA extracts were sequenced using SOLiD sequencing technology. A total of 21 million reads over six sequencing runs were obtained. Reads from the fraction containing RNA <40 nt were trimmed to 30 nt length since we expected a high false sequencing error at the 3′ end of these reads. We applied quality filtering to the reads to ensure that reads which very likely contain sequencing errors are not used in further analysis. A total of 71% of reads were retained after filtering. Detailed filtering counts are listed in supplementary table S4. Application of our sRNA pipeline on the data yields a total of 711 sRNA candidates for further analysis.

### Transcription start site detection

A specific pattern, creating a large pileup of reads with identical starting positions, located shortly upstream of annotated genes and operons, was a common structure seen in our data. Fig. 2 indicates such a read pattern before the gene *dnaA*. Its location and well-defined start was a hint, that these read patterns represent the transcription start sites (TSS) of the corresponding downstream gene or operon. An alignment of 20 randomly chosen samples of putative TSS from our data with TSS data from Wurtzel and colleagues [4] was performed to verify this assumption. Unfortunately it is impossible to clearly identify TSS solely based on the data at hand. However, we consistently found our putative TSS to be within 1 nt from those described by Wurtzel and coworkers [4], confirming that these patterns indicate TSS. Furthermore, we cannot distinguish between independent sRNAs and processed TSS's. Hence we removed all sRNAs identified as possible TSS from our later analysis.

### Identification and validation of sRNAs in the sequence data

The high coverage with a total of 21 million SOLiD reads of 50 nt length enabled us to compare all of the 263 known sRNAs in *L. monocytogenes*, that were identified previously [2–4,18,20]. 142 of the 711 automatically identified sRNA candidates from this study were previously identified by three studies [2–4], as represented in Fig. 3. While these 142 (55%) known sRNAs were recovered by the automatic pipeline, a manual revision of known sRNAs specifically aiming at sRNAs, which were missed due to either the conservative coverage threshold applied or a filter discarding candidates too close to, or overlapping with annotated genes, increased the recovery rate to 90% of the previously described small RNAs in at least one of the two conditions and at least one of the 3 corresponding size fractions. When classifying the sRNAs automatically and manually according to their location and read patterns, we found 82 of the known sRNAs to represent UTRs of downstream genes rather than independently transcribed sRNAs in intergenic space. Furthermore, allowing for minor differences in size we found that most known sRNA match our findings. Notably, with all the differences between studies, there seemed to be a general consensus on the 5′ end of sRNAs, hence the transcription start site, often varying only by 1 or 2 nt, while the 3′ end and hence the transcription termination site of the same sRNA identified by different studies often varied extensively. Both, methodical limitation in the 3′ accuracy as well as biological

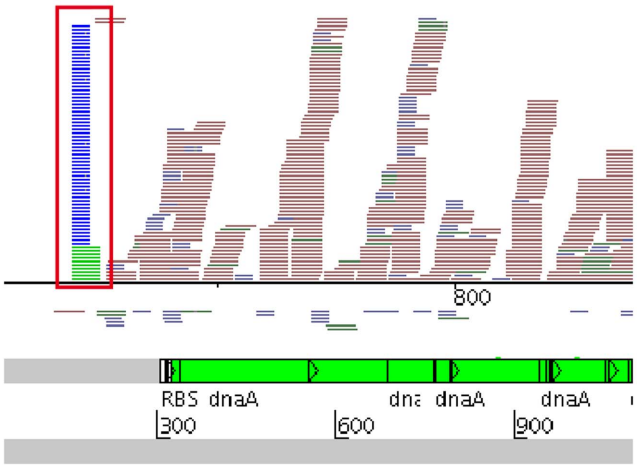


**Figure 2. Pileup of reads representing the TSS of the *dnaA* gene of *L. monocytogenes*.** Reads are mapped onto the *L. monocytogenes* genome and depicted as horizontal lines in the top half of the figure. Forward reads are mapped above, reverse reads below the base line. Blue reads are from the sample containing RNA fragments <40 nt, green reads from the sample containing RNA between 40 nt and 150 nt, red reads from the fraction containing RNA >150 nt. The lower half of the figure shows the corresponding annotation at this genome location, with the beginning of the *dnaA* gene at position 318. Artemis [39] was used to illustrate the mapped reads and annotation of the genome.
doi:10.1371/journal.pone.0083979.g002

variation due to unspecific termination of transcription may be a possible explanation for this observation. We summarized our findings in supplementary table S1, which contains a comprehensive list of known sRNAs and their features as well as their class indicated by our study.

The automated classification of sRNA candidates by our pipeline revealed that 70% of our sRNA candidates resemble TSS and long UTRs (>150 nt) instead of independent small transcripts. We removed those candidates and all known sRNAs from further analysis. The remaining 172 yet undescribed candidates where manually analyzed for their potential to resemble new sRNAs on the *L. monocytogenes* genome. Supplementary table S2 lists these 172 candidates and their individual automated and manual classification. Most of the 172 candidates identified by automated methods were dismissed after a manual inspection for one of several reasons: (1) probable origin as TSS, alternative TSS or 3′ UTR of a regular gene or annotated ORF, due to their location and read pattern, (2) expression below the local noise level, and (3) expression peaks on lowly expressed genes. The individual reasons to dismiss certain RNAs are also given in supplementary table S2. However, we propose nine new sRNAs candidates within the *L. monocytogenes* genome. These candidates show sufficient expression above the noise level and indications of independent expression.

## Nine new asRNAs

Analysis of the SOLiD sequencing data lead to the discovery of new small RNAs mostly transcribed anti-sense of annotated *L. monocytogenes* genes. We have picked nine candidates for further analysis. All nine candidates showed expression opposite of an annotated gene and therefore were classified as antisense RNAs. Fig. 4 and Fig. S1 show the read mappings of these nine asRNAs, which are listed in table 1. For some of the corresponding genes, a biological function is annotated, allowing us to infer a possible function of asRNAs.

Conservation analysis was performed using the MAUVE multiple genome alignment tool [25]. Of the nine candidates, most were well conserved within other *Listeria* species. anti0055 however, was specific for *L. monocytogenes* and anti2330 was found to be only conserved in *L. innocua and L. welshimeri*.

The asRNA anti0055 is located antisense of *lmo0055* or *purA*, an adenylosuccinate synthetase, important in the *de novo* synthesis of purine nucleobases, which also plays roles in infection [26] and intracellular growth [27]. Transcription of the antisense RNA starts 365 nt downstream of the TSS of *purA* in the opposite direction. The exact length of the transcript cannot be assessed, but additional reads downstream of the sRNAs TSS suggest a length of at least 289 nt. See Fig. 4(A) for read mappings in this locus. Significant expression of both, the *purA* gene as well as its asRNA can only be detected in the extracellular sample. Expression in the intracellular sample is very low and not above the expected noise level.

Another newly identified asRNA is transcribed opposite of *lmo2225*, a putative fumarate hydratase according to the KEGG database and based on orthology assumed to be active within the

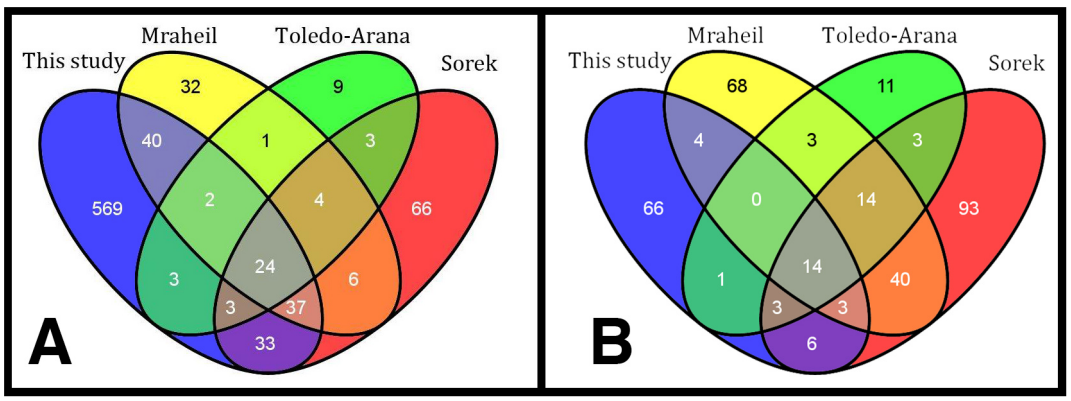


**Figure 3. sRNAs identified by different studies [2–4] and this study and their overlap. sRNAs for this study were identified via automatic identification with our newly developed pipeline.** 144 (55%) known sRNAs were recovered with the automated method. Of the 711 sRNAs identified in total, 569 were yet undescribed. The majority of these, however, were later removed due to their likely origin as transcription start site and 5′ UTR of known genes. Most of sRNAs, which were not recalled by the automated method, were found by manual reevaluation, increasing the total recall rate to 90%.
doi:10.1371/journal.pone.0083979.g003

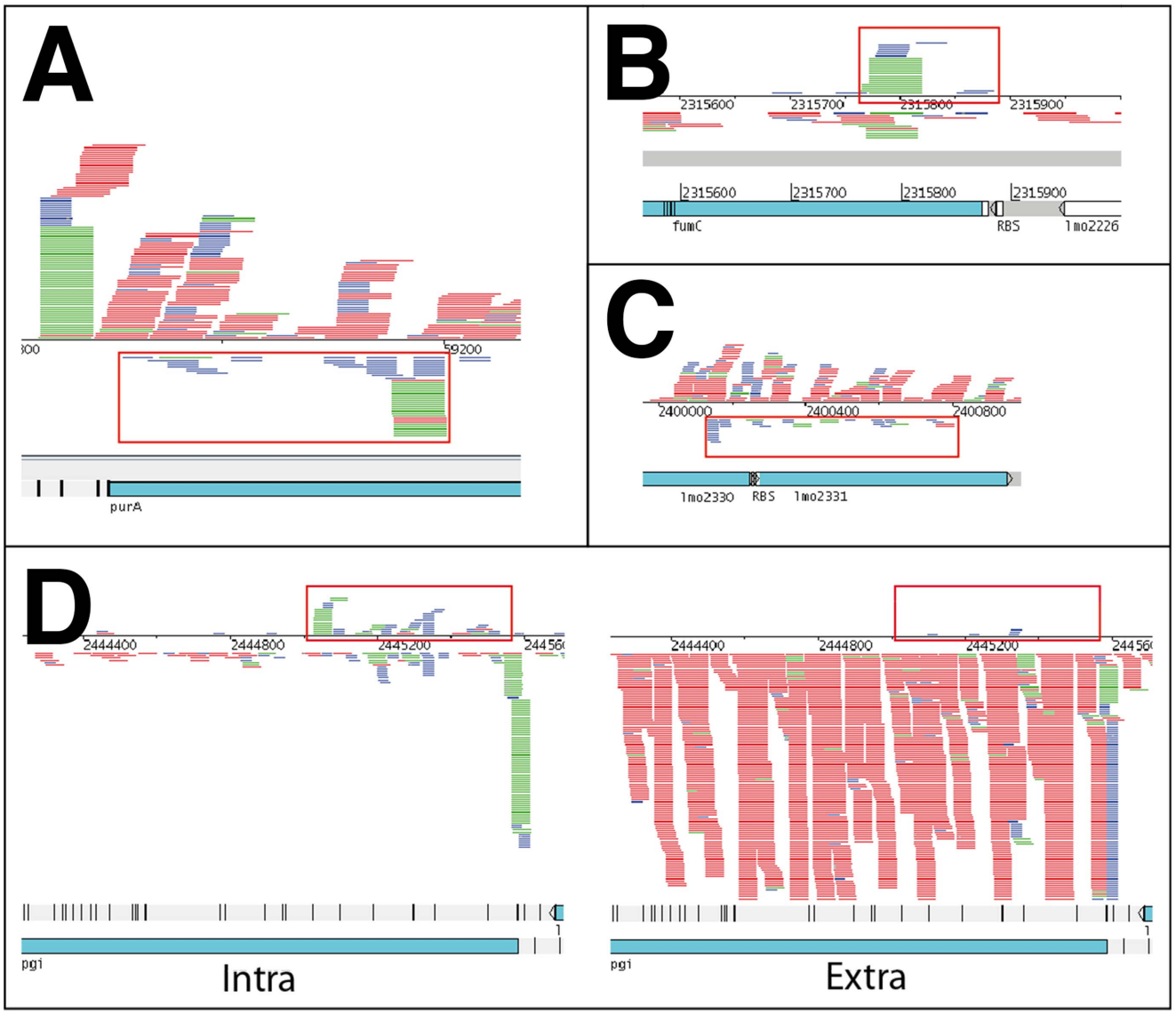


**Figure 4. Pileup of reads representing four newly identified asRNAs of *L. monocytogenes*.** Putative sRNAs are marked with red boxes. Each colored line represents a mapped read either on the forward strand (above the line) or the reverse strand (below the line). Blue reads are from the sample containing RNA fragments <40 nt, green reads from the sample containing RNA between 40 and150 nt. Red reads from the sample of RNAs >150 nt. The lower half of each figure shows the corresponding annotation at this genome location. (A) anti0055 (*purA*). Shown is the extracellular condition. (B) anti2225 (*fumC*). Shown is the extracellular condition. (C) anti2330 (*lmo2331*) in phage locus of *L. monocytogenes*. Shown is the extracellular condition. (D) anti2367 (*pgi*). Shown is the intracellular and extracellular condition respectively. Expression of the *pgi* gene and the boxed antisense RNA is mutual exclusive between the two conditions.
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citrate cycle. Its putative TSS is 110 nt upstream of the beginning of the *fumC* gene, for which no independent TSS could be identified. Again, the length of the transcript cannot be determined with certainty, but additional reads suggest around 110 nt of length. Expression of anti_fumC can be found in intra- and extracellular sample. However expression is roughly 10-fold higher in the intracellular sample (see also Fig. 4(B)). Differential expression analysis found this locus to be differentially expressed with a p-value of 0.064. *L. monocytogenes* harbors a prophage locus with genes from *lmo2271* until *lmo2332* [28], which at the very end contains weak, but consistent expression of an antisense RNA. It covers parts of the genes *lmo2330* and *lmo2331* and stretches from near the 3′ end of *lmo2331* until the 3′ end of *lmo2330*. Expression can be detected in both extracellular and intracellular condition. See Fig. 4(C) for a mapping of reads onto the corresponding locus.

