Department for Farm Animals and Veterinary Public Health

University of Veterinary Medicine Vienna

Institute of Food Safety, Food Technology and Veterinary Public Health

(Head: Dipl.ECVPH Univ.-Prof. Dr.med.vet. Martin Wagner)

Characterization of the RHS protein in bacterial competition of *Listeria monocytogenes*

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Theresa Harm

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Supervision: Dr.rer.nat. Kathrin Kober-Rychli

Unit of Food Microbiology

Institute of Food Safety, Food Technology and Veterinary Public Health

University of Veterinary Medicine Vienna

Veterinärplatz 1, 1210 Vienna; Austria

Kathrin.rychli@vetmeduni.ac.at

Reviewer: Dr.rer.nat. Tom Grunert

Unit of Functional Microbiology

Institute of Microbiology

University of Veterinary Medicine Vienna

Veterinärplatz 1, 1210 Vienna; Austria

tom.grunert@vetmeduni.ac.at

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1. Introduction

1.1 *Listeria monocytogenes*

The genus *Listeria spp.* comprises currently at least 17 species of rod-shaped gram-positive bacteria, including *Listeria* (*L.*) *monocytogenes*. All of these species are facultative anaerobe, nonsporulating and are able to grow and survive at low temperatures, low and high pH and high salt concentrations. Focussing on *L. monocytogenes*, this *Listeria* species grows between -0.4 °C and 50 °C and pH-values from 4.5 to 7.0. It possesses peritrichous flagellation, which allow *L. monocytogenes* tumbling motility between 20 °C and 25 °C. At higher temperatures the expression of the flagella and thus motility is reduced.

L. monocytogenes is able to survive many environmental stresses due to its physiological adaptations and can grow in different environments, such as soil, water and animals. This great adaptability allows *L. monocytogenes* to counteract different immune defence mechanisms and even cross the blood-brain-barrier or the placenta in infected individuals. As *L. monocytogenes* can grow and persist in the food processing environment, too, its adaptability and potent pathogenicity make it a great concern for human health (Farber and Peterkin 1991, Gandhi and Chikindas 2007, Orsi and Wiedmann 2016, Rodríguez-Auad 2018).

1.1.2 Pathogenicity of *Listeria monocytogenes*

Only two out of the 17 species forming the genus *Listeria spp.*, *L. monocytogenes* and *L. ivanovii*, are pathogenic. *L. ivanovii* is predominately pathogenic for animals such as ruminants, but can rarely infect humans, too, whereas *L. monocytogenes* causes infections in humans and more than 40 different animal species. *L. monocytogenes* is a facultative intracellular pathogen, which causes a rare, but severe human illness called listeriosis (Orsi and Wiedmann 2016).

There are four different evolutionary lineages of *L. monocytogenes*, I, II, II and IV, with differences in virulence genes, ribotyping and DNA arrays, but overlapping ecological niches. Whereas lineage III and IV are rare and predominately found in animals, lineage I and II strains are more often associated with human listeriosis. These two lineages I and II are commonly

found in spoiled food, but human listeriosis cases are more often caused by *L. monocytogenes* belonging to the lineage I. To differentiate single *L. monocytogenes* strains a typing system based on somatic (O) and flagellar (H) antigens is used. Based on this method 13 serotypes can be discriminated, but only four of them are responsible for most of the human listeriosis cases. These include serotype 4b and 1/2b belonging to the lineage I and serotype 1/2a and 1/2c belonging to the lineage II (Cossart 2011, Orsi et al. 2011).

The food-borne pathogen *L. monocytogenes* causes listeriosis, which generally occurs in sporadic cases, but small epidemics are also possible. Sporadic cases of listeriosis are associated with dietary risk factors, including consumption of raw or undercooked foods and cold cuts. The main foods implicated are milk, unpasteurized soft cheeses, ready-to-eat prepared meats, undercooked poultry and fish, unwashed raw vegetables and even contaminated fruits. Unlike most other foodborne pathogens, *L. monocytogenes* can grow in food with low moisture content, high salt concentration and at low temperatures, which make it hard to control (Noordhout et al. 2014, Rodríguez-Auad 2018).

Listeriosis is potentially lethal especially in immunocompromised and newborn or unborn individuals. The mortality rate among infected individuals is about 20-30 %, which is very high, but the annual number of infections is rather low. In 2018 a total of 2,549 cases of human listeriosis were reported in the EU, corresponding to a notification rate of 0.47 confirmed cases per 100,000 people. Compared to 2017 this led to a slight increase of infections of 2.75 %. The overall fatality rate in the EU among cases with known outcome was 15.6 % in 2018, which increased from 13.6 % in 2017. Infections with *L. monocytogenes* were most commonly reported for elderly people, whose age was above 64 years and particularly over 84 years (European Food Safety Authority and European Centre for Disease Prevention and Control 2019).

L. monocytogenes causes gastroenteritis in healthy individuals, but can cause severe illnesses, like sepsis, meningitis or encephalitis, in immunocompromised individuals. Infections during pregnancy can result in abortions, stillbirths or preterm birth. Unborn babies and neonates can be infected through vertical transmission from the mother to the baby, but also during birth by ascending colonisation from the vagina. In rare cases, cutaneous listeriosis may also occur, which manifests itself through eczematous skin infections (Allerberger and Wagner 2010, Cossart 2011, Noordhout et al. 2014).

As contaminated food is the major source of infection with *L. monocytogenes* the gastrointestinal tract is the primary entry site of the pathogens into the host. Before reaching the intestine the ingested bacteria must withstand the adverse environment of the gastrointestinal tract and adapt to available nutrients and various physical stresses such as changes in pH, osmolarity and desiccation and *L. monocytogenes* has to overcome a variety of host immune barriers, too. Once reached the intestinal tract, *L. monocytogenes* penetrates the mucosal tissue either directly, via invasion of enterocytes, or indirectly, via active penetration of the Peyer's patches. Bacteria, that crossed this barrier, are carried by blood or lymph to the lymph node, the spleen and the liver. The principal site of bacterial multiplication are the hepatocytes of the liver then. In immunocompromised individuals *L. monocytogenes* is also able to cross the blood-brain barrier or penetration of the placental barrier may occur in pregnant individuals. The infection process and its effects are shown in Fig. 1 (Barbuddhe and Chakraborty 2009, Johansson and Freitag 2019, Vázquez-Boland et al. 2001).

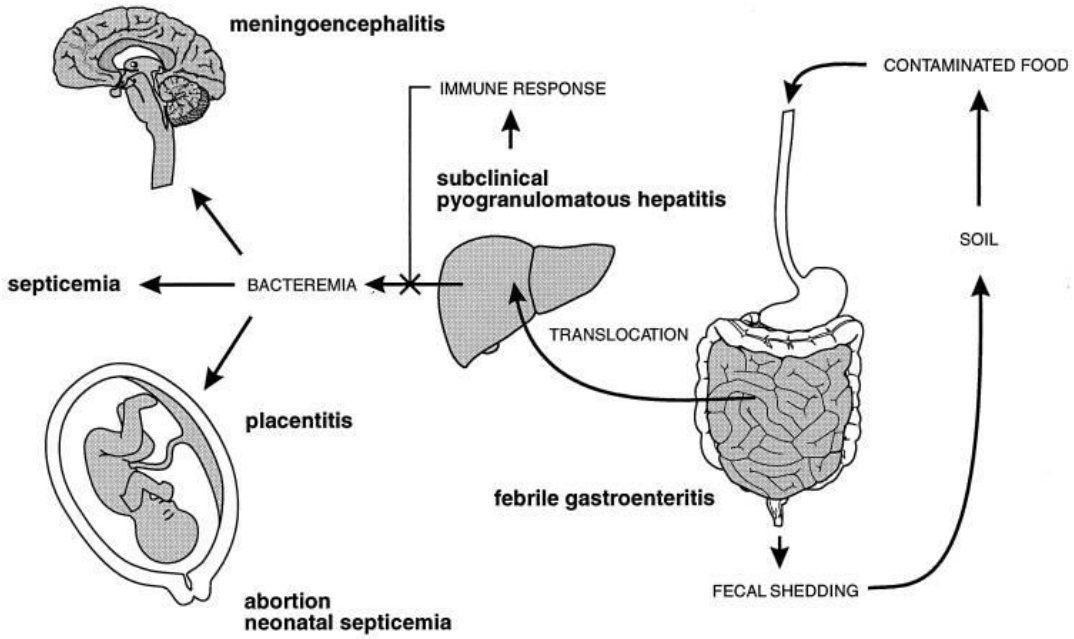

Figure 1: **Infection pathway of *Listeria monocytogenes*** (adapted from Vázquez-Boland et al. 2001)