Most notably among the nine new asRNAs is anti2367 opposite of *lmo2367* or *pgi*, coding for a glucose-6-phosphate-isomerase with suggested function in the pentose-phosphate-pathway and glycolysis (see KEGG-database). Expression starts 568 nt upstream and on the opposite strand of the putative TSS for *pgi*. Its length can be estimated between 325 and 700 nt and expression can only be detected in the intracellular sample. Its differential expression p-value is 0.026 with a normalized fold change of 10.

## Experimental confirmation of novel asRNA candidates

To confirm the transcriptional regulation of several new asRNAs (≥50 nt) in our study we selected anti0055, anti2106,

**Table 1.** List of the nine newly identified sRNAs in *L. monocytogenes*, which are classified as asRNAs with the corresponding antisense gene given.

| name | start | end | strand | length | class | corresponding gene |
|---|---|---|---|---|---|---|
| anti0055 | 59153 | 59203 | − | 50 | asRNA | *lmo0055/purA* |
| anti0466 | 503060 | 503108 | − | 48 | asRNA | *lmo0466* |
| anti2106 | 2186912 | 2187025 | + | 113 | asRNA | *lmo2106* |
| anti2130 | 2213928 | 2213976 | + | 48 | asRNA | *lmo2130* |
| anti2224-2 | 2314018 | 2314047 | + | 29 | asRNA | *lmo2224* |
| anti2225 | 2315763 | 2315820 | + | 57 | asRNA | *lmo2225/fumC* |
| anti2330 | 2400131 | 2400197 | − | 66 | asRNA | *lmo2331* |
| anti2367 | 2445029 | 2445120 | + | 91 | asRNA | *lmo2367/pgi* |
| anti2378 | 2454760 | 2454790 | − | 30 | asRNA | *lmo2378* |

doi:10.1371/journal.pone.0083979.t001

anti2225, anti2330 and anti2367 for performing qRT-PCR analysis. The results showed that all selected asRNAs are differentially expressed under intra- and extracellular growth conditions (see Fig. 5). In addition we could confirm by using northern blot analysis that anti0055 is up-regulated during intracellular growth (see Fig. 5(B)).

In the case of anti2673 which is up-regulated during intracellular growth, the corresponding gene *lmo2673* (*pgi)* on the other hand is down-regulated in the intracellular growth condition. See Fig. 4 (D) for the alignment of intracellular and extracellular reads to the *L. monocytogenes* genome, showing mutual exclusive expression of *pgi* and the corresponding asRNA.

### Long antisense RNAs

We were able to confirm the expression of five from six proposed lasRNAs in our sequence data and were able to identify asRNA candidates that have similar properties. These asRNAs have been previously reported, but in this study we found these are likely to resemble much longer lasRNAs. Specifically the asRNAs anti2046, anti2259, anti2677 and anti2717 all stretch over several genes and potentially form lasRNAs. Also see the comments of the corresponding asRNAs in supplementary table S1 for additional information on these lasRNAs. Supplementary Fig. S2 shows the mapping for all of the aforementioned possible lasRNAs in the artemis viewer.

## Discussion

Small RNAs in *L. monocytogenes* have been subject to intensive research over the last years. Improving technologies with increased sensitivity lead to the identification of 257 sRNAs in total by several studies using different techniques [2–4,18,20]. This study re-evaluates these small RNAs with focus on their probable origin and functional properties, and proposes nine new non-coding sRNAs, making use of an extensive transcriptome dataset, compiling a total of 21 million SOLiD sequencing reads. Five of these nine new asRNA could be confirmed via qRT-PCR and one candidate (anti0055) could also be validated in northern blot experiments by performing three biological independent experiments to show their biological relevance.

### Computational prediction of sRNAs by a new pipeline

We implemented a specialized analysis pipeline for the identification of sRNAs in SOLiD sequencing data. In contrast to existing pipelines and analysis tools, this pipeline exploits the specific properties of fractionated RNAseq data to identify sRNAs with increased sensitivity and specificity. The pipeline makes use of fractionated RNA data, to improve on the distinction between degradation products of large RNA molecules and independent small non-coding RNAs. Since distinction between long UTRs and sRNAs located 5′ of genes or polycistronic transcription and intergenic sRNAs is often inaccurate based solely on annotational data and read-pileup-shapes, a manual analysis of the data is still advised where the complete context of gene expression in an area can be assessed.

The pipeline was designed for use with SOLiD specific color-coded sequencing data as an input, but is easily usable with other next generation sequencing technologies as well, making it universally applicable. While it is possible to analyze and identify sRNAs based on a single RNA-Seq experiment with this pipeline, particularly projects with a multitude of datasets with RNA of different size fractions will strongly benefit from the pipelines capabilities of integrating information from between different datasets. Furthermore downstream analysis tools integrated into the pipeline help in the fast interpretation of acquired data. They include a clustering algorithm to identify the same sRNAs in different samples or studies, an automated sRNA classification system based on size, position, and read pattern of a candidate, as well as differential expression analysis to compare data taken under different conditions. The pipeline can be easily modified to meet a wide range of requirement for the analysis of transcriptomic data.

### lasRNA

Long antisense RNAs are a type of non-coding RNAs that have been described previously [3,4]. These lasRNAs are significantly longer than typical, short asRNAs and typically stretch over whole genes instead of just covering the UTR of a gene. Wurtzel and colleagues proposed some of these lasRNAs have a double function both as mRNA and asRNA and introduced a related structure called excludon [4]. In this structure, two adjacent, yet oppositely arranged genes overlap with the other gene with their corresponding transcript and forms corresponding lasRNAs. This structure has the potential to create an expression regulation by mutual exclusion, where one gene cannot be expressed while the other is, as the transcript for one gene will also act as asRNA for the other.

We were able to identify four previously known asRNAs [3,4] showing similar properties: anti2046, anti2259, anti2678 and anti2717 were all found to be significantly longer than originally

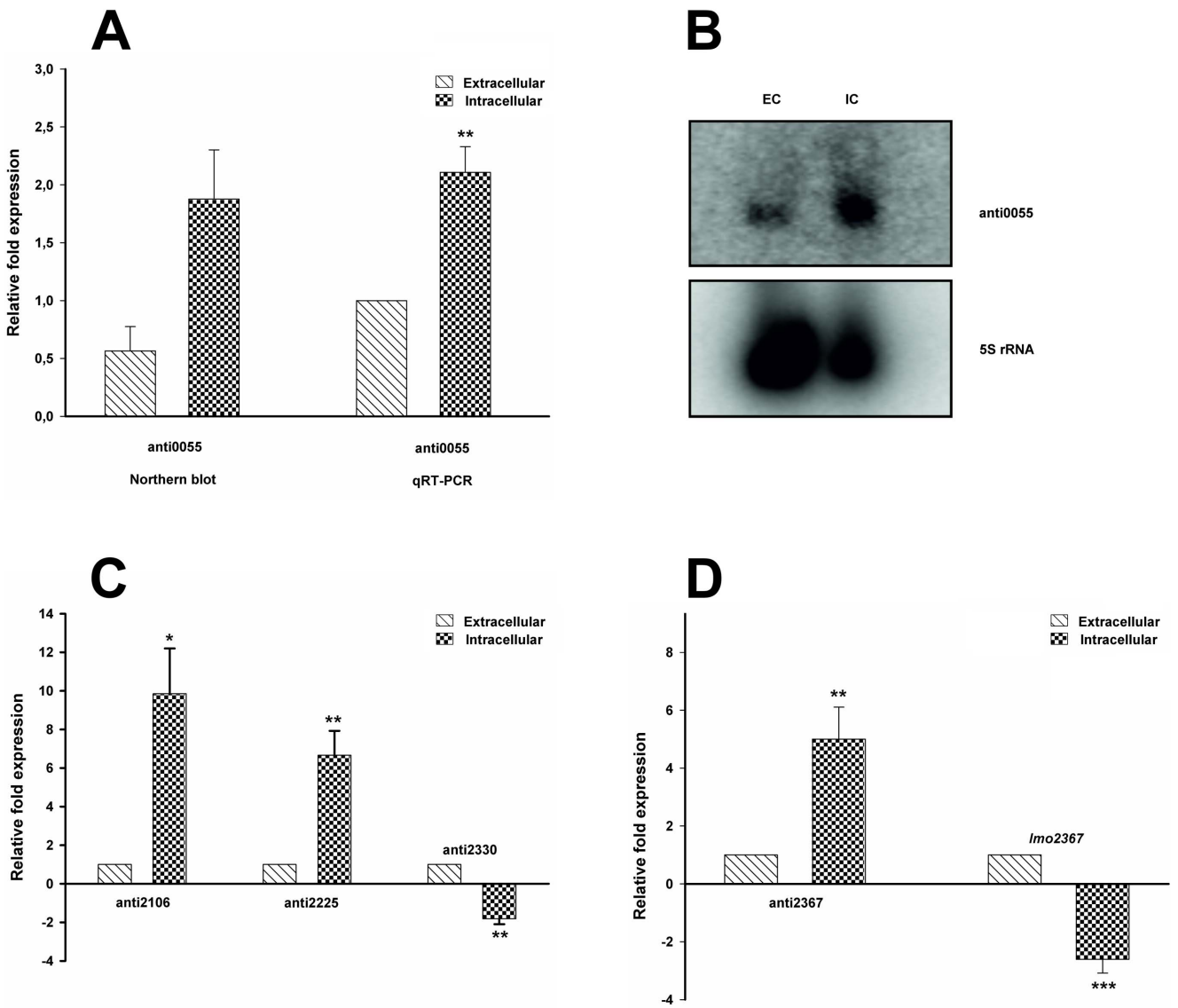


**Figure 5. Validation of new asRNA transcripts from *L.* monocytogenes and their effect on gene regulation after transition to the intracellular growth conditions.** A) The antisense RNA transcript anti0055 (*purA*) is validated by northern blot analysis and strand-specific qRT-PCR. The graph shows intracellular up-regulation of anti0055. B) Northern blot images of anti0055 and control 5S rRNA EC: Extracellular, IC: Intracellular. C) The presence of antisense transcripts anti2106 (*lmo2106*), anti2225 (*fumC*), and anti2330 (*lmo2330*) was determined by strand-specific qRT-PCR. anti2330 is down-regulated, anti2106 and anti2225 are up-regulated significantly. D) Strand-specific qRT-PCR analysis confirmed the existence and up-regulation of antisense RNA transcript anti2367. *pgi* (*lmo2367*) was down-regulated, which indicates the possible role of anti2367 in *pgi* gene regulation. '*' $P \leq 0.05$; '**' $P \leq 0.01$; '***' $P \leq 0.001$.
doi:10.1371/journal.pone.0083979.g005

proposed. All four candidates have been originally described to cover part of a single gene, but in our data were found to cover four to six genes instead. See corresponding comments in supplementary table S1 and the read pileups in supplemental Fig. S2. Given the length of the lasRNAs, structures comparable to the excludons described by Wurtzel and colleagues [4] are likely for these lasRNAs. The most likely reason for us to identify those sRNAs as significantly longer than before described, is the higher sequencing coverage in our experiments. It enables us to identify weekly but consistently transcribed areas better than before, leading to the discovery of previously unidentified long transcripts that were originally thought to be distinct or shorter.

## Identification of nine new sRNA candidates

Automated identification of asRNA in the data and manual refinement of results revealed nine new sRNAs candidates in *L. monocytogenes*. Most notably among these are four asRNAs opposite of the genes *lmo2225* (*fumC*), *lmo2330*, *lmo0055* (*purA)* and *lmo2367* (*pgi*).

The prophage A118 can be found in the *L. monocytogenes* EGD-e genome inserted between the genes *lmo2271* and *lmo2332* [22]. At the very end of this prophage region, covering the 3′ end of *lmo2331* and the 5′ end of *lmo2330* we identified another down-regulated asRNA (see Fig. 5 (C)). *lmo2331* is predicted to encode a cell wall lipoprotein, while *lmo2330* is similar to the phage protein *gp33*. Antisense transcription of the prophage genes has previously been reported and this might be an additional case of such [2–4].

Apart from this general antisense transcription it might represent specific and active repression of phage gene expression, as phage control by means of antisense transcription is a long known phenomenon [29]. More recently Irnov and colleagues also reported the expression of asRNA in prophages of *Bacillus subtilis* and suggested a function in maintaining the phage host equilibrium [14].