1.1.3 *Listeria monocytogenes* in the food processing environment

Due to its great adaptability *L. monocytogenes* is able to survive in the food processing environment. Among a great diversity of *L. monocytogenes* strains found in the food

production, particularly strains belonging to sequence type (ST) 121 are prevalent. *L. monocytogenes* strains of ST 121, belonging to the lineage II and serotype 1/2a, can persist up to several years in food processing environments, because persistent strains commonly colonize sites, which are difficult to clean. Thus the risk of food contamination and listeriosis is increased as these persistent strains can continuously contaminate food. Among all sequence types ST121 is the most prevalent, predominately found in food and the food processing environment. These facts suggest that *L. monocytogenes* ST121 strains might harbour unique genotypic and phenotypic features facilitating survival and growth in the food processing environment. However, it is still unknown which molecular mechanisms contribute to this niche-specific adaptation.

L. monocytogenes strains isolated from the food processing environment show often resistance to environmental stresses including antimicrobials and heavy metals as well as adaptation to cold, salt, acid, oxidative stresses and the ability to form biofilms. Mobile elements such as prophages and genomic island as well as plasmids, which are not evenly scattered among the genome, but clustered in hypervariable hotspots, comprise new genetic information and facilitate adaptation to new niches such as the food production environment for certain *L. monocytogenes* strains. All ST121 genomes show a high degree of conservation among prophages and especially among plasmids, but differences exist in prophage content and prophage conservation. This high level of conservation suggests strong selective pressure acting on ST121 and these plasmids and prophages might provide important adaptations for the persistence in the food processing environment. It has been already shown that some *L. monocytogenes* ST121 plasmids contribute to tolerance against elevated temperature, salinity, acidic environments, oxidative stress and disinfectants. The ST121 genomes also contain an undescribed insertion harbouring recombination hotspot (RHS) repeat proteins, which might be involved in bacterial competition (Harter et al. 2017b, Naditz et al. 2019, Pasquali et al. 2018, Rychli et al. 2017, Schmitz-Esser et al. 2015).

1.2 Other bacterial species found in the food processing environment

The production of microbiologically safe and high-quality foods requires the control of many different factors such as the quality of raw ingredients, food-processing conditions, hygiene and

storage conditions as there are many different bacterial species that survive in diverse niches. Thus the removal of undesirable microorganisms such as *L. monocytogenes*, *Salmonella*, pathogenic *Escherichia* (*E.*) *coli*, *Campylobacter*, and *Staphylococcus aureus* as well as vegetative spoilage bacteria such as *Lactobacillus* and *Pseudomonas* species is very important to prevent food-borne diseases. Depending on the product the prevalence of certain species is different. For example the most abundant bacteria causing spoilage of refrigerated beef and pork are *Brochothrix thermospacta*, *Carnobacterium* spp., clostridia, *Enterobacteriaceae*, *Lactobacillus* spp., *Pseudomonas* spp. and *Weissella* spp. (den Besten et al. 2018, Stellato et al. 2016).

1.2.1 *Lactococcus piscium*

Lactococcus (*L.*) *piscium* is a psychrotrophic lactic acid bacterium. It is Gram-positive, nonsporulating, facultative anaerobe and produces lactic acid as the principal end metabolite from carbohydrate fermentation. *L. piscium* forms nonmotile cocci from 0.5 to 1 µm in diameter, which appear individually, in pairs or in short chains. The optimal pH for growth is neutral and the optimal temperature is 24-26 °C, although it is also able to grow at 0 °C. Growth at 30 °C is weak and strain-dependent. *L. piscium* is adapted to different environmental conditions and can survive different environmental stresses caused by various steps during food processing such as low temperature, high salt concentrations and presence of preservative agents. The adaptation to low temperatures provides an important advantage for bacterial competition against pathogenic psychrotroph bacteria. *L. piscium* has been isolated in a variety of chilled, modified atmosphere and vacuum packed food including meat, seafood and vegetables, but can be found in dairy products, too.

L. piscium acts either as a bioprotective or a spoilage microorganism depending on the strain and the food matrix. For example in raw salmon *L. piscium* doesn't spoil the product, but in pork it has a lightly spoiling effect. The protective effect of *L. piscium* was shown in vacuum packed shrimps. However, it is strain-dependent and varies according to the interaction of *L. piscium* with the spoiling microorganisms

L. piscium also shows antimicrobial activity against Gram-positive and Gram-negative bacteria such as *Brochotrix*, *Lactobacillus*, *Enterococcus*, *Pseudomonas* and *Serratia*. These

antimicrobial activities are commonly associated with the production of antimicrobial peptides, organic acids and hydrogen peroxide and nutrient competition, too. *L. piscium* is also able to inhibit the growth of pathogens or opportunistic pathogens such as *E. coli*, *Salmonella* and *L. monocytogenes*. As *L. monocytogenes* is frequently isolated from meat and seafood the antagonistic activity of *L. piscium* is important there. To prevent growth of *L. monocytogenes* *L. piscium* CNCM I-4031, the only currently known strain to inhibit growth of *L. monocytogenes*, uses a cell-to-cell contact-dependent mechanism. *L. piscium* CNCM I-4031 inhibits different *L. monocytogenes* strains, but at different sensitivities. Inhibition occurs, when *L. piscium* reaches its maximum population density. In addition to growth inhibition it reduces the virulence of *L. monocytogenes* (Saraoui et al. 2016, Saraoui et al. 2018).

1.2.2 *Pseudomonas fragi*

Pseudomonas (*P.*) *fragi* is a psychrotrophic, Gram-negative bacterium that grows at temperatures ranging from 2 to 35 °C. Members of the genus *Pseudomonas* are commonly considered aerobic, but they can also survive in microaerophilic and anaerobic environments.
P. fragi belongs to the main players recognised as food spoilers and is currently recognised as one of the most threatening ones. It produces several types of enzymes like lipases and proteases, which are responsible for the spoilage of particularly meat and dairy products, but also of fish and vegetables. As *P. fragi* can survive under microaerophilic conditions, too, it spoils not only aerobically stored food, but also vacuum packed and modified atmosphere condition products, but the spoilage activity of *P. fragi* is lower in not aerobically stored food. However, the food spoiling potential is strain dependent. The spoiling potential is affected by different colonization capabilities of the food processing environment, being a result of resistance to routine cleaning of surfaces and the capability of biofilm formation (Filippis et al. 2018, Stellato et al. 2017, Wang et al. 2017).

1.2.3 *Pseudomonas fluorescens*

P. fluorescens is a psychrotrophic, Gram-negative, rod-shaped and motile bacterium. It is able to grow at pH values ranging from 4 to 8 and temperatures from 4 to 32 °C. However *P. fluorescens* strains isolated from humans and other mammalians are able to grow at 37 °C.

Like *P. fragi P. fluorescens* is aerobic, but can also survive in microaerophilic and anaerobic environments.

P. fluorescens is one of the most threatening food spoilers. It can perfectly grow in food, such as meat, seafood and dairy products, stored aerobically and at low temperatures. The food spoiling potential of *P. fluorescens* is strain dependent and depends on its adaptions to the food processing environment like in *P. fragi*. Once the food matrix is colonized by this *Pseudomonas* specie, slime and malodour is produced.