Antisense of the *purA* gene we were able to identify an asRNA at the 5′ end of the gene. The *purA* gene encodes a putative adenylosuccinate synthetase with assumed function in the de novo purine synthesis pathway, making it an essential enzyme in the synthesis pathway of purine nucleobases. Purine synthesis seemingly plays an important role for intracellular growth of *L. monocytogenes* [27] and a *L. monocytogenes* serotype 4b strain with a mutation of *purA* is known to be strongly attenuated in the infection of mice [26]. This makes a lifestyle dependent regulation of *purA* very likely, and asRNAs are known to play a major role in the adaption to rapid environmental changes in general [30] as well as the transition of *L. monocytogenes* from saprophytic to virulent lifestyle in particular [31]. However, no classical or obvious regulation pattern could be found when analyzing expression of both the *purA* gene and its corresponding asRNA within the RNA-Seq data which could be also observed by qRT-PCR (data not shown). We observed increased expression of asRNA anti0055 under intracellular versus extracellular growth condition using qRT-PCR analysis as well as northern blot analysis (see Fig. 5 (A and B)). The biological relevance of this up-regulated asRNA has to be characterized in future.

We identified a new asRNA anti2225 opposite of the *fumC* gene, coding for a fumarate hydratase typically with central function in the TCA-cycle. Interestingly, an antisense transcript of the homologous gene has also been found in the Gram-negative *Helicobacter pylori* and experimentally verified by northern blot and RT-PCR [32]. In addition, many asRNAs of housekeeping genes of *Cyanobacterium synechocystis* have been identified [33], demonstrating that such asRNAs are a common mechanism of transcriptional regulation. Furthermore *L. monocytogenes* is already suspected to have an interrupted TCA-cycle [34]. Also it shown that even an interrupted TCA-cycle may serve as an essential generator for purine for which we already propose a regulation by means of PurA [35]. Furthermore Schauer and coworkers have shown the central role of purine biosynthesis for intracellular growth [27]. Here we could show that expression of the *fumC* gene (data not shown) as wells as anti2225 (see Fig. 5(C)) is up-regulated after transition to the intracellular lifestyle. Biological interpretation of these finding is challenging at this point and needs further experimental validation. Signs of classical asRNA regulation patterns can be found expressed opposite of the gene *lmo2367/pgi* for anti2367. Inspecting the sequencing data of the intracellular and extracellular growth condition, the expression of either the gene or the asRNA seems to be mutually exclusive, giving a hint for a causal link and a possible regulation mechanism interfering with the expression of *pgi* on the transcriptional level. This pattern is clearly visible in Fig. 4(D) and Fig. 5(D) showing the mapped reads for both the intracellular and the extracellular condition which could be also confirmed by qRT-PCR analysis. Expression of the *pgi* gene is low for the intracellular growth, and high for the extracellular growth, while expression of the corresponding asRNA on the opposite strand is high for the intracellular and low for the extracellular condition (see Fig. 5(D)). *lmo2367/pgi*, encodes a glucose-6-phosphate isomerase with central function in the interface between glycolysis and the pentose phosphate pathway. Previous reports link the transition from extracellular to intracellular growth of *L. monocytogenes* to a reduced expression of *pgi* [36] and a corresponding shift in metabolic pathways leading to the degradation of glucose phosphate by the pentose phosphate pathway [1]. Furthermore a proteomic study was able to identify the *pgi* corresponding peptides under two different extracellular conditions but not within intracellular conditions of *L. monocytogenes* [37]. As a housekeeping gene, *pgi* is under the control of a housekeeping promoter, and hence requires promoter independent specific regulation of this gene. The identification of anti2367 sheds lights on the metabolic adaptation on transcriptional level by antisense RNAs in *L. monocytogenes*.

## Conclusion

The high coverage and strong strand specificity of our data revealed a substantial amount of general antisense transcription over the *L. monocytogenes* genome. Similar general antisense transcription has been described previously [33,38]. The biological relevance of this phenomenon is not yet fully understood, but the finding of such in another bacterial organism underlines its importance of further inquiry of the matter. Given the high number of newly identified asRNAs as well as the identification of exceptionally long non coding antisense RNAs, lasRNAs, it is obvious that antisense transcription is an important factor in the regulatory network of *L. monocytogenes* and it should be investigated whether similar types of regulation are common in other bacterial species.

## Supporting Information

**Table S1 List of previously identified sRNAs.**
(XLS)

**Table S2 List of newly identified sRNAs.**
(XLS)

**Table S3 Oligonucleotides used in this study.**
(DOCX)

**Table S4 Reads count summary of experimental transcriptome data.**
(XLS)

**File S1 Detailed description of data analysis pipeline.**
(DOC)

**Figure S1 Read mapping of asRNA anti0466, anti2106, anti2130, anti2224-2 and anti2378.**
(PDF)

**Figure S2 Read mappings of lasRNA like structures.**
(PDF)

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## Author Contributions

Conceived and designed the experiments: NK MAM TH . Performed the experiments: NK MAM GKM XQ RM TH. Analyzed the data: SB SW TR GKM XQ RM MAM TH. Wrote the paper: SB SW GKM RM NK MAM TR TH.

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# Detection of Very Long Antisense Transcripts by Whole Transcriptome RNA-Seq Analysis of *Listeria monocytogenes* by Semiconductor Sequencing Technology

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## Abstract

The Gram-positive bacterium *Listeria monocytogenes* is the causative agent of listeriosis, a severe food-borne infection characterised by abortion, septicaemia, or meningoencephalitis. *L. monocytogenes* causes outbreaks of febrile gastroenteritis and accounts for community-acquired bacterial meningitis in humans. Listeriosis has one of the highest mortality rates (up to 30%) of all food-borne infections. This human pathogenic bacterium is an important model organism for biomedical research to investigate cell-mediated immunity. *L. monocytogenes* is also one of the best characterised bacterial systems for the molecular analysis of intracellular parasitism. Recently several transcriptomic studies have also made the ubiquitous distributed bacterium as a model to understand mechanisms of gene regulation from the environment to the infected host on the level of mRNA and non-coding RNAs (ncRNAs). We have used semiconductor sequencing technology for RNA-seq to investigate the repertoire of listerial ncRNAs under extra- and intracellular growth conditions. Furthermore, we applied a new bioinformatic analysis pipeline for detection, comparative genomics and structural conservation to identify ncRNAs. With this work, in total, 741 ncRNA locations of potential ncRNA candidates are now known for *L. monocytogenes*, of which 611 ncRNA candidates were identified by RNA-seq. 441 transcribed ncRNAs have never been described before. Among these, we identified novel long non-coding antisense RNAs with a length of up to 5,400 nt e.g. opposite to genes coding for internalins, methylases or a high-affinity potassium uptake system, namely the *kdpABC* operon, which were confirmed by qRT-PCR analysis. RNA-seq, comparative genomics and structural conservation of *L. monocytogenes* ncRNAs illustrate that this human pathogen uses a large number and repertoire of ncRNA including novel long antisense RNAs, which could be important for intracellular survival within the infected eukaryotic host.

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**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. The data have been uploaded to the ENA at the EBI with the accession number PRJEB6949 (http://www.ebi.ac.uk/ena/data/view/PRJEB6949).

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## Introduction

*Listeria monocytogenes* is a non-sporulating, Gram-positive soil bacterium which has a low GC content. The ubiquitous nature of the bacterium enables it to enter the human food chain via food-processing environments. In addition, the ability of *L. monocytogenes* to grow at low temperatures and to resist harsh preservation techniques increases the risk of food contamination. By uptake via contaminated food products, *L. monocytogenes* can cause listerial infection known as listeriosis. Listeriosis often manifests with clinical symptoms such as meningitis, meningoencephalitis, septicaemia, abortion, prenatal infection and also gastroenteritis. Furthermore, high mortality rates of up to 20–30% in humans which are diseased with listeriosis (especially pregnant women, elderly and immunocompromised persons) makes *L. monocytogenes* a serious life-threatening human pathogen [1,2].

The genus *Listeria* consists of ten species, *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. marthii*, *L. welshimeri*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grayi* and *L. fleischmannii*. *L. monocytogenes* and *L. ivanovii* are the only known pathogens of this group [3–8].

Comparative whole genome sequencing of representative strains comprising the entire species of *L. monocytogenes* was performed by Kuenne *et al.* [9]. In the genus *Listeria*, genome reduction has led to the generation of non-pathogenic species from pathogenic progenitor strains [10]. Indeed, many of the genomic regions specific for pathogenic species (such as *L. monocytogenes*) represent

genes which are absent in non-pathogenic species (such as *L. innocua* and *L. welshimeri*) [10]. This also effects the number of conserved non-coding RNAs (ncRNAs) within the genus *Listeria* [9,11]. Recently genome sequencing of different *L. monocytogenes* serotypes has been accompanied by transcriptional profiling using whole genome microarrays and RNA-seq. This has been done to examine the adaptive changes of *L. monocytogenes* to grow in different natural environments and to identify responsible genes and ncRNAs mediating transcriptional responses [9,11–15]. For *L. monocytogenes*, 262 ncRNAs have been identified yet including 134 putative sRNAs, 86 antisense RNAs (asRNAs) and 42 riboswitches [16]. Also in other bacteria, asRNA transcripts could be observed for 10% up to 50% of protein-coding genes, e.g. in *Escherichia coli*, *Synechocystis* sp. PCC6803, *Helicobacter pylori* [17], *Bacillus subtilis* [18] and *Mycobaterium tuberculosis* [19].

In this study we present information on transcriptomic profiling using RNA-seq, comparative genomics and structural conservation of *L. monocytogenes* ncRNAs. The bacterial strains have been grown in BHI broth (extracellular conditions) and in the cytosolic environment of the host cell (intracellular condition). To our best knowledge, this is the first time that Ion Torrents Personal Genome Machine (PGM) (Life Technologies) was used for RNA-seq analysis of a bacterial human pathogen by next generation semiconductor sequencing technology to detect novel small and long ncRNAs. Using this technology, we found antisense transcripts in *Listeria* with a length up to 5,400 nt.

## Materials and Methods

### Bacterial strains and growth conditions

The strains *L. monocytogenes* EGD-e [20], *L. monocytogenes* 1043S [21] and *L. monocytogenes* EGD-e Δ*prfA* [22] were grown in BHI broth (VWR) overnight at 37°C with shaking at 180 rpm (Unitron, Infors). Overnight cultures were diluted 1:50 in 20 ml fresh BHI broth using a 100 ml Erlenmeyer flask and were incubated at the same conditions mentioned above until $OD_{600\ nm}$ 1.0.

### Cell culture and infection model

P388D1 murine macrophage cells (ATCC CCL-46) were cultured in RPMI1640 (Gibco) supplemented with 10% fetal calf serum (PAA Laboratories) in 85-mm-diameter tissue culture plates. For intracellular growth assays bacteria were added to P388D1 murine macrophages monolayer at a multiplicity of infection (MOI) of 10 *Listeria* per eukaryotic cell. The intracellular growth assays were performed as described in [23].

### RNA isolation

For RNA extraction from *L. monocytogenes* grown extracellularly in BHI, we applied aliquots of 0.5 ml from the same *Listeria* culture grown until mid-exponential phase used to infect P388D1 macrophages. The bacterial cells were treated with 1.0 ml RNA protect (Qiagen) for 5 min and were collected by centrifugation for 10 min (8000 g). The bacterial pellets were stored at −80°C until use. RNA extraction from intracellularly grown *L. monocytogenes* in macrophages, 4 h post infection, was performed as described previously [23]. Briefly, infected host cells (see above: Cell culture and infection model part) were lysed using cold mix of 0.1% (wt/vol) sodium dodecyl sulfate, 1.0% (vol/vol) acidic phenol and 19% (vol/vol) ethanol in water. The bacterial pellets were collected by centrifugation for 3 min (16,000 g). Total RNA was extracted using miRNeasy kit (Qiagen) with some modifications [11]. The collected pellets were washed with SET buffer (50 mM NaCl, 5 mM EDTA and 30 mM Tris-HCl (pH 7.0)). After centrifugation at 16000 g for 3 min pellets were resuspended into 0.1 ml Tris-HCl (pH 6.5) containing 50 mg/ml lysozyme (Sigma), 25 U of mutanolysin (Sigma), 40 U of SUPERase (Ambion), 0.2 mg of proteinase K (Ambion). The incubation for 30 min was carried out on a thermo mixer at 37°C and with shaking (350 rpm). QIAzol (Qiagen) was added, mixed gently and incubated for 3 min at room temperature. An additional incubation for 2 min at room temperature was done after adding 0.2 volume chloroform followed by centrifugation at 16000 g at 4°C for 15 min. The upper aqueous phase, containing RNA, was transferred to a new collection tube and 1.5 volumes of 100% ethanol was added and mixed thoroughly. The probes containing RNA were transferred into columns supplied with the miRNeasy Kit (Qiagen) and treated according to the manual including an on-column DNase digestion (RNase-Free DNase, Qiagen). RNA was eluted by RNase-free water and stored at −80°C until needed. The quantity of the isolated total RNA was determined by absorbance at 260 nm and 280 nm, and the quality was assessed using Nano-chips for Agilents 2100 Bioanalyzer.

### RNA sequencing

To deplete bacterial rRNA we applied the Ribo-Zero Magnetic Kit (Bacteria) (Epicentre) and treated the depleted RNA with tobaco acid pyrophosphatase (Epicentre) as recommended by the manufacturer.