P. fluorescens is not only responsible for food spoilage, but it is also a human pathogen. It is an indigenous, but low abundant, member of the microbiota of the mouth, stomach and lungs. Although *P. fluorescens* is far less virulent than *Pseudomonas aeruginosa*, it can cause acute opportunistic infections in humans. The most common site of infection is the blood, where *P. fluorescens* causes bacteraemia as a result of transfusion of contaminated blood products or the use of contaminated equipment for intravenous infusions. The most intriguing association between human disease and *P. fluorescens* is that approximately 50 % of the Morbus Crohn patients have serum antibodies to an antigen of *P. fluorescens* (Gonçalves, Letícia Dias Dos Anjos et al. 2017, Scales et al. 2014, Stellato et al. 2017).

1.2.4 *Acinetobacter harbinensis*

Acinetobacter (*A.*) *harbinensis* is a Gram-negative, psychrotolerant, strictly aerobic non-motile coccobacillus. It is able to grow at temperatures from 2 to 35 °C with an optimal growth temperature between 8 and 20 °C. *A. harbinensis* grows at pH values ranging from 6 to 8.5 with an optimum pH of 7.2. It is a heterotrophic bacterium and able to grow in media with acetate as the sole carbon source and ammonia as the sole source of nitrogen.

A. harbinensis is also a nitrification bacterium and capable of removing ammonium and organic chemicals at 2 °C. Thus it is important for the ammonium removal from source water and water sanitation improvement in cold areas. It can be used in biologically enhanced activated carbon filters to treat source water in winter, because it shows stronger ammonium removal activity at lower temperatures (Li et al. 2014, Ma Yin Peng et al. 2019, Zhang et al. 2015).

1.3 Toxin-antitoxin systems in bacteria

Toxin-antitoxin (TA) loci are widely distributed in prokaryotic genomes and encompass a diverse range of genetic elements that are found on plasmids, phages or chromosomes of bacteria. All this systems encode a stable toxin and a highly instable antitoxin; the toxin being able of manipulating vital processes of the bacteria such as transcription, translation, DNA replication and membrane homeostasis. Under normal growth conditions, which means low cellular stress, the antitoxin inhibits the activity of the toxin, but under conditions of stress the cognate antitoxins are selectively degraded and thus the toxins are able to inhibit cellular processes or induce cell death. This inhibition results in rapid growth arrest of the bacterial cells. First TA systems were thought to be cell suicide factors, but now it is know that they act as a stress response system (Hall et al. 2017, Page and Peti 2016, Slayden et al. 2018).

Antibiotics are no omnipotent weapons against bacteria and will possibly fail in some cases. One reason for failure is the fact that target bacteria acquire resistance mutations, but bacteria themselves can use endogenous mechanisms to evade stress, too, and one of this mechanisms is persistence. In the last years TA systems became more interesting as the play an important role in the induction of the bacterial persister phenotype. Bacterial persister cells form a subpopulation of genetically identical, slow or not growing cells, which are highly tolerant to environmental stresses such as antibiotics. Antibiotics typically target the cellular processes of actively growing and replicating cells and thus are rendered ineffective against persister cells, which is a major health concern. In the persister state bacteria are able to survive until the environmental stress is removed and revert back to actively growing cells once it is gone. This can lead to a relapse of infection or to latent infections. The bacterial persister phenotype has been observed in clinically important pathogens such as *E. coli*, *Salmonella enterica* and *Mycobacterium tuberculosis*. For wild-type *E. coli* the frequency of persister cells is about one in a million in planktonic populations, but can increase steeply to one in 100 in stationary cultures and biofilms, which are highly resistant to antibiotics (Hall et al. 2017, Page and Peti 2016, Slayden et al. 2018).

TA systems are not only associated with infections, but they also occur in the course of bacterial competition. Colonization of niches means struggle for nutrients and space and thus bacteria have evolved weapons to restrict growth of competing eukaryotic and prokaryotic cells. In case of bacterial competition TA systems are also called polymorphic toxin systems (PTS). They are

composed of a secreted multidomain toxin, a protective immunity protein – the antitoxin, protecting from autointoxication and intoxication by clonemates – and multiple loci for alternative toxic domains. The bacterial toxins can be used against competing bacteria, but also against the host in case of pathogenic species. The rearrangement hotspot (RHS) family of toxins belongs to the group of PTS for example (Jamet and Nassif 2015).

TA systems are currently classified into six distinct types based on the nature and activity of the antitoxin and the way the antitoxin neutralizes the activity of the toxins. While toxins are typically proteins the antitoxins are either low-molecular-weight proteins in TA systems class II, IV, V and VI or small regulatory RNAs in TA systems class I and III.

TA systems of class I have a noncoding RNA antitoxin and a protein toxin. The antitoxin small regulatory antisense RNA (sRNA) base-pairs to the mRNA of the toxin and thus inhibits its translation. Under normal growth conditions the RNA duplex is degraded by a RNase, but when environmental stresses occur the antitoxin sRNAs are reduced and so the toxin can be translated. The toxins insert in bacterial membranes and produce pores, resulting in inhibition of ATP synthesis and loss of membrane potential.

In type II systems, the antitoxin is a small, unstable protein that is very susceptible to proteolysis in contrast to the toxin. The antitoxin binds and inhibits the cognate toxin by protein complex formation. When the concentration of TA complexes is reduced due to antitoxin degradation, the expression of the TA operon is supressed. Under stress conditions the antitoxin is degraded and the free toxin can exert its inhibitory effects.

As in type I systems, in type III systems the antitoxin is a sRNA. These sRNAs form pseudoknots that directly bind to the toxin, not to its mRNA, and thus inhibiting it. Type III toxins are endoRNase that cleave mRNA at specific sites.

TA systems of class IV have proteinaceous toxins and antitoxins. Toxin and antitoxin never interact with each other, because the antitoxin inhibits binding of the toxin to its target. Under environmental stress toxins affect cellular morphology by binding and inhibiting the polymerization of the bacterial cytoskeletal proteins. In contrast, the antitoxin stabilizes bundling of the cytoskeletal filaments.

Currently there is only one known TA system of class V with the antitoxin endoRNase GhoS and the toxin GhoT. Under normal growth conditions the antitoxin cleaves the mRNA of the

toxin but under environmental stress the mRNA of the antitoxin is degraded by a type II TA system. The toxin is then functional to lyse the cell membrane.

As in type II systems antitoxin and toxin of type VI TA systems are of proteinaceous nature and there is currently only one such system known. The antitoxin socA is a proteolytic adapter protein that neutralizes the toxin socB, which would block replication elongation, by enhancing its degradation by another protein ClpXP (Page and Peti 2016, Yang and Walsh 2017).

An overview of the different classes of TA systems is shown in Fig. 2.