Afterwards, the RNA was fragmented by RNase III (Applied Biosystems) at 37°C for 4 min. The yield and size distribution of the fragmented RNA was assessed using Quant-iT RNA assay kit with Qubit Fluorometer (Invitrogen) and the Agilent RNA 6000 Pico Chip kit with Agilent 2100 Bioanalyzer instrument. Size distribution of RNase III fragmented RNA delivered median size of 200 nt. For the cDNA library preparation, Ion Total RNA-seq kit v2 (Ion Torrent, Life Technologies) was used as recommended by the manufacturer. The libraries were purified by AMPure XP Reagent (Beckman Coulter). The yield and size distribution of the amplified cDNA were assessed by Qubit Fluorometer (Invitrogen) and DNA 1000 kit (Agilent). In the next step, clonally amplified Ion Sphere Particles (ISPs) containing the amplified cDNA were prepared using the Ion OneTouch System (Life Technologies). The amplified libraries were diluted to 8.3 nM and loaded on 316 Chip of the Ion Torrent semiconductor sequencing instrument personal genome machine (PGM) (Life Technologies).

### Real-time-RT-PCR

Reverse transcription to produce cDNA was performed by SuperScript II Reverse Transcriptase (Invitrogen) using 1 μg RNA. The probes were subjected to quantitative real-time PCR in a final volume of 25 μl using QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturers instruction. A standard curve was generated for the used primer pairs (see supplemental material) using different copy numbers of genomic DNA from *L. monocytogenes* EGD-e. For each primer pair a negative control (water), RNA sample without reverse transcriptase (to determine genomic DNA contamination) and a sample with known amount of copy numbers (to test the efficiency of the reaction) were included as controls during cDNA quantification. After real-time PCR all samples were run on a 1.5% agarose gel to verify that only a single band was produced. The expression level of each gene was calculated by normalizing its mRNA quantity to the quantity of the mRNA of *gyrB* encoding gyrase B [24] for the same sample using a mathematical model for relative quantification in real-time PCR published by Pfaffl [25].

**Table 1.** Scoring system.

| Criterion | Score | | | | | |
|---|---|---|---|---|---|---|
| Length (nt) | >50 | +0.25 | >75 | +0.25 | >100 | +0.5 |
| Reads | >9 | +1 | >100 | +1 | | |
| GC (%) | >40 | +0.25 | >50 | +0.25 | | |
| RNALfold | | +0.25 | | | | |
| POMAGO | = 13 | +0.25 | | | | |
| RNAz (p) | >0.9 | +0.25 | | | | |

For evaluation of the ncRNA candidates, a scoring system retrieved from known ncRNAs (Rfam, [13,15,26], see supplemental material) was developed. For increasing length, number of reads and GC content, scores are summed up along the column; for example, an ncRNA candidate of length 100 nt receives a score of +1. The higher the score of a candidate, the higher its probability to be an ncRNA.
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## *In silico* Genome Data Analysis

In order to analyze the genome of *L. monocytogenes* (NC_003210) with RNA-seq data and to detect potential novel ncRNAs, we investigated the genome searching for: (a) proteins, (b) known ncRNAs, (c) conserved regions, (d) locally stable structures, (e) possible *de novo* ncRNAs, and (f) positions of known potential small RNAs from literature [13,15,26].

**Annotation of known proteins.** Protein annotation from NCBI (NC_003210) was extended by a *de novo* protein prediction with BacProt [27] based on homologous proteins of other firmicutes. Furthermore, BacProt predicts species specific novel proteins based on *Listeria* specific information on Shine-Dalgarno sequences and TATA boxes gained from the homology search.

**Annotation of known ncRNAs.** tRNAs were annotated using tRNAscan-SE (v.1.23) [28] with parameters -omlfrF. For the annotation of ribosomal RNAs (rRNAs), we used rnammer (v.1.2) [29] with the parameters -S bac -m lsu,ssu,tsu.

For the other ncRNA classes, homology searches using BLAST (v.2.2.21) [30] (E-Value: $E<10^{-4}$) and infernal (v.1.0.2) [31] were performed. Known sequences of the corresponding classes, which were downloaded from Rfam database (v.10.0) [32], were used as input.

**Conserved regions: multiple genome-wide alignment.** The multiple genome-wide alignment was calculated using POMAGO [33] with *L. monocytogenes* EGD-e as reference species. The following organisms were included into the multiple genome-wide alignment analysis: *L. monocytogenes* ATCC 19117, *L. monocytogenes* CLIP80459, *L. monocytogenes* FSL J1-208, *L. monocytogenes* L99, *L. monocytogenes* SLCC2482, *L. monocytogenes* SLCC2372, *L. monocytogenes* SLCC2376, *L. monocytogenes* SLCC2378, *L. monocytogenes* SLCC2479, *L. monocytogenes* SLCC2540, *L. monocytogenes* SLCC2755 and *L. monocytogenes* SLCC7179.

**Annotation of *de novo* ncRNAs via RNAz.** Based on the calculated multiple genome-wide alignment an RNAz-analysis --cutoff = 0.5 (v.2.1) [34] was performed.

**Locally stable secondary structures.** Locally stable secondary structures are indicating positions for small RNAs. Those structures were calculated with RNALfold (v.2.0.7) [35] using parameters -d 2 -L 120. Hits with a total length less than 50 nt were discarded. A dinucluotide shuffling of each sequence with shuffle -d -n 1000 was performed to predict thermodynamically stable RNA structures. For further analyses only extraordinarily stable structures with a Z-score cut-off $\leq -3.0$ (top 5% of stable structures) were taken into account.

## Transcriptome data analysis

Reads were clipped with fastx-clipper (v. 0.0.13) (http://hannonlab.cshl.edu/fastx_toolkit/). All reads from one growth condition were merged to one library and then mapped to the *L. monocytogenes* EGD-e genome (NC_003210) by segemehl (v.0.1.3–335) [36] using standard paramaters (-A 85 -e 5). For normalisation the number of all mapped reads (except rRNAs and tRNAs) of the two libraries were used.

**Detection of possible *de novo* non-coding RNAs.** For the detection of potential novel non-coding RNAs, all intergenic regions with a minimum length of 10 nt and a minimum coverage of ten reads were defined as 'seeds'. For the analysis of long (antisense) non-coding RNAs, we merged seed regions, with a distance less than 100 nt. All candidates were scored according to

**Table 2.** Overview of RNA-seq libraries.

| Library | Number of reads | | Read length | Mean read length | |
|---|---|---|---|---|---|
| | before clipping | after clipping | | before clipping | after clipping |
| intra-1 | 3,253,920 | 3,151,751 | 6–368 | 106.613 | 85.7815 |
| intra-2 | 3,412,934 | 3,322,309 | 8–374 | 156.797 | 116.062 |
| intra-3 | 3,748,637 | 3,660,315 | 8–385 | 150.629 | 107.838 |
| extra-1 | 3,165,988 | 3,079,495 | 6–365 | 108.007 | 82.53 |
| extra-2 | 3,322,796 | 3,247,113 | 6–371 | 138.98 | 102.825 |
| extra-3 | 3,710,603 | 3,660,845 | 6–362 | 157.823 | 114.506 |

Libraries were retrieved by next generation semiconductor sequencing technology. Number of reads before and after clipping and their mean length.
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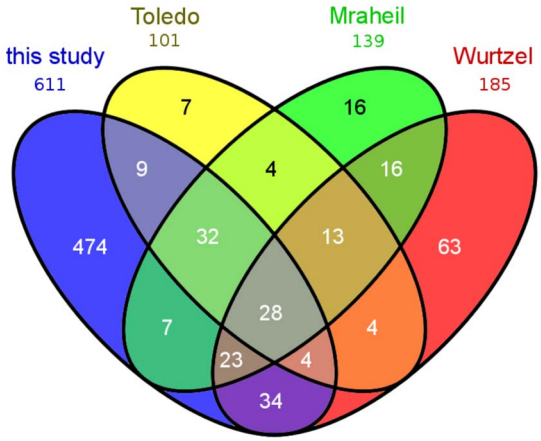


**Figure 1. Comparative analysis of ncRNA transcriptome data**: Comparison of our ncRNA candidates with results of previous studies performed by Toledo-Arana *et al.* [13], Mraheil *et al.* [11] and Wurtzel *et al.* [15]. Note that whenever an ncRNA prediction of this study overlaps with multiple previously described candidates, it is a single hit in the diagram. Altogether, including previous literature, Rfam and this work, now 741 putative ncRNAs are described. In this work we defined 611 to be putative ncRNAs, of which 474 ncRNAs are not part of previous literature, 33 of them known ncRNAs from Rfam.
doi:10.1371/journal.pone.0108639.g001

the characteristics of known ncRNAs of Rfam [37] and from previously identified ncRNAs [13,15,26] to indicate possible novel ncRNAs (Tab. 1, supplemental material (http://www.rna.uni-jena.de/supplements/listeria/).

For further analyses, we took only candidates with a score of 2.5 or higher into account. Additionally, we checked our candidates for possible overlaps with the 5′UTR predicted by Wurtzel *et al.* [15].

## Results and Discussion

### Full ncRNA candidate set

In this study we analyzed the transciptomes of *L. monocytogenes* grown extracellularly in BHI broth and *L. monocytogenes* grown intracellularly in murine macrophages. Our analysis was based on three independent biological replicates for each condition resulting in six RNA-seq libraries produced by the Ion torrent (PGM) next generation sequencing platform. We obtained 3.1–3.7 million reads up to a length of 385 nt (see Tab. 2).

The experimental approach was combined with comprehensive *in silico* studies. To detect novel ncRNAs, we investigated various characteristic features of ncRNAs in the *L. monocytogenes* genome and transcriptome: seeds, GC-content, secondary structure, conservation and multiple genome-wide alignment.

(1) A *seed* is defined by an intergenic region covered by ≥10 reads for ≥10 nt. We searched for seeds and merged them to one candidate if they were at most 100 nt apart. We received 2074 candidate ncRNA locations. Locations longer than 50 nt, 75 nt and 100 nt were rewarded by +0.25, +0.5 and +1 respectively (see Tab. 1). If the number of reads was at least ten, the score of the ncRNA candidate was increased by 1. If the number of reads even exceeded 100, the score was again increased by 1.

(2) We analyzed the GC-content. The whole genome of *L. monocytogenes* EGD-e has an GC content of 38%. The ncRNAs of Rfam identified in *L. monocytogenes* EGD-e were found to have an GC content of 52% and 44% (with and without rRNAs/tRNAs). We decided to reward ncRNAs with GC content above 40% with 0.25, and another 0.25 points for GC content above 50%. However, previously reported ncRNAs [13,15,26] showed a lower GC content (on average 37%, 37.8% and 37.6% respectively).

(3) Using RNALfold we searched for locally stable secondary structures. For 87/143 ncRNAs described in Rfam and 118/260 ncRNA candidates previously described in the literature [13,15,26], we found a region which was identified by RNALfold as locally stable secondary structure. If a candidate was predicted to contain a locally stable secondary structure region, we rewarded this candidate by adding +0.25 to its score.

(4) Another hint for an (ncRNA) gene is its conservation among closely related species. Therefore, we computed a genome-wide multiple sequence alignment comparing *L. monocytogenes* EGD-e with 12 other *L. monocytogenes* serotypes. If the candidate region was present in all other serotypes, the candidate was rewarded by adding another +0.25 to its score.

(5) The multiple genome-wide alignment was used as input for RNAz to predict novel ncRNAs. If a candidate was identified to be a novel ncRNA with probability above 0.9, we added another +0.25 to its score.

For the further analysis we took only those novel ncRNA candidates into account that exceeded a given threshold. We chose this threshold by checking how many of the previously described ncRNAs would have been selected. For a threshold of 2.5, 132/143 of the ncRNAs described in Rfam and 137/260 of the previously putative ncRNAs described in the literature, would have been selected. Using this threshold, we present a set of 441 potential novel ncRNA candidates. To get a full set of ncRNA locations, we added the previously described ncRNAs to our set of novel ncRNA candidates. This results in 741 ncRNA locations (since both sets are overlapping), ranging from to 10–5,347 nt (mean: 239 nt) length for *L. monocytogenes*. If we use our threshold also for the previously described ncRNA locations, we get a set of 611 ncRNA candidates. The list of all candidates, their genomic locations and features as described above, as well as overlaps to previously described ncRNAs and adjacent proteins is given in the supplemental material.

### Comparison to previous studies

As mentioned above, 260 locations of ncRNA candidates (including start- and stop positions) were previously described in the literature [11,13,15]. We compared our 611 ncRNA candidates with the results of these previous studies (see Fig. 1).

In 2009, Toledo-Arana *et al.* [13] used tiling arrays and RNAs from wild type and mutants grown *in vitro*, *ex vivo*, and *in vivo*, to present a complete operon map of *L. monocytogenes*. In this study, 100 ncRNA candidates were suggested. Of this 100 putative sRNAs, 77 locations were also confirmed by our observations, whereas 23 locations had a score ≤2.5 or were not even identified as seeds.

Mraheil *et al.* [11] reported 150 putative regulatory RNAs identified by deep sequencing with cDNA obtained from extracellularly grown bacteria and from *L. monocytogenes* isolated from infected macrophages using 454 pyrosequencing. From these 150 putative regulatory RNAs, we identified 102 using our method and a score threshold of 2.5. More than half of the remaining 48 ncRNAs were covered with less than 10 reads and were not part of our seeds.