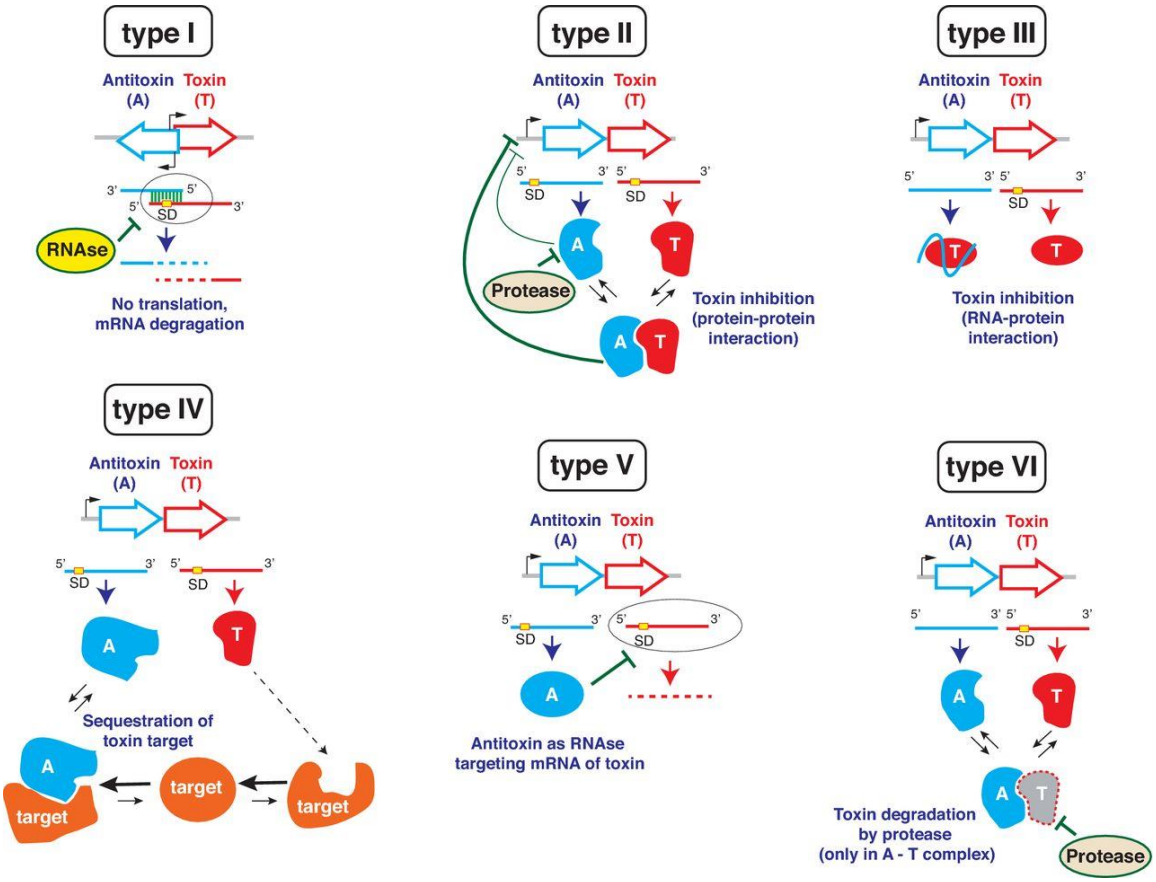

Figure 2: **The different classes of toxin-antitoxin systems** (adapted from Lobato-Márquez et al. 2016)

1.4 The RHS protein in bacterial competition

Bacteria live in community where they have to cooperate and compete with bacteria of the same or other species. Apart from quorum sensing, there are also other modes of communication based on direct cell-to-cell contact like contact-dependent growth inhibition (CDI). CDI was first discovered in *E. coli* EC93, which inhibited the growth of other *E. coli* strains in co-culture

experiments by direct cell-to-cell contact with the target cells. But CDI is not exclusively a mode of communication of Gram-negative bacteria, but can also be found in Gram-positive bacteria.

The CDI system in *E. coli* EC93 encodes toxin and antitoxin and represents a TA system of class V. The CDI locus is comprised of three genes. The general organization of a typical locus encoding a toxin with its cognate antitoxin is shown in Fig. 3. CdiB is an outer membrane β-barrel protein that is needed for the secretion and presentation of CdiA on the cell surface. CdiA contains a growth inhibitory activity localized on its C-terminal region, which is neutralized by the CdiI immunity protein to prevent autoinhibition. The transport domain of CdiA is located on the N-terminus followed by a hemagglutinin repeat region. CdiA is delivered to its target cell and incorporated by specific receptors, where it induces DNA or RNA degradation to inhibit growth. The CDI pathway is shown in Fig. 4. Many CDI systems also contain orphan modules, which are tandem arrays of TA gene pairs downstream from the main TA gene cluster lacking initiation signals. Nevertheless these orphan modules encode a functional toxin and its cognate antitoxin and are able to mediate CDI, too. To be actually expressed these orphan modules have to undergo translocation and fusion to the normal TA gene cluster, resulting in recombination and a TA chimera. This interchangeability of orphan modules represents a toxin diversity that gives a growth advantage to bacteria expressing them (Hayes et al. 2014, Jamet and Nassif 2015, Poole et al. 2011).

Figure 3: **General organization of a locus encoding toxin and its cognate antitoxin** (adapted from Jamet and Nassif 2015)

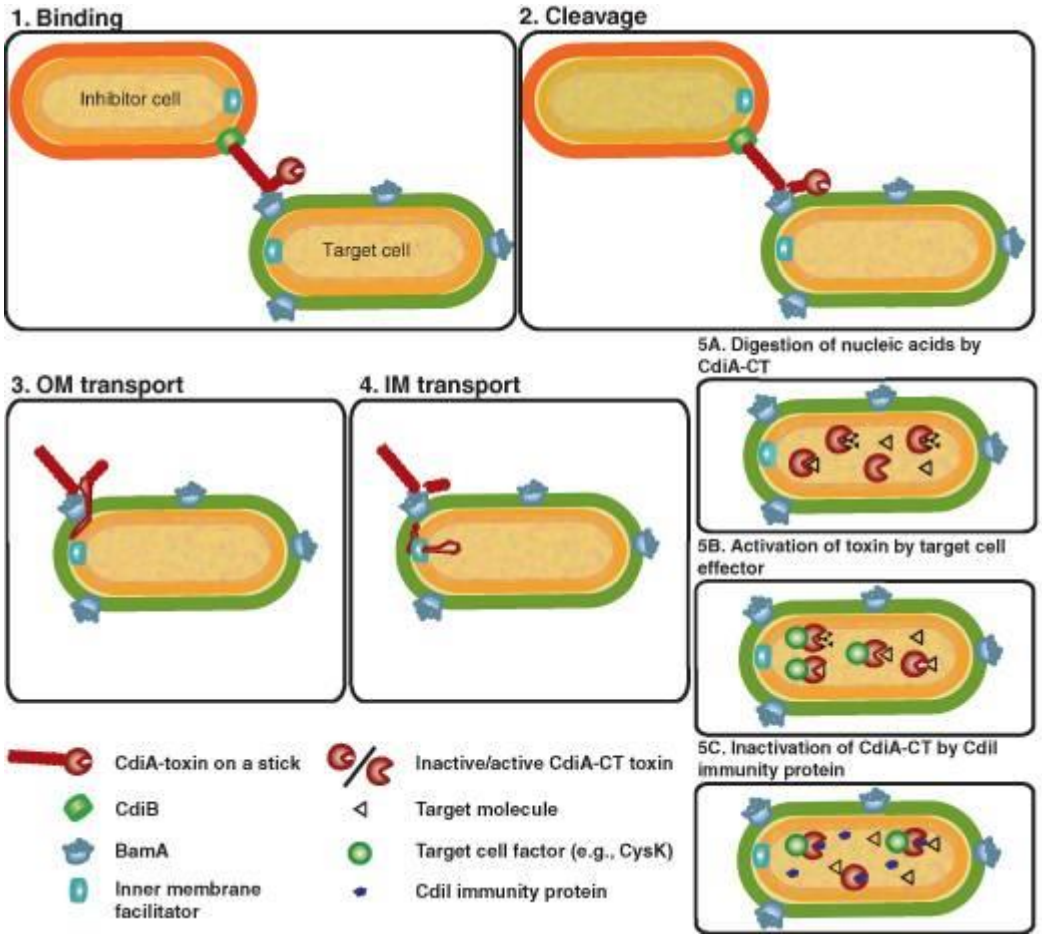

Figure 4: **The major steps involved in contact-dependent growth inhibition** (adapted from Hayes et al. 2014)

The CDI proteins resemble the rearrangement hotspot (RHS) protein family in various characteristics. Members of the RHS protein family are widely distributed throughout the eubacteria. They are large, filamentous proteins, differing in size between Gram-negative bacteria, where they are composed of ~1,500 residues, and Gram-positive bacteria, where they can reach over 2,000 residues. Characteristic for RHS proteins is a central Tyrosine-Aspartic acid (YD) repeat resembling the hemagglutinin repeats of CdiA. These highly conserved repeats are located in the N-terminal core regions of the RHS protein and in contrast to that the C-terminus is highly variable. The genetic organization of RHS proteins and CDI systems is similar, too, as many *rhs* loci contain numerous silent cassettes resembling orphan modules that also undergo rearrangement. According to their chromosomal location there exist eight

different *rhs* elements, *rhsA* to *rhsH*, which are differently present in different species. *E. coli* for example harbours all of them (Jamet and Nassif 2015, Poole et al. 2011, Wang et al. 1998). As CdiA the RHS proteins of many bacterial species are exported to the cell surface and are another form of contact-depended cell-to-cell communication used in intercellular competition. However, they are not secreted through a class V TA system as the proteins of the CDI system. As *rhs* loci do not encode for any CdiB homologue, the transport protein for CdiA in the CDI system, nor do RHS proteins harbour any leader sequence for CdiB-dependent transport they need to be transported via a TA system class VI. The role in intercellular competition has already been shown for *Dickeya* (*D.*) *dadantii* and *Bacillus subtilis*. *D. dadantii* harbours two different RHS proteins, RhsA and RhsB, to inhibit competing cells and protects itself with immunity proteins. To inhibit growth of other bacterial cells *D. dadantii* has to have contact to its target cells, because the soluble RHS protein is not inhibitory. Once delivered to its target cell, both RHS proteins degrade the DNA and thus inhibit further growth (Benz and Meinhart 2014, Jamet and Nassif 2015, Koskiniemi et al. 2013, Poole et al. 2011).