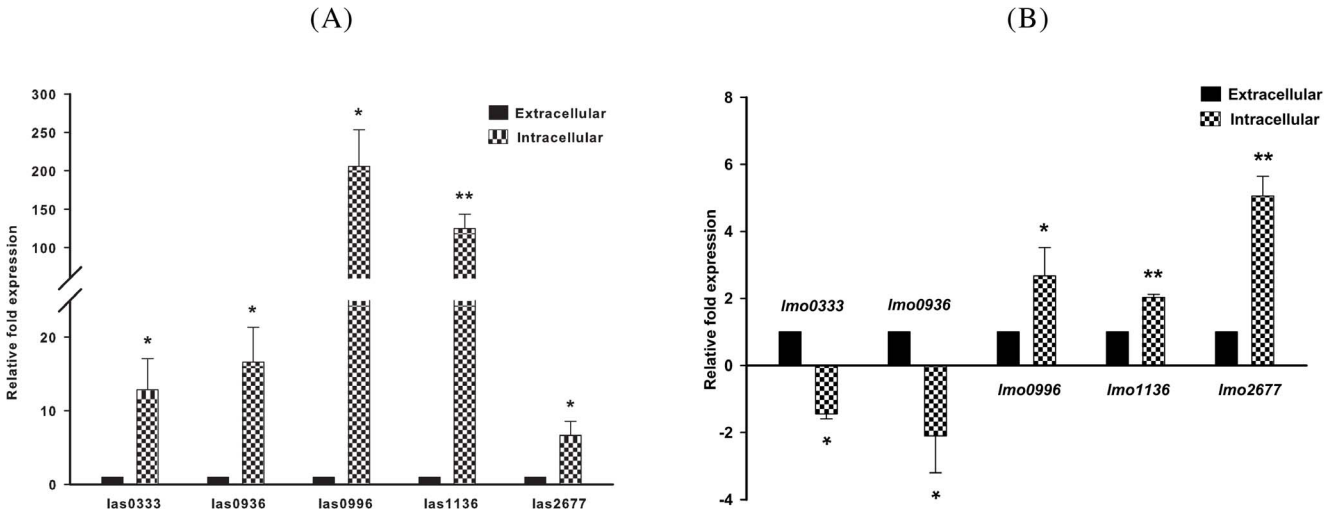


**Figure 2. Validation of new long antisense (las) RNAs in *L. monocytogenes* by qRT-PCR analysis.** (A) The presence of las transcripts was determined by strand-specific qRT-PCR analysis. Supporting the results of RNA-seq, the qRT-PCR analysis indicated that the novel lasRNA transcripts las0333, las0936, las0996, las1136 and las2677 were significantly up-regulated in intracellular conditions. '*' $-P \leq 0.05$ '**' $-P \leq 0.01$. (B) Strand specific qRT-PCR analysis of las respective target genes shows significant downregulation of *lmo0333* (internalin), and *lmo0936* (nitroflavin reductase), upregulation of *lmo0996* (methyltransferase), *lmo1136* (internalin) and *lmo2677* (esterase) in intracellular growth condtions. '*' $-P \leq 0.05$; '**' $-P \leq 0.01$. Primers used for qRT-PCR are available at the online Supplemental Material.
doi:10.1371/journal.pone.0108639.g002

Wurtzel *et al.* [15] performed a comparative study of *L. monocytogenes* and the non-pathogenic *L. innocua* using strand-specific cDNA sequencing. This resulted in genome-wide transcription start site maps and the identification of 183 ncRNAs. From the 183 reported ncRNAs, 100 were identified by our method, whereas half of the remaining ncRNAs were lacking expression.

Interestingly, there were a few examples where Wurtzel *et al.* [15] described a long candidate, which was covered by two or more candidates from our putative ncRNA set. These regions were discovered as several candidates by our method, since the expression pattern dropped down in between the candidates. The most noticeable example is anti1846 with a described length of 1371 nt, which overlaps with four of our candidates (216 nt, 141 nt, 23 nt and 227 nt).

In general, our method rather predicted longer ncRNAs which overlap with two or more previously described ncRNAs. For example, *LhrC-1–LhrC-4* were reported earlier as four ncRNA candidates [15] and have been merged by our approach to a single putative ncRNA, which conforms to the first description of this ncRNA by Christiansen *et al.* [38] in 2006. But even though the complete region was covered, the expression was not continuously on the same level.

Nevertheless, we missed a few of the ncRNA candidates described in previous studies (see Fig. 1). This can be attributed to the differences in the experimental setup: we used a different sequencing technology, different organisms at different expression time points, and a different subsequent *in silico* scoring. From the previously reported ncRNA candidates that were actually covered by reads, only a small fraction was rejected by our filtering steps.

From the 611 ncRNAs detected by our method, 474 were identified here by RNA-seq for the first time. From these, 33 candidates were already known from Rfam and 441 have, as far as we know, never been reported before.

In our set of predicted ncRNAs we found some highly interesting (long-)antisense ncRNAs (lasRNAs) with up to 5,400 nt, which were induced under intracellular conditions. Most of the lasRNAs described below were validated by qRT-PCR (Fig. 2).

## Internalins are very likely controlled by our detected lasRNAs

Two long ncRNA candidates were detected as antisense transcripts of two genes coding for the proteins *lmo0333* and *lmo1136* (see Tab. 3, and Fig. 3A,B). Both proteins *lmo0333* and *lmo1136* are similar to internalin proteins (according to NCBI annotation) and contain an LRR-LPXTG-motif.

Internalins (Inls) are a large group of proteins containing leucine-rich-repeats (LRR) and are known to play an important role in host-pathogen-interactions. The bacterial cell-surface anchored proteins InlA and InlB are required for cell-, tissue- and organ-specific invasion of *L. monocytogenes*. InlA engages the cell-junction protein E-Cadherin as its cellular receptor and InlB uses the hepatocyte growth factor receptor (HGFR, c-Met) for internalization [39]. Another cell-surface bound internalin is InlK, which binds to the Major Vault Protein (MVP) and thereby shields the bacterium from autophagy [40]. The secreted internalin InlC interacts directly with IKK$\alpha$, a subunit of the I$\kappa$B kinase complex, which is critical for the phosphorylation of I$\kappa$B and activation of NF-$\kappa$B, to suppress the inflammatory response [41].

The regulation of internalins is relevant to understand the virulence of *L. monocytogenes*. Previous studies showed that the master virulence regulatory protein PrfA regulates several internalins, e.g., *inlAB* and *inlC* [42]. Moreover, transcriptional regulation by the alternative sigma factor SigB was reported for several internalins, e.g., *inlA*, *inlB*, *lmo0263* and *lmo0610* [43,44].

Using RNA-seq, we showed in this study that internalins encoded by *lmo0333* (*inlI*) and *lmo1136* are subject of antisense transcriptional regulation by long non-coding antisense RNAs (lasRNAs) las0333 and las1136. *Lmo1136* is presumed to encode an internalin [20] which has not been studied so far. InlI was recently described and investigated by Sabet *et al.* [45] in the mouse infection model, but a knockout mutant for the *inlI* gene

**Table 3.** Selected candidates.

| Fig. | UpOrf | | DownOrf | | Start | Stop | S | cL(Size) | GC | IC | EC | P | RNAz | RNAL | Antisense | Score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antisense transcript of proteins with LRR+LPXTG motif | | | | | | | | | | | | | | | | |
| 3A | lmo0333 | + | lmo0334 | + | 361885 | 362047 | − | 163(163) | 0.410 | 28 | 0 | 13 | . | −18.80 | lmo0333 | 3 |
| 3A | lmo0333 | + | lmo0334 | + | 363242 | 363256 | − | 15(15) | 0.420 | 121 | 0 | 13 | . | . | lmo0333 | 2.5 |
| 3B | lmo1136 | + | lmo1137 | − | 1171054 | 1171546 | − | 493(493) | 0.449 | 87 | 0 | 13 | . | . | lmo1136 | 2.75 |
| Novel long antisense transcript (2,400 nt–3,800 nt) | | | | | | | | | | | | | | | | |
| 3C | lmo0537 | + | lmo0538 | + | 575365 | 575502 | − | 138(138) | 0.475 | 16 | 0 | 13 | . | . | lmo0537 | 2.5 |
| 3C | lmo0537 | + | lmo0538 | + | 575671 | 575866 | − | 136(196) | 0.478 | 13 | 0 | 13 | . | . | lmo0537 | 2.5 |
| 3C | lmo0538 | + | lmo0539 | − | 576528 | 577231 | − | 541(704) | 0.487 | 65 | 0 | 13 | . | −17.70 | lmo0538 | 3 |
| 3C | lmo0538 | + | lmo0539 | − | 577367 | 577631 | − | 226(265) | 0.444 | 499 | 388 | 13 | . | −22.60 | lmo0538 | 4 |
| Antisense transcripts of methylases | | | | | | | | | | | | | | | | |
| 3D | lmo0581 | − | iap | − | 617842 | 618101 | + | 164(260) | 0.403 | 55 | 0 | 13 | . | −21.20 | lmo0581 | 3 |
| 3D | lmo0581 | − | iap | − | 618619 | 618728 | + | 110(110) | 0.442 | 16 | 0 | 13 | . | . | lmo0581 | 2.5 |
| 3E | lmo0934 | + | lmo0935 | + | 971926 | 972112 | − | 140(187) | 0.501 | 16 | 0 | 13 | 0.974 | . | lmo0934 | 3 |
| 3E | lmo0935 | + | lmo0936 | + | 972702 | 972980 | − | 221(279) | 0.407 | 39 | 0 | 13 | . | . | lmo0935/lmo0936 | 2.75 |
| 3E | lmo0936 | + | lmo0937 | − | 973369 | 973508 | − | 91(140) | 0.391 | 1750 | 222 | 13 | 0.975 | . | lmo0936 | 3 |
| 3F | lmo0996 | + | clpE | − | 1026660 | 1026870 | − | 104(211) | 0.400 | 400 | 12 | 13 | . | −23.40 | lmo0996 | 3.5 |
| Antisense transcript of *kdpABCD* operon | | | | | | | | | | | | | | | | |
| 4 | lmo2676 | + | lmo2677 | − | 2748401 | 2748684 | − | 223(284) | 0.380 | 25 | 42 | 13 | 0.990 | −13.00 | lmo2676 | 3 |
| 4 | lmo2676 | + | lmo2677 | − | 2748684 | 2754031 | + | 4647(5348) | 0.429 | 228 | 35 | 13 | 0.973 | −17.96 | lmo2677/lmo2678/lmo2679/kdpC/kdpB | 4.25 |

Selected asRNAs and their genomic location, syntenic genes (UpOrf, DownOrf), corresponding GC-content and length (in brackets extended lengths for asRNA detection). IC – number of reads mapped to this region from intracellular library; EC – number of reads mapped to this region from extracellular library; P – number of closely related *Listeria* serotypes, with a homologous region identified in a genome-wide multiple sequence alignment; RNAz – p-Value of *de novo* ncRNA prediction of RNAz; RNAL – MFE of locally stable secondary structures, calculated by RNALfold; Score – Score assigned in this study. The complete list of all novel ncRNA candidates can be viewed at the supplemental page.