There are also RHS proteins with toxic activity against eukaryotic cells like the insecticidal toxin C protein of entomopathogen bacteria or the RHS toxin of *Pseudomonas aeruginosa* PSE9. *Pseudomonas* starts the expression of the RHS toxin upon contact with phagocytic cells. The RHS toxin was then exposed on the bacterial surface and transported in the phagocytic cells, where it induced inflammosme-mediated death. In a mouse model of acute pneumonia an infection with *Pseudomonas aeruginosa* lacking the RHS toxin resulted in enhanced animal survival thus making the RHS toxin a virulence factor (Jamet and Nassif 2015, Kung et al. 2012).

L. monocytogenes strains of the sequence type 121 also harbour an *rhs* element with 29 RHS repeats in their genome. It is located on an insertion between the EGDe *lmo2753* and *lmo2754* homologs. The function of the RHS protein in *L. monocytogenes* has to be determined yet, but it is hypothesized that it is involved in contact-depended cell-cell interactions and competition against other bacteria as the already characterized RHS proteins of other species such as *D. dadantii* or *E. coli*. It might be involved in persistence or survival in the food processing environment of *L. monocytogenes*, too (Schmitz-Esser et al. 2015). The arrangement of the *rhs* locus and its surroundings is shown in Fig. 5.

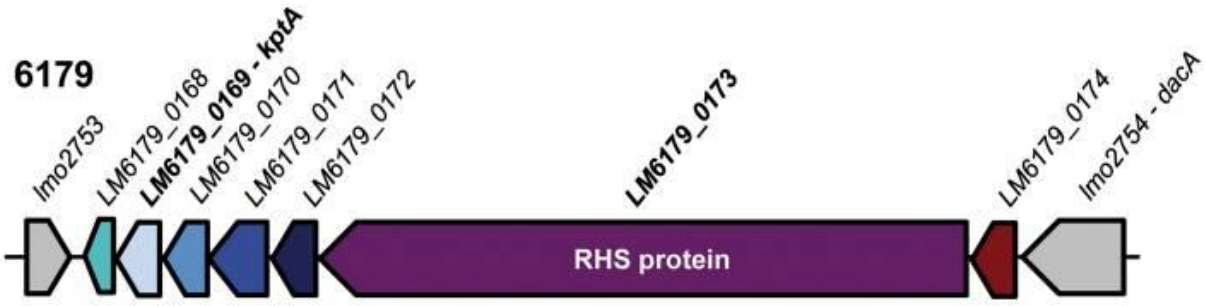

Figure 5: **Organization of the *rhs* locus and surroundings in *Listeria monocytogenes* 6179** (adapted from Schmitz-Esser et al. 2015)

1.5 Aims

The aim of this bachelor thesis is to determine the role of the RHS protein of *Listeria monocytogenes* in the competition against other bacteria present in the food producing environment.

To measure the expression of the RHS protein a *L. monocytogenes* 6179 strain with eGFP-tagged promotor of the *rhs* operon (*L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$) was created at the Unit of Food Microbiology of the University of Veterinary Medicine Vienna and used for co-cultivation experiments.

All strains used in this thesis are available at the Unit of Food Microbiology. Inhibitor and target strains will be mixed at different ratios and co-cultured in nutrient rich and nutrient poor media at 10 °C and 20 °C, mimicking temperatures found in food production plants.

First the best culture medium and the most appropriate time points for the fluorescence measurement have to be determined. Second it has to be determined if *L. monocytogenes* 6179 harbouring the plasmid pIMK2(eGFP) is a good positive control

Our hypothesis is that the RHS protein is involved in bacterial competition of *L. monocytogenes* resulting in growth competition against other highly prevalent bacteria in the food producing environment.

2. Material and Methods

2.1 *Listeria monocytogenes* growth conditions

Materials:

- Internal cryogenic strain collection
- Trypto-Casein Soy Agar; TSA (Biokar Diagnostics; France)
- Yeast Extract (Biokar Diagnostics; France)
- CASO-Bouillon (Carl Roth; Germay)

Equipment:

- Friocell 222 Incubator (MMM Group; Germany)
- Incubator EHRET Type BK 4266 L13214 – Gewerk M13 (Ehret; Germany)
- KS-15 Shaker (Edmund Bühler GmbH; Germany)

L. monocytogenes strains were taken from the internal cryogenic strain collection. All following steps were performed carefully near the flame of a Bunsen burner to prevent contamination of the *L. monocytogenes* cryogenic cultures and the *L. monocytogenes* cultures on the TSA plates. First a small amount of cells from the cryogenic culture was smeared on a TSA plate by T-streak using a separately packaged loop. The plates were incubated at 37 °C overnight. After incubation the plates were stored in the fridge at -4 °C to prevent the agar from drying out.
The next step was the activation of the bacterial cells. Working near Bunsen burner to prevent contamination a single colony was taken with a loop and transferred to 10 ml Tryptic Soy Broth with Yeast (TSB-Y), vortexed and incubated at 37 °C overnight shaking.

2.2 *Lactococcus piscium*, *Pseudomonas fragi*, *Pseudomonas fluorescenes* and *Acinetobacter harbinensis* growth conditions

Materials:

- Internal cryogenic strain collection
- Trypto-Casein Soy Agar; TSA (Biokar Diagnostics; France)
- Yeast Extract (Biokar Diagnostics; France)
- CASO-Bouillon (Carl Roth; Germay)

Equipment:

- Platform shaker Unimax 1010 (Heidolph Instruments; Germany)

L. piscium, *P. fragi*, *P. fluorescenes* and *A. harbinensis* were taken from the internal cryogenic strain collection. All following steps were performed carefully near the flame of a Bunsen burner to prevent contamination of the cryogenic stocks and the cultures on the TSA plates. First a small amount of cells from the cryogenic culture was smeared on a TSA plate by T-streak using a separately packaged loop. The plates were incubated at room temperature overnight. After incubation the plates were stored in the fridge at +4 °C to prevent the agar from drying out.

The next step was the activation of the bacterial cells. Working near Bunsen burner to prevent contamination a single colony was taken with a loop and transferred to 10 ml TSB-Y, vortexed and incubated at room temperature overnight shaking.

2.3 Identification of the most suitable culture medium

Materials:

- CASO-Bouillon (Carl Roth; Germany)
- Yeast Extract (Biokar Diagnostics; France)
- Luria Bertani Broth; LB (Himedia; India)
- Brain Heart Broth; BHI (Biokar Diagnostics; France)
- Roswell Park Memorial Institute-1640 medium; RPMI (with phenol red) (HyClone; USA)
- RPMI medium 1640 (without phenol red) (Gibco; USA)
- Iron citrate (internal stock solution)
- Phosphate buffered saline; PBS (Gibco; USA)
- Kanamycin Sulfate (AppliChem; Germany)
- Clear 96-well plate (Sarstedt AG & Co. KG; Germany)
- Black 96-well plate (Thermo Fisher Scientific; USA)

Equipment:

- Microplate Reader Infinite F200 (Tecan; Switzerland)

- Software i-control 1.5 (for infinite reader)

The culture media PBS, Brain Heart Broth with yeast (BHI-Y), LB, RPMI with phenol red, RPMI without phenol red, TSB-Y and TSB-Y diluted 1:10 with demineralized water were tested for their autofluorescence. 200 µl of every culture media were dispensed in six wells of a black 96-well plate respectively. The plate was placed in the Microplate Reader and the lid was removed. Fluorescence was determined at an excitation wavelength of 488 nm and an emission wavelength of 510 nm. The plate type was chosen to be "Greiner flat/black".
The fluorescence measurement was performed after 0 h, 4 h and 24 h. Between the measurements the 96-well plate was put in the incubator at 20 °C.