doi:10.1371/journal.pone.0108639.t003

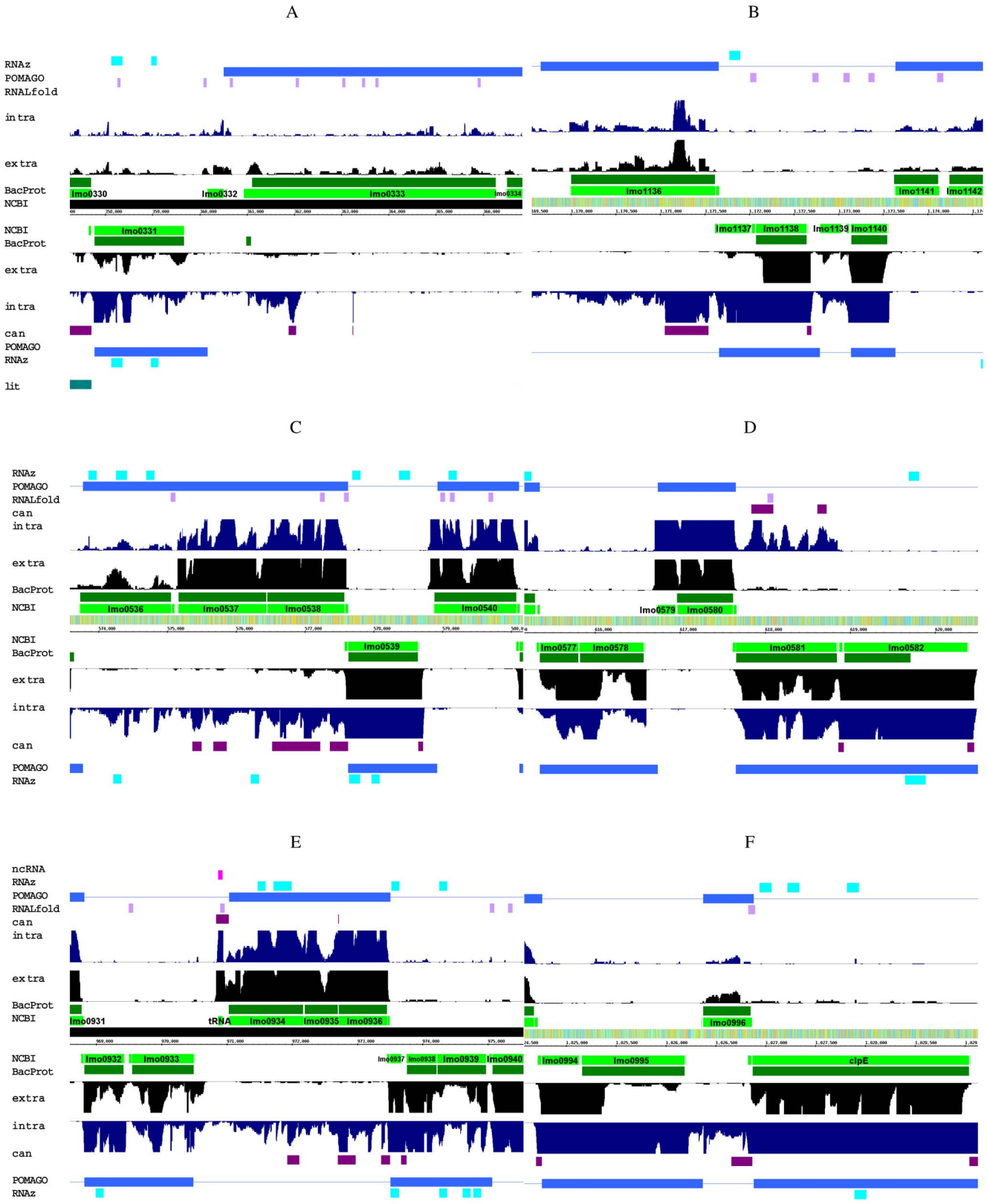


**Figure 3. Transcription of selected long asRNAs (lasRNAs)**: (A) Internalin protein; (B) Internalin protein (note the different scales of x-axis); (C) a novel long antisense transcript with more than 2,400–3,800 nt; (D) predicted SAM-dependent methyltransferase; (E) a rRNA methylase homolog; (F) similar to a methylated DNA protein cystein methyltransferase (note the different scales of x-axis). The upper half of each transcription profile represents the plus strand and the lower one the minus strand. Number of displayed reads is limited to 20. Dark purple – detected ncRNA candidates; lightgreen – NCBI annotation; darkgreen – BacProt annotation; black – reads of the extracellular library; dark blue – reads of the intracellular library; violet – locally stable secondary structure (analyzed with RNALfold); blue – conserved region among other *L. monocytogenes* serotypes (analyzed with

POMAGO); cyan blue – potential new ncRNAs predicted by RNAz; pink – annotated ncRNAs. A better resolution of the figure can be found in the supplement.
doi:10.1371/journal.pone.0108639.g003

did not exhibit any difference in virulence when compared to the wild type [45].

The long antisense transcripts of internalin have a length of 163 nt and 493 nt (Tab. 3). According to the expression levels those transcripts are presumably even longer, 1214 nt and 1617 nt respectively (see Fig. 3A,B). For *lmo0333* another antisense transcript of only 15 nt length, which is covered by 121 uniquely mapped reads, was detected. The number of reads mapping to the proposed lasRNAs varies between 28 and 121 reads. Interestingly transcription seems to be specific for *Listeria* grown in macrophages (intracellular) as for the extracellular condition no expression was observed.

We quantified the extra- and intracellular expression levels by qRT-PCR for all five selected lasRNAs (see Fig. 2A) and their corresponding mRNA transcripts (see Fig. 2B). All lasRNAs were up-regulated in the intracellular compartment. mRNA targets of *las0333* and *las0936* were repressed, whereas transcription of *lmo0996*, *lmo1136* and *lmo2677* was induced under intracellular conditions. This might indicate that these newly identified lasRNAs are involved in depression of target mRNAs (*lmo0333* and *lmo0936*) and stabilization of mRNA transcripts (*lmo0996*, *lmo1136* and *lmo2677*), what has been also reported for other lasRNA transcripts, e.g. from *Prochlorococcus* [46].

## Novel long antisense transcript (2,400 nt–3,800 nt)

An extremely long antisense transcript, spanning at least 2,400 nt (see Tab. 3), was observed antisense to *lmo0537* and *lmo0538*. Gene *lmo0537* codes for an amidohydrolase including a dimerization domain. The transcript contains four asRNA candidate loci, which might be also a single long antisense transcript. It is likely that the detected lasRNA influences its antisense genes *lmo0538* and *lmo0537*. However, this cannot be proven yet. Nevertheless, a rough inverse transcript pattern of the proteins and their expected antisense regulators is observable (see Fig. 3C). The antisense transcript of *lmo0537* seems to be specific for intracellular conditions.

## Antisense transcripts to methylases

Another example that caught our attention are antisense transcripts of various methylases, namely *lmo0581* (a predicted SAM-dependent methyltransferase, see Fig. 3D), *lmo0935* (CspR protein, a rRNA methylase homolog, see Fig. 3E) and *lmo0996* (similar to a methylated DNA protein cystein methyltransferase, see Fig. 3F).

The antisense transcript of *lmo0581* was mainly observed for the intracellular condition (see Fig. 3D). Even though the expression is very low in some parts, it is spanning *lmo0581* (1161 nt) completely. Gene *lmo0581* itself is transcribed under extracellular and intracellular growth conditions.

The second putative lasRNA spans three genes (see Fig. 3E): it was detected antisense to *lmo0936* (similar to nitroflavin-reductase), *lmo0935* (SpoU, rRNA methylase) and *lmo0934* (uncharacterized Fe-S protein, energy production and conversion). One striking feature of this candidate is its length of 2,500 nt. Even though the transcription rate is very low in some regions, an antisense transcript of this length is remarkable. Whereas the transcription of the lasRNA is specific for intracellular grown *Listeria*, the genes are covered with reads originating from both growth conditions.

The third methyltransferase having putative asRNA transcripts is *lmo0996* (see Fig. 3F), which is similar to methylated DNA-protein-cystein methyltransferase. This asRNA is an intergenic transcript and appears to be transcribed continuously with its syntenic genes *lmo0997* (*clpE*, ATP-dependent protease) and *lmo0995* (predicted acetyltransferase). The intergenic transcription is observed only in intracellularly grown *Listeria*. This indicates that the reads cannot be simply attributed to extended 5′ or 3′ UTRs, but are rather a putative specific intracellular ncRNA. We observed only very low transcription for the protein gene *lmo0996*, neither for extracellular nor for intracellular conditions.

All of the above mentioned antisense transcripts are short (91–221 nt) and covered by 16–1750 reads (see Tab. 3). The read pattern of the ncRNA candidates is rather unsteady. A direct influence of the lasRNAs to the methylases can be only hypothesized.

## The *kdpEDABC* operon is controlled by an extremely long non-coding antisense RNA

Among the newly detected lasRNAs we have identified a very long antisense RNA of about 5,400 nt which completely covers the region from *lmo2677* up to *lmo2680* and partially the gene *kdpB* (see Tab. 3 and Fig. 4). This lasRNA is strongly activated during the intracellular growth phase of the pathogen and was confirmed by qRT-PCR (see Fig. 2) analysis. Previously Wurtzel *et al.* [15] described an asRNA for *lmo2678*, which is transcribed under exponential growth at 37°C and is controlled by SigB. The gene *lmo2678* encodes the response regulator (KdpE) of a two component system (TCS) together with a cognate histidine kinase (KdpD) encoded by *lmo2679* [47]. Under high-osmolarity conditions the KdpED TCS regulates the adjacent *kdpABC* operon which is responsible for high-affinity potassium uptake as previously reported for *Escherichia coli* [48]. Several different reports described KdpED to be involved in intracellular survival of pathogenic bacteria, for example *Staphylococcus aureus*, enterohaemorrhagic *E. coli*, *Salmonella typhimurium* and *Yersinia pestis* [49]. In *L. monocytogenes*, however, it does not seem to play an important role in virulence [50]. This is supported by the observation that the entire locus *lmo2677–lmo2681(kdpB)* is down-regulated by massive antisense transcription. This suggests that alternative uptake systems exist to ensure potassium uptake. Such systems have been already reported for *B. subtilis* [51]. It is, however, unclear why this long asRNAs is necessary to block the *kdpED* TCS and *kdpABC* operon under intracellular conditions. Why is a short asRNA, as described by Wurtzel *et al.* [15], produced during extracellular growth conditions, not sufficient to stop transcription of *lmo2678* and the *kdpED* TCS/*kdpABC* operon? We speculate that these asRNAs do not only stringently regulate transcription in *cis*, but also in *trans*.

Recently Mellin *et al.* [16] reported that in the presence of vitamin $B_{12}$, the corresponding riboswitch induces transcriptional termination. This causes an antisense RNA *aspocR* to be transcribed as a short transcript. In the absence of vitamin $B_{12}$, *aspocR* is transcribed as a long antisense RNA, inhibiting *pocR* expression [16]. A similar non-classical function could be also assumed for the *kdpEDABC* interfering las2677/las2678 RNAs.

Furthermore, there seems to be a correlation between the asRNA read pattern and the start and stop sites of the operon genes. For example, for *lmo2678/kdpE* there is an increase and decrease correlating with the start and stop positions of this (see

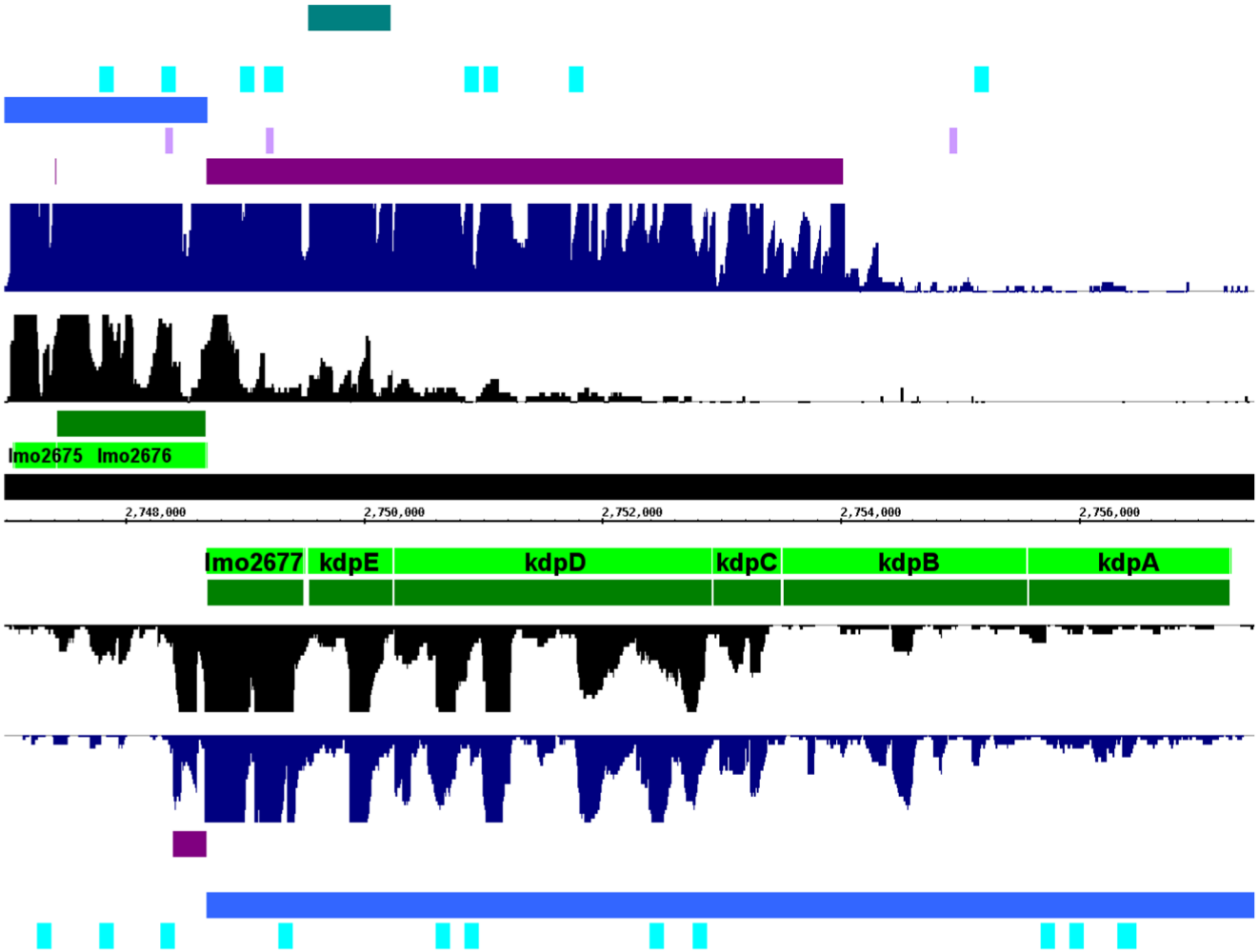


**Figure 4. Transcription of a selected long asRNA (lasRNA)**: *kdpABCD* operon. Number of displayed reads is limited to 20. Dark purple – detected ncRNA candidates; lightgreen – NCBI annotation; darkgreen – BacProt annotation; black – reads of the extracellular library; dark blue – reads of the intracellular library; violet – locally stable secondary structure (analyzed with RNALfold); blue – conserved region among other *L. monocytogenes* serotypes (analyzed with POMAGO); cyan blue – potential new ncRNAs predicted by RNAz; pink – annotated ncRNAs; teal green – ncRNA candidates of previous studies.
doi:10.1371/journal.pone.0108639.g004

Fig. 4). It is tempting to speculate whether this lasRNA is originating from *lmo2676* or not. In case it is originating from *lmo2676*, the transcript might resemble an excludon. Interestingly another ncRNA candidate was detected directly downstream to *lmo2677* (see Fig. 4). Nevertheless, this seems to be a separate transcript and not an extended 3′UTR, since there is an obvious decrease of reads at the end of *lmo2677*. This ~300 nt RNA antisense to the 5′part of *lmo2676* is stronger expressed under extracellular conditions.