2.4 Identification of the best culture medium for growth of the bacterial strains

2.4.1 Growth conditions of the bacterial cultures

Materials:

- CASO-Bouillon (Carl Roth; Germany)
- Yeast Extract (Biokar Diagnostics; France)
- Kanamycin Sulfate (AppliChem; Germany)

Equipment:

- Friocell 222 Incubator (MMM Group; Germany)
- Incubator EHRET Type BK 4266 L13214 – Gewerk M13 (Ehret; Germany)
- KS-15 Shaker (Edmund Bühler GmbH; Germany)
- Platform shaker Unimax 1010 (Heidolph Instruments; Germany)

A single colony of *L. monocytogenes* 6179 harbouring the plasmid pIMK2(eGFP) (*L. monocytogenes* + pIMK2(eGFP)), was activated in 10 ml TSB-Y with 10 µl of Kanamycin (25 mg/ml) added overnight shaking at 37 °C. Single colonies of *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ and *L. monocytogenes* 6179 wildtype (*L. monocytogenes* 6179 WT) were activated in 10 ml TSB-Y overnight shaking at 37 °C. Single colonies of *L. piscium*, *P. fragi*,

P. fluorescenes and *A. harbinensis* were activated in 10 ml TSB-Y overnight shaking at room temperature.

2.4.2 Measurement of growth

Materials:

- Overnight cultures of *L. monocytogenes* + pIMK2(eGFP), *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$, *L. monocytogenes* 6179 WT, *L. piscium*, *P. fragi*, *P. fluorescenes* and *A. harbinensis*
- Semi-micro cuvettes (Greiner Bio-One; Austria)
- CASO-Bouillon (Carl Roth; Germany)
- Yeast Extract (Biokar Diagnostics; France)
- Roswell Park Memorial Institute-1640 medium; RPMI (with phenol red) (HyClone; USA)
- Phosphate buffered saline; PBS (Gibco; USA)
- Clear 96-well plate (Sarstedt AG & Co. KG; Germany)

Equipment:

- UV Spectrophotometer UV-1800 (Shimadzu; Japan)
- Software UVProbe 2.42
- Centrifuge 5810 R (Eppendorf AG; Germany)
- Microplate Reader Infinite F200 (Tecan; Switzerland)
- Software i-control 1.5 (for infinite reader)
- Friocell 222 Incubator (MMM Group; Germany)

To prevent contamination all following steps were performed near a Bunsen burner flame.
The optical density of the bacterial cultures was determined at 600 nm (OD_{600}) after vortexing them. Based on this measurement the required volume of bacterial culture for an OD_{600} of 0.1 in 6 ml TSB-Y diluted 1:10 was calculated using this formula:

$$required\ volume\ of\ bacterial\ culture = \frac{required\ OD_{600} * required\ volume\ of\ TSB{-}Y}{measured\ OD_{600}}$$

Then the cultures were centrifuged for 10 minutes at 4000 rounds per minute (rpm). The supernatant was discarded and the pellets were resuspended in 10 ml TSB-Y diluted 1:10.
Separately 6 ml of TSB-Y diluted 1:10, PBS and RPMI, respectively, were prepared for each strain and the calculated volume for an OD_{600} of 0.1 was taken out. Then the same volume of bacterial culture resuspended in 10 ml TSB-Y diluted 1:10 was added. All cultures were vortexed thoroughly after doing that.
For every culture medium 200 µl of bacterial culture were dispensed in six wells of a 96-well plate respectively, doing so for all of the tested species. In six wells respectively 200 µl of TSB-Y diluted 1:10, RPMI and PBS were dispensed and served as blanks. To determine the growth of the bacterial cultures the optical density at 610 nm (OD_{610}) was measured using the plate reader. The plate type was chosen to be "Greiner flat/transparent". Before the plate was placed in the Microplate Reader the lid was removed.
The measurement of the OD_{610} was performed after 0 h, 4 h and 24 h of growth at 20 °C.

2.5 Co-cultivation of bacterial species isolated from the food processing environment with *Listeria monocytogenes* strains

2.5.1 Growth conditions of the bacterial cultures

Materials:

- CASO-Bouillon (Carl Roth; Germany)
- Yeast Extract (Biokar Diagnostics; France)
- Kanamycin Sulfate (AppliChem; Germany)

Equipment:

- Friocell 222 Incubator (MMM Group; Germany)
- Incubator EHRET Type BK 4266 L13214 – Gewerk M13 (Ehret; Germany)
- KS-15 Shaker (Edmund Bühler GmbH; Germany)
- Platform shaker Unimax 1010 (Heidolph Instruments; Germany)

A single colony of *L. monocytogenes* 6179 harbouring the plasmid pIMK2(eGFP) (*L. monocytogenes* + pIMK2(eGFP)), was activated in 10 ml TSB-Y with 10 µl of Kanamycin

(25 mg/ml) added overnight shaking at 37 °C. Single colonies of *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ and *L. monocytogenes* 6179 wildtype (*L. monocytogenes* 6179 WT) were activated in 10 ml TSB-Y overnight shaking at 37 °C. Single colonies of *L. piscium*, *P. fragi*, *P. fluorescenes* and *A. harbinensis* were activated in 10 ml TSB-Y overnight shaking at room temperature.

2.5.2 Preparation of pure cultures and co-cultures

Materials:

- Cultures of *L. monocytogenes* + pIMK2(eGFP), *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$, *L. monocytogenes* 6179 WT, *L. piscium*, *P. fragi*, *P. fluorescenes* and *A. harbinensis* with an OD_{600} adjusted to 0.1 in RPMI without phenol red
- Black 96-well plate (Thermo Fisher Scientific; USA)
- Clear 96-well plate (Sarstedt AG & Co. KG; Germany)

The black and the clear 96-well plate were prepared in the same way. For the pure cultures 200 µl of bacterial culture were dispensed in six wells respectively, doing so for all of the tested species.

Following co-cultures were tested:

- *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ with *L. piscium*
- *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ with *P. fragi*
- *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ with *P. fluorescenes*
- *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ with *A. harbinensis*

The co-cultures were prepared at a ratio of 1:1. So in six wells respectively 100 µl of *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ were mixed with 100 µl of the other species. Then the co-cultures were mixed thoroughly by pipetting up and down. In six wells respectively 200 µl of RPMI without phenol red were dispensed and served as blank.

2.5.3 Measurement of growth and fluorescence

Materials:

- Prepared black and clear 96-well plate
- Sealable plastic box

Equipment:

- Microplate Reader SpectraMax (Molecular Devices LLC; USA) (Institute of Microbiology; University of Veterinary Medicine of Vienna)
- Software SoftmaxPro
- Friocell 222 Incubator (MMM Group; Germany)

The 96-well plates were transported in a sealable plastic box to the Institute of Microbiology of the University of Veterinary Medicine in Vienna were the measurement was performed. To determine the growth of the bacterial cultures the OD_{600} was measured using the clear 96-well plate. Before the plate was placed in the Microplate Reader the lid was removed. The computer was used to choose the following settings for the measurement:

- Mode: Absorbance
- Wavelength: 600 nm
- Plate type: 96 Well Greiner clear
- Mode: Top Read

The fluorescence of the bacterial cultures was determined by measuring the black 96-well plate. Before the plate was placed in the Microplate Reader the lid was removed. The computer was used to choose the following settings for the measurement:

- Mode: Fluorescence
- Wavelength:
    - Excitation: 488 nm
    - Emission: 510 nm
- Plate type: 96 Well Greiner blk/clrbtm
- Mode: Top Read

The measurement was performed at different time points according to the requirements of the single experiments. Between the measurements at these different time points the 96-well plates

were transported back to the Unit of Food Microbiology of the University of Veterinary Medicine in Vienna using the sealable plastic box. There the plates were stored in an incubator at 20 °C until the next measurement.

2.6 Cloning into *Listeria monocytogenes* – Chromosomal integration of the pPL2 vector

2.6.1 Gradient PCR (targeting tRNA-Arg-TCT)

Materials:

- DreamTaq Green PCR Master Mix (2x) (Thermo Fisher Scientific; USA)
- Forward primer tRNA-Arg-TCT_fwd (Microsynth Austria GmbH ; Austria)
    - Sequence of the forward primer: 5'-TGG ACC GAG ATG ACA ACG AA-3'
- Reverse primer tRNA-Arg-TCT_rev (Microsynth Austria GmbH ; Austria)
    - Sequence of the reverse primer: 5'-ACC AAG GCT TTT CAA TGC TTT-3'
- Sterile water (from the DreamTaq Green PCR Master Mix (2x) Kit) (Thermo Fisher Scientific; USA)
- *L. monocytogenes* 6179 WT DNA (internal stock at -20 °C)
- PCR tubes

Equipment:

- Thermocycler T100 Thermal Cycler (Bio-Rad Laboratories, Inc.; USA)
- Eppendorf Centrifuge 5424 (Eppendorf AG; Germany)

The primers were designed using the software Primer3. The gradient PCR was performed to determine the optimal annealing temperature of the primers. They flank the integration site of the pPL2 bacteriophage in *L. monocytogenes* 6179 WT, the tRNA-Arg-TCT site, which is shown in Tab. 1, and they should be used if an insertion of pPL2 occurred. But at the beginning the optimal annealing temperature of the primers had to be determined.

Table 1: tRNA-Arg-TCT gene (upper cases) with flanking regions (lower cases)

>fig|1639.130.rna.12 tRNA-Arg-TCT [Listeria monocytogenes 6179_finished]

aaaagaatgaaattagccaccgtgctaatgcgattaaacaattagaaaaagatttagcagaagtagtagaaaaagtaaccaaaaagt
gagaaaataagtaacagtatcacgggggaggaaacaaaatgaaattattagtagttagcgacagccactcagaacgtgactgctta
attcatcttaaagaaaaatacaaaaatacagtcgatgcgatgattcactgtggggattctgaattagaagctgacgatcctgcgattca
gggatttcatactgttcgaggcaactgtgactttggcggaggctttccaaatgattgggtaggagaagtagatggctaccgtattttca
ccacgcacggacatctgtataacatcaaaatgacactcatgaatttacggtatcgcgcacgtgaactaaacgcagattttgccttttttg
gtcactctcatgaattaggagtagacatgctagacgacaccatcattttaaacccaggaagcatttccttaccaagagggcgcatccg
tatcaaaacatacgctcttatcgattcaacaccagaaggcatccaagttcgattcatggaccgagatgacaacgaactaacagacct
aacccaaaccttcccattaacgaagcgtaactaggtcaaaagacacccgaaaaagaaaaaatgcaataacttaaagaaaaccattg
acaaacaagcgatttaaacataaaatggtatttggctgttgaaaagacagtgccatttGTCCTGATAGCTCAGCTGG
ATAGAGCAACGGCCTTCTAAGCCGTCGGTCGGGGGTTCGAATCCCTCTCAGGA
CGtaatatagttaaatttaaaaatctctaaagcattgaaaagccttggtattaaagggtgaatgaatgttttagagatttttttatatcttat
aatacccgttttattccgttatttttgtggcatttgtggcaaaatttgtggtattttcatccgtttttagtgtgaaaaaagcatctactttagact
gattatgttgacgtaaattagaacttaggtggctataatattttaatgttgtattaatatcatcatgaccaagtctatcagctacataaatgat
atccatgcctgcctcaacgcatagtcctgtgtgagtgtgtcgtaacttatgcaatgtcactggttcagaattaattgtactgcatatctttttt
caacgctttattacatgatgcgttatccactggtttattgtgataggtgatgaataataacatttgtggatttttataccatattcttttatata
agcagaatgccacgcgagataagactgtaaatattgaactgtggagttatcaatatagatcacacgtgacttttttgtcttggtatcaat
gaatgtattagtgtacttataatcccacgctttattgactgttatagaacgtttagcgaaattaatatctttctttgttagtgcaataatctcttc
gaacctcatacctgtttgcactgctagaaagataactgctcgtgatatagaatgaaaatttgcaagttct

At first the primers (100 µM) were diluted 1:10 with water from the DreamTaq Kit and the *L. monocytogenes* 6179 WT DNA was thawed at room temperature. The master mix for the PCR was prepared according to the instructions in Tab. 2.

Table 2: PCR master mix

	Volume (Vol.) for 1 reaction [µl]
DreamTaq Green PCR Master Mix (2x)	12.5
Primer forward (10 µM)	1
Primer reverse (10 µM)	1
Water	8.5
DNA template	2

The DreamTaq Green PCR Master Mix is a ready-to-use solution containing DreamTaq DNA polymerase, optimized DreamTaq Green buffer, $MgCl_2$ and dNTPs and also allows direct loading of the PCR product on a gel.

All components were vortexed well before added to the master mix and so was the complete master mix as well. In eight PCR tubes 23 µl of master mix were dispensed respectively. In four tubes 2 µl of DNA were added and in the other four tubes 2 µl of water were added, which then served as negative control. Before placed in the thermocycler the PCR tubes were centrifuge for a few seconds to mix the content well. The PCR machine was programed as shown in Tab. 3.

Table 3: cycler program

	T (°C)	**Time**
1x	94	5 minutes (min)
30x	94	30 seconds (sec)
	Gradient	30 sec
	72	2 min
Final elongation	72	5 min
1x	12	Hold

For every temperature of the gradient (54 °C – 56 °C – 58 °C – 60 °C) one PCR tube containing DNA template and one containing water was placed in the thermocycler.

2.6.2 Transformation into competent cells of *Listeria monocytogenes*

Materials:

- Competent *L. monocytogenes* 6179 cells (internal cryogenic strain collection)
- Vector pPL2-eGFP "pPL2-eGFP plasmid from *E. coli* colony 1" (internal storage)
- Brain Heart Broth; BHI (Biokar Diagnostics; France)
- Yeast extract (Biokar Diagnostics; France)
- D(+)-Sucrose for biochemistry (Acros Organics B.V.B.A.; USA)
- Trypto-Casein Soy Agar; TSA (Biokar Diagnostics; France)
- Chloramphenicol (AppliChem; Germany)
- Electroporation cuvette 2 mm (Biozym Biotech Trading GmbH; Austria)

Equipment:

- Electroporation device MicroPulser™ (Bio-Rad Laboratories, Inc.; USA)
- Incubator EHRET Type BK 4266 L13214 – Gewerk M13 (Ehret; Germany)

First the competent cells were thawed on ice. Then 5 µl of pPL2-eGFP were added to the cells and mixed by pipetting up and down. The mixture was transferred to an electroporation cuvette and put on ice. The cells were electroporated using the settings shown in Tab. 4.

Table 4: electroporation program

Voltage	2 kV
Resistance	400 Ω
Capacity	25 µF
Time	5 ms

After the electroporation 1 ml of pre-warmed BHI-Y supplemented with 0.5 M sucrose medium was added. The cuvette was incubated for a minimum of 1 h at 30 °C. Then 150 µl of

the suspension were plated on three TSA plates supplemented with 10 µg/ml of chloramphenicol and on three TSA plates supplemented with 25 µg/ml chloramphenicol respectively. The plates were incubated at 30 °C for four days.