To confirm our newly identified asRNAs in another *L. monocytogenes* serotype 1/2a strain, we have preformed additional RNA-seq experiments (unpublished RNA-seq data, online supplementary material) with the commonly used *L. monocytogenes* strain 10403S grown under extra- and intracellular conditions. Comparison of presence/absence of the las0333, las0936, las0996, las1136 and las2677 showed a similar occurrence of these asRNAs between *L. monocytogenes* strain 10403S and EGD-e. This implicates a conserved expression mechanism for *L. monocytogenes* serotype 1/2a strains for these selected asRNA candidates.

In addition, we have also tested the transcription regulator mutant of *L. monocytogenes* EGD-e Δ*prfA* under the same experimental conditions described above. Our RNA-seq analysis (unpublished RNA-seq data, online supplementary material) showed that all above mentioned asRNAs were independently controlled by the master virulence regulator PrfA. Furthermore, these new RNA-seq data warrant detailed investigation in future.

## Conclusion

We systematically used the semiconductor sequencing technology for RNA-seq to identify ncRNAs and determine the difference of extra- and intracellular growth conditions. We reported bacterial antisense transcripts with a size up to 5,400 nt. It would be interesting to use our pipeline to examine whether similar transcripts can be observed in other bacteria. Further work has to be done to fully understand the functional role of these long non-coding antisense RNAs in bacterial physiology. Particularly in the case of the *kdpABCD* operon, the regulation of $K^+$ by long non-coding antisense RNAs now deserves further attention.

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## Author Contributions

Conceived and designed the experiments: TH MM. Performed the experiments: SW GKM XQ RM. Analyzed the data: SW MAM TH MM. Contributed reagents/materials/analysis tools: SW GKM XQ RM TC MAM TH MM. Wrote the paper: SW GKM XQ RM TC MAM TH MM.

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# Current status of antisense RNA-mediated gene regulation in *Listeria monocytogenes*

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*Listeria monocytogenes* is a Gram-positive human-pathogen bacterium that served as an experimental model for investigating fundamental processes of adaptive immunity and virulence. Recent novel technologies allowed the identification of several hundred non-coding RNAs (ncRNAs) in the *Listeria* genome and provided insight into an unexpected complex transcriptional machinery. In this review, we discuss ncRNAs that are encoded on the opposite strand of the target gene and are therefore termed antisense RNAs (asRNAs). We highlight mechanistic and functional concepts of asRNAs in *L. monocytogenes* and put these in context of asRNAs in other bacteria. Understanding asRNAs will further broaden our knowledge of RNA-mediated gene regulation and may provide targets for diagnostic and antimicrobial development.

**Keywords: *Listeria monocytogenes*, antisense RNA, asRNA, regulation, next generation sequencing, bacteria**

## INTRODUCTION

*Listeria monocytogenes* is a Gram-positive, facultative foodborne pathogen that causes a severe life-threatening disease (listeriosis) in susceptible humans and animals. Complex regulatory mechanisms allow *L. monocytogenes* to adapt and survive in a wide range of environmental conditions (e.g., low temperature, high pH, and high-salt conditions) and infect a variety of hosts including mammalia and insects (Cossart and Toledo-Arana, 2008). Furthermore, *L. monocytogenes* was used as a model pathogen for the investigation of key elements of cell mediated immunity (Witte et al., 2012). Given its implications as public health concern, versatility as a bacterium and experimental model, significant effort has been undertaken to characterize genomic and transcription regulation in *L. monocytogenes* (Cossart and Lebreton, 2014).

Genomic studies uncovered crucial genes regulating listerial pathogenesis, such as the ∼9 kb virulence gene locus *Listeria* pathogenicity island-1 (LIPI-1) in which the major virulence determinants are organized (Chakraborty et al., 2000; Glaser et al., 2001). However, interpretation of genome-wide gene regulation in *Listeria* remains challenging due to the complex regulatory networks that are controlled by transcription regulators and alternative sigma factors (e.g., PrfA, $\sigma^B$, and CodY) (Chaturongakul et al., 2011; Lobel et al., 2012; Xayarath and Freitag, 2012).

The recent discovery of the presence of non-coding RNA (ncRNA) elements in various bacterial genomes added a further layer of complexity in our understanding of bacterial gene regulation. In the last decade a myriad of non-coding RNAs (ncRNAs) of different genomic origin, length, function, and mechanisms of gene regulation were identified (Gottesman and Storz, 2011; Storz et al., 2011; Caldelari et al., 2013).

Although ncRNAs represent a heterogeneous group, they can roughly be divided into three categories. The first category consists of regulatory elements that are located in the 5′UTR of their targets (e.g., riboswitches, thermosensors, or pH-sensors). An important example in *L. monocytogenes* is a thermosensor that controls the major virulence regulator PrfA of LIPI-1. At low temperatures (∼30°C) the thermosensor forms a complex secondary structure that prevents translation of PrfA by interfering with the Shine-Dalgarno (SD) region (Johansson et al., 2002).

*Trans*-encoded small RNA (sRNA) could be considered as the second category. Those transcripts regulate genes located elsewhere on the genome and share only limited complementarity with the target. They often interact with multiple different target transcripts, and therefore function analogous to human microRNA (Gottesman, 2005). To date, 154 sRNA were identified in the genome of *L. monocytogenes* and primarily termed as rli (Mandin et al., 2007; Wurtzel et al., 2012).

The last group of ncRNAs, designated as *cis*-encoded antisense RNAs (asRNAs), is located on the opposite DNA strand of their target and therefore share a high degree of complementarity with it. There is growing evidence that asRNAs are present in several

Gram-positive and Gram-negative bacterial species and families with a high variability in prevalence and genomic density (Georg and Hess, 2011). The fraction of genes with a reported asRNA varies significantly with ∼75% in cyanobacterium *Prochlorococcus* (Voigt et al., 2014), ∼46% in *Helicobacter pylori* (Sharma et al., 2010) compared to ∼20% in *Escherichia coli* (Georg and Hess, 2011).

In this review, we focus on the current status of reported asRNAs in *L. monocytogenes*, their function and outline mechanisms where applicable. A general review of the function of ncRNAs in *Listeria* is outside of the scope of this review and is summarized elsewhere (Izar et al., 2011; Cossart and Lebreton, 2014).

## IDENTIFICATION OF asRNAs IN *L. MONOCYTOGENES*

The reliable detection of antisense RNA is challenging because of technical difficulties. A major problem using microarrays, for instance, is artificially generated products during cDNA synthesis from RNA (Perocchi et al., 2007). Recently, major technical developments for generating and analyzing high-throughput data contributed to an increase in quantity and quality of information on asRNA. Until 2009, only a few asRNAs were described for *L. monocytogenes* by means of classical methods (Mandin et al., 2007). With the advent of whole genome tiling arrays and next-generation sequencing methods the number of asRNAs expanded exponentially (Toledo-Arana et al., 2009; Mraheil et al., 2011; Wurtzel et al., 2012; Behrens et al., 2014). Toledo-Arana et al. identified 21 novel asRNAs as well as 50 sRNAs (defined as <500 nucleotides), including seven that were located on the opposite strand of another transcript (Toledo-Arana et al., 2009). Applying a whole genome tiling array approach, this group investigated transcription profiles in several settings, such as growth of *L. monocytogenes* in different phases (exponential and stationary phase), distinct media, and organs (rich media, blood, and intestine) and under stress conditions (hypoxia and low temperature). This study demonstrated the influence of regulatory RNAs in response to different microenvironments.

Using 454 pyrosequencing, Mraheil et al. revealed a large portion of known regulatory RNAs. In total the 150 discovered regulatory RNA elements, of which 71 were previously unknown, include 29 asRNAs (Mraheil et al., 2011). Comparing expression profiles of extracellular bacteria to that in the intracellular compartment of murine macrophages, the authors found differential expression of asRNAs. This observation supports the notion that expression of regulatory RNAs (such as asRNAs) changes in response to extrinsic stimuli and therefore contribute to an adaptive expression program.

Another next generation sequencing platform, namely Illumina was used by Wurtzel and colleagues. In a RNA-seq experiment with transcription start site (TSS)-detection they identified 86 additional ncRNAs, including 50 novel asRNAs (Wurtzel et al., 2012). Comparing the transcriptome of *L. monocytogenes* with the closely related non-pathogenic *Listeria* species, the authors found significant divergence in the repertoire of regulatory RNAs. Furthermore, this study identified long asRNAs that are complimentary to genes but also function as sense transcripts for divergently oriented genes. Those unprecedented constructs were named "excludons" (Wurtzel et al., 2012).

The last study to date was performed by Behrens and colleagues. Using the SOLiD ultra deep sequencing platform and choosing similar conditions as Mraheil et al. (2011), 90% of known regulatory RNAs were confirmed and additional nine asRNAs were identified (Behrens et al., 2014). Moreover, four asRNAs previously described (Toledo-Arana et al., 2009; Wurtzel et al., 2012) were confirmed in this study and—likely as a consequence of higher coverage rate—were predicted to be even longer than initially reported.

In summary, using different array and sequencing methods more than hundred asRNAs were described in *L. monocytogenes* to date.

## CLASSIFICATION AND MECHANISTIC CONCEPTS OF asRNAs

Antisense RNA derives from promoters located on the complementary strand of a gene or operon they target. Reported asRNAs in *L. monocytogens* comprise a heterogeneous group of transcripts with significant variability in length (30 to thousands of nucleotides), differences in origin and mechanisms (Mandin et al., 2007; Toledo-Arana et al., 2009; Mraheil et al., 2011; Wurtzel et al., 2012).

According to these characteristics asRNAs can roughly be classified in five categories: (i) short, (ii) long, (iii) 3′UTR, (iv) 5′UTR, and (v) excludon (**Figure 1**).

Short asRNA that are antisense to genes in *L. monocytogenes* are for example rliE, rli23, rli25, rli29, rli30, and rli35 (Toledo-Arana et al., 2009).

Besides this, a remarkable example of sRNAs oriented antisense to each other was described for rli112. This sRNA is encoded in the intergenic region between *lmo2709* and *lmo2710* and is located antisense to the sRNA rli50 (Mraheil et al., 2011). Furthermore, another asRNA (rli28/29) is predicted to be antisense to rli78, which shares 94% homology with rli112 (Mraheil et al., 2011). To date, eight additional pairs or even groups of sRNA oriented antisense to each other have been described: rliC&rli125/rli85, rli42&sbrA/rli89, rli94&rli44, rliF&rli95, rli45&rli46, rli138&rli139, rli98&rli48, rli99&rli140 (Mandin et al., 2007; Toledo-Arana et al., 2009; Mraheil et al., 2011; Wurtzel et al., 2012).

Long asRNAs are transcripts of several hundred nucleotides that overlap more than one ORF. A representative of this class covers *lmo2095–lmo2098* (Toledo-Arana et al., 2009). Interestingly, using tiling array and northern blot analysis, two different antisense transcripts with the same transcription start side but alternative termination sites were detected. While one transcript (RNA1) was 255 nucleotides in length and located exclusively antisense to *lmo2095*, the second transcript (RNA2) was 2149 nucleotides in length and spans across neighboring genes partially including *lmo2098* (**Figure 1B**). The same study reported two other long asRNA that were slightly shorter but still span multiple ORFs (anti2095–2098 and anti2394–2395). Four additional potential long asRNAs (anti2046, anti2259, anti2677, und anti2717) overlapping to multiple ORFs were recently described (Behrens et al., 2014).

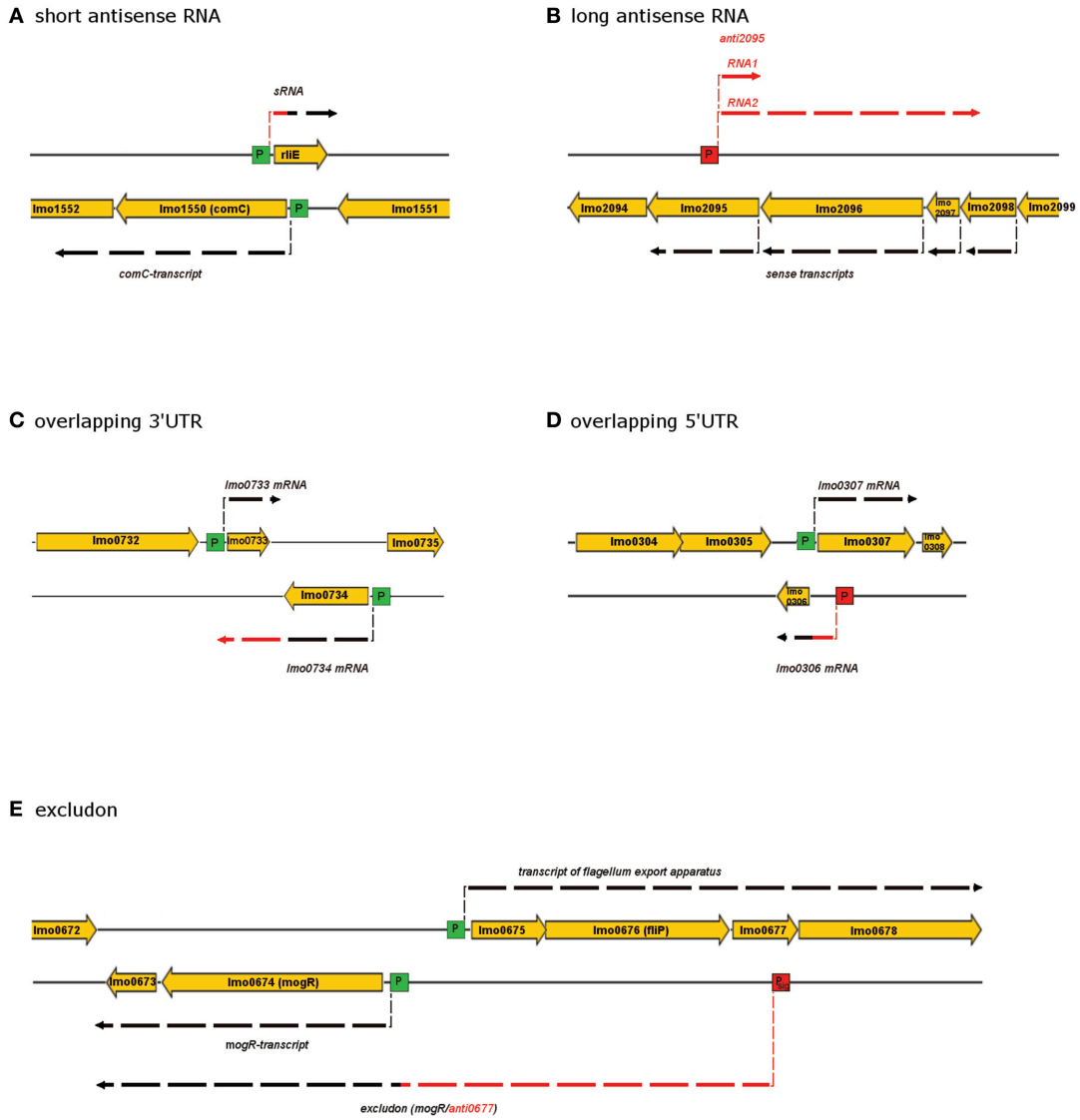


**FIGURE 1 | Classes of *cis*-mediated antisense regulation found in *L. monocytogenes*.** Genes are depicted as yellow arrows while transcripts are illustrated by dashed lines. Red color of dashed lines highlights regions antisense to other transcripts. Schematic views of a short antisense RNA regulation **(A)**, a long antisense RNA regulation **(B)**, overlapping 3′UTR **(C)**, overlapping 5′UTR **(D)**, and the excludon concept **(E)** are given.

The concept of 5′-UTR overlapping asRNAs were found for some adjacent genes that are divergently oriented (transcription takes place in opposing direction starting from proximal promoters). It might represents an effective way to regulate neighboring genes. For example, transcription of *lmo0306* starts in the 5′UTR and thereby overlaps with the transcript of *lmo0307* (**Figure 1D**).

3′UTR asRNA are conceptually similar to 5′UTRs, however, the involved genes are located in a convergent orientation (distal promoters on opposite strands with converging transcription direction). For example, *lmo0733* and *lmo0743* both encoding putative transcription regulators, interact through 3′UTR (**Figure 1C**). The transcripts of *lmo0734* substantially overlap the ORF of the divergent oriented *lmo0733* with 750 nucleotides (Toledo-Arana et al., 2009). Thus, asRNAs deriving from both 5′- and from 3′UTR of adjacent genes exemplify a way to link the expression of two neighboring genes.

Most recently, a new antisense RNA-mediated concept of gene regulation was discovered in *L. monocytogenes*—the excludon (Wurtzel et al., 2012). An excludon is a remarkably long asRNA extending over multiple neighboring genes, which are organized in two sets—one set of genes being divergent orientated to the other (**Figure 1E**). The asRNA overlaps with one set of genes and thereby prevents expression of those by complementation, while it serves as a coding sequence for the other. Ipso facto, expression of the overlapping gene is inhibited, while expression of the opposite divergent gene is increased. Genes regulated by excludons often have related or opposite function, thus, it is most likely that

excludons serve as asRNA-mediated biological switches (Wurtzel et al., 2012).

## MECHANISMS OF asRNA IN *L. MONOCYTOGENES*

Although next generation sequencing was instrumental in the identification of several novel asRNAs in *L. monocytogenes*, precise mechanisms of action of asRNAs remain largely unknown. Based on limited mechanistic knowledge in *L. monocytogens* and mechanisms of asRNAs in other bacteria, some concepts have emerged. asRNA/target interactions can occur on different levels: (i) transcription, (ii) transcript stability, or (iii) translation.

On a transcriptional level, two mechanisms, transcription interference, and transcription attenuation were described. In transcription interference, the transcription of the target sequence is hindered by parallel transcription of the asRNA from a promoter locate opposite convergent from the sense promoter. The resulting asRNA is likely just a byproduct of this mechanism and the process of asRNA transcription itself rather than the intrinsic asRNA function represents the regulatory mechanism (Brantl and Wagner, 2000; Callen et al., 2004). In transcription attenuation sense transcription is prematurely stopped by a termination structure that forms upon interaction of the asRNA with the mRNA (Brantl and Wagner, 2000; Stork et al., 2007). To date, these mechanisms were confirmed in *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Vibrio anguillarum* (Stork et al., 2007; Brantl and Bruckner, 2014), but not in *L. monocytogenes*.

asRNA-mediated alteration of transcript stability could occur by complementation with subsequent RNase-mediated degradation of the sense/antisense RNA duplex as shown in *Salmonella typhimurium*, *S. aureus*, and in *Synechocystis* sp. (Duhring et al., 2006; Lee and Groisman, 2010; Lasa et al., 2012). Although most asRNA/mRNA interactions are thought to result in degradation of the target sequence, asRNAs have also the potential to stabilize a sense transcript. Mechanisms involve the stabilization of transcripts by inducing cleavage of unstable polycistronic transcripts. A striking example of this case was demonstrated in *Escherichia coli* for *gadXW* (Opdyke et al., 2004, 2011; Tramonti et al., 2008).

Another stabilizing mechanism shown in *Prochlorococcus* sp. MED4 and *Synechocystis* sp. PCC 6803 functions via the masking of the RNases cleavage sites and thereby prevent degradation of a target transcript by formation of the asRNA/mRNA duplex (Stazic et al., 2011; Sakurai et al., 2012). So far, none of these regulatory mechanisms were demonstrated in *L. monocytogenes*.

Besides those mechanisms, some asRNA are supposed to also function in *trans*. Therefore, these transcripts can interact with genes encoded at different sites in the chromosome.

The asRNA rliE in *L. monocytogenes* is illustrative of this class. rliE overlaps with the gene *comC* and thereby likely acts as *cis*-regulator. In addition, as possible targets for rliE in *trans comEA-EB-EC*, *comFA-FC,* and *lmo0945* were found (Mandin et al., 2007). Similar to *comC*, all of these genes are putatively involved in competence, thus, rliE may represent a global regulator of this machinery.

At a more distal level, asRNAs can prevent translation by binding to the SD sequence of the target mRNA (Kawano et al., 2007). Inability of the ribosome to bind the SD region obstructs translation of the sense sequence.

## WHAT ARE THE MAIN FUNCTIONS OF ANTISENSE RNA IN *LISTERIA MONOCYTOGENES*?

Reports on precise biologic functions of asRNAs in *L. monocytogenes* remain scarce and knowledge on asRNAs is mostly of descriptive nature. Reviewing functions of asRNA for bacteria it has been reported that antisense RNA regulation is frequently used for distinct purposes. In detail, asRNA is used to repress transcription of transposases or genes that encode for toxins as well as to control the expression of transcription regulators (Thomason and Storz, 2010). This is consistent with three asRNAs rli23, rli25, and rli35 described in *L. monocytogenes,* which overlap the transposase genes *lmo0172*, *lmo0330,* and *lmo0828,* respectively (Toledo-Arana et al., 2009). Furthermore, asRNAs that target transcription regulators are abundantly found in the *Listeria* genome, such as the above mentioned *lmo0733* and *lmo0734* (**Figure 1C**) (Toledo-Arana et al., 2009). In total, ∼10% of all asRNA described for *L. monocytogenes* to date are thought to be involved in regulating transcription regulators.

Besides this, the well-investigated asRNA in *L. monocytogenes* are implicated in the control of metabolism, virulence, bacterial architecture and different transporting systems (Toledo-Arana et al., 2009; Mraheil et al., 2011; Wurtzel et al., 2012; Mellin et al., 2013; Behrens et al., 2014) and presage significant involvement of asRNAs in different domains of bacteria.

The best-established function was described for anti0677 controlling the flagellum biosynthesis excludon, which downregulates *lmo0675-0676-0677* encoding for the flagellum export apparatus and contributing to expression of the motility gene repressor MogR (*lmo0674*) (Toledo-Arana et al., 2009). The anti0677 promoter is responsive to the stress and temperature-activated transcription regulator RNA polymerase factor $\sigma^B$. Temperature-induced MogR-mediated flagellum biosynthesis suppression was shown to be important for virulence of *L. monocytogenes* (Grundling et al., 2004). Although disputed in literature, flagellum expression has been suggested to induce the host inflammatory response (Hayashi et al., 2001). Thus, anti0677 inhibits expression of the flagellum export apparatus and promotes MogR expression and might thereby also contribute to abrogating the host response to *L. monocytogenes*.

Recently, Mellin et al. described a vitamin B12-binding riboswitch-regulated asRNA (Mellin et al., 2013). The *pocR* gene (*lmo1150*) encodes a transcriptional regulator, which activates transcription of the neighboring *pdu* and *cob* genes in the presence of propanediol. Pdu and Cob are essential for the catabolism of 1,2-propanediol catabolism and vitamin $B_{12}$ biosynthesis. Propanediol is a byproduct of the metabolism of commensal intestinal bacteria. The ability to metabolize propanediol is important for pathogenicity and provides a survival advantage for bacterial during infection. In the process of propanediol catabolism vitamin $B_{12}$ is required as a cofactor for involved enzymes. The reported asRNA anti1150 (*aspocR*) overlaps with the *pocR* gene. Interestingly, *aspocR* is controlled by a vitamin $B_{12}$ dependent riboswitch that prematurely terminates transcription of *aspocR* in presence of vitamin $B_{12}$ and thereby generates

only a small transcript previously known as rli39. Subsequently, PCR-based experiments confirmed that *pocR* transcription was negatively regulated by *aspocR*. Additional experiments using ectopically transcribed *aspocR* showed inhibitory action in *trans* on *pocR* expression. These findings emphasize that the utilized mechanism is rather transcription attenuation or inhibition of translation than transcription interference or modulation of transcript stability in this case. Given that *pocR* is important for vitamin $B_{12}$ biosynthesis, here antisense regulation seems to be rather a fine-tuning mechanism than an on- off-switch (Mellin et al., 2013).

Two further reported excludons, anti1846 and anti0605, affect the regulation of a permease-efflux pumps and a putative permease-efflux pump, respectively (Wurtzel et al., 2012). Notably, the promoter of the anti0605-controlled excludon is sigB responsive. These excludons might represent a biologic switch to change between cellular uptake and release of components based on the extracellular environment.

Another reported excludon (anti0424) is most likely involved in regulating central metabolic pathways in *L. monocytogenes*. As it spans two divergently oriented genes encoding for enzymes necessary for the usage of different carbon utilization, it might represent a possibility for a selective switching between those pathways (Wurtzel et al., 2012).

## CONCLUSION

Technological and methodological advances transformed the field of RNA-mediated gene regulation in bacteria and provided insight into an unexpected complexity. In *L. monocytogenes* hundreds of ncRNAs, including even more than hundred asRNAs possibly implicated in the regulation of 102 *Listeria* genes, were discovered to date.

This number seems rather low compared to the scope reported from other bacteria and will presumably rise with further studies. Yet, as recent findings in *L. monocytogenes* show the dependency of some antisense transcripts on transcription factors or even the absence of a metabolite, the importance of experimental conditions is highlighted.

Also despite the rather low extent of asRNAs reported to date, *L. monocytogenes* has proven to be a valuable model organism for studying asRNA regulation and given rise to novel discoveries like the excludon concept that could then be transferred to other bacteria.

It might be speculated that asRNAs in *L. monocytogenes* likely act through different mechanisms and could either function as an on-off switch or fine regulators of a particular network. Thereby, asRNAs might be involved in regulating metabolic processes, virulence and determinants of host inflammatory response. In addition, the impact of asRNA regulation is spread as many targets of antisense regulation then again affect the expression of other genes (e.g., transcriptional regulators).

However, our understanding about mechanisms and function remains limited to few individual transcripts. Mechanistic and functional validation of ncRNAs, including asRNAs, will shed further light into the extent of RNA-mediated regulation in bacteria. This understanding may then allow to develop new approaches for therapeutics.

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