2.6.3 Colony-PCR to determine the chromosomal integration of eGFP

Materials:

- DreamTaq Green PCR Master Mix (2x) (Thermo Fisher Scientific; USA)
- Forward primer tRNA-Arg-TCT_fwd (Microsynth Austria GmbH ; Austria)
    - Sequence of the forward primer: 5'-TGG ACC GAG ATG ACA ACG AA-3'
- Reverse primer tRNA-Arg-TCT_rev (Microsynth Austria GmbH ; Austria)
    - Sequence of the reverse primer: 5'-ACC AAG GCT TTT CAA TGC TTT-3'
- Sterile water (from the DreamTaq Green PCR Master Mix (2x) Kit) (Thermo Fisher Scientific; USA)
- *L. monocytogenes* 6179 WT DNA (internal stock at -20 °C)
- PCR tubes
- Trypto-Casein Soy Agar; TSA (Biokar Diagnostics; France)
- Chloramphenicol (AppliChem; Germany)
- Cultures of electroporated *L. monocytogenes* 6179 + pPL2-eGFP
- 10% IGEPAL® CA-630 (Sigma Aldrich; USA)

Equipment:

- Thermocycler T100 Thermal Cycler (Bio-Rad Laboratories, Inc.; USA)
- Eppendorf Centrifuge 5424 (Eppendorf AG; Germany)
- Incubator EHRET Type BK 4266 L13214 – Gewerk M13 (Ehret; Germany)

The Colony-PCR was performed to verify if pPL2 really integrated in the colonies that grew on the TSA plates supplemented with chloramphenicol. pPL2 harbours a chloramphenicol resistance gene and thus allows bacteria, which have integrated it, to grow in presence of chloramphenicol. The resulting PCR product for the pPL2 vector integrated into the tRNA-Arg-TCT site of *L. monocytogenes* should be larger than the product of the *L. monocytogenes* 6179

WT negative control and the PCR product of colonies in which the integration was not successful. *L. monocytogenes* 6179 WT DNA was used as a control.

First half of a colony of the electroporated *L. monocytogenes* was picked with a sterile toothpick and dissolved in 10 µl IGEPAL in a PCR tube. The other half of the colony was streaked out on a fresh TSA plate supplemented with the same concentration of chloramphenicol as the original plate using T-streak and working near the flame of a Bunsen burner. These plates were put in the incubator at 30 °C. In total ten colonies from different plates were picked. The PCR tubes with IGEPAL were then incubated at 95 °C in the thermocycler to dissolve the colonies. In the meantime the master mix was prepared and all components were vortexed well before added to the master mix and so was the complete master mix as well. The master mix for the PCR was prepared according to the instructions shown in Tab. 5.

Table 5: PCR master mix

	Vol. for 1 reaction [µl]
DreamTaq Green PCR Master Mix (2x)	12.5
Primer forward 1:10 (final concentration: 10 µM)	0.75
Primer reverse 1:10 (final concentration: 10 µM)	0.75
Water	1
DNA template	10

After the incubation 15 µl of PCR master mix were added to the colonies dissolved in IGEPAL and pipetted up and down to mix well. Two PCR tubes were prepared separately and in each 15 µl master mix were dispensed. Both of them served as negative control and in one of them 10 µl of sterile water were added and in the other 10 µl of *L. monocytogenes* 6179 WT DNA were added. All of the PCR tubes were centrifuged for a few seconds before placed in the PCR machine. The PCR machine was programed as shown in Tab. 6.

Table 6: cycler program

	T (°C)	Time
1x	94	5 min
30x	94	30 sec
	60 (determined by gradient PCR)	30 sec
	72	2 min
Final elongation	72	5 min
1x	12	Hold

2.6.4 Agarose Gel Electrophoresis

Materials:

- peqGOLD universal Agarose (peqlab a VWR brand; Germany)
- 1x TAE buffer (Sigma-Aldrich; USA, Merck; Germany)
- GeneRuler 1kb DNA Ladder (Thermo Scientific; USA)
- peqGREEN (peqlab a VWR brand; Germany)
- PCR products

Equipment:

- GelDoc 2000 75S/03005 (Bio-Rad Laboratories, Inc.; USA)
- Microwave (Silva Schneider HandelsgesmbH; Austria)
- Power supply Power Pac 1000 (Bio-Rad Laboratories, Inc.; USA)
- Printer P91W (Mitsubishi Electric; Japan)

To analyse the PCR products a 1 % agarose gel was used. Therefor 1 g Agarose was dissolved in 100 ml TAE buffer in a piston. The content of the piston was boiled in the microwave for 3 min. Every time the content began to boil, the piston was taken out of the microwave and shaken per hand for a few seconds to avoid a boiling delay. After boiling the gel was cooled under cold water. Then 2 µl peqGreen, which is a DNA- and RNA dye and a safe alternative to ethidium bromide, were added. The fluid gel was loaded into a mold with an inserted comb and

cooled to its solid form for at least 30 min. When solid, the comb was removed and the gel was transferred to the electrophoresis chamber.
The first and last pocket of the gel were filled with 3 µl 1 kb DNA Ladder and the others with 10 µl PCR products. The condition for the gel electrophoresis was constant 100 Volt (V) for 30 min.
To see the resulting bands on the gel the GelDoc was used. The agarose gel was exposed to ultraviolet light and a picture was taken.

2.6.5 Colony-PCR to confirm the presence of pPL2(eGFP)

Materials:

- DreamTaq Green PCR Master Mix (2x) (Thermo Fisher Scientific; USA)
- Forward primer eGFP (Microsynth Austria GmbH ; Austria)
    - Sequence of the forward primer: 5- CGG CCA TGG TGA GCA AGG GCG AGG AG-3
- Reverse primer eGFP (Microsynth Austria GmbH ; Austria)
    - Sequence of the reverse primer: 5-CGG CTG CAG CAG CTT GAT GAG TAC AAG CA-3
- Sterile water (from the DreamTaq Green PCR Master Mix (2x) Kit) (Thermo Fisher Scientific; USA)
- *L. monocytogenes* 6179 WT DNA (internal stock at -20 °C)
- PCR tubes
- 10% IGEPAL® CA-630 (Sigma Aldrich; USA)
- Replica streaks of cultures of electroporated *L. monocytogenes* 6179
- Culture of *L. monocytogenes* 6179 + pIMK2(eGFP)

Equipment:

- Thermocycler T100 Thermal Cycler (Bio-Rad Laboratories, Inc.; USA)
- Eppendorf Centrifuge 5424 (Eppendorf AG; Germany)

The Colony-PCR to show if an integration of the vector pPL2 occurred showed that this was not the case. As phages sometimes do not integrate in the genome immediately but nevertheless

are present in the bacterial cell we wanted to know if pPL2 is in the *L. monocytogenes* 6179 cells but hasn't integrated yet. Therefore the *eGFP* gene was tagged as it is part of pPL2.
Single colonies of electroporated *L. monocytogenes* from different replica plates were picked with a sterile toothpick and dissolved in 10 µl IGEPAL in PCR tubes. A single colony of *L. monocytogenes* 6179 + pIMK2(eGFP) was dissolved in 10 µl IGEPAL, too. Because the plasmid pIMK2 also harbours the *eGFP* gene *L. monocytogenes* 6179 + pIMK2(eGFP) was chosen as positive control. The PCR tubes were then incubated at 95 °C in the thermocycler to dissolve the colonies. In the meantime the master mix was prepared and all components were vortexed well before added to the master mix and so was the complete master mix as well. The master mix for the PCR was prepared according to the instructions shown in Tab. 7.

Table 7: PCR master mix

	Vol. for 1 reaction [µl]
DreamTaq Green PCR Master Mix (2x)	12.5
Primer forward (10 µM)	0.75
Primer reverse (10 µM)	0.75
Water	1
DNA template	10

After the incubation 15 µl of PCR master mix were added to the colonies dissolved in IGEPAL and pipetted up and down to mix well. One PCR tube was prepared separately and 15 µl master mix were dispensed into it. It served as negative control thus 10 µl of sterile water were added to the master mix. All of the PCR tubes were centrifuged for a few seconds before placed in the PCR machine. The PCR machine was programed as shown in Tab. 8.

Table 8: cycler program

	T (°C)	**Time**
1x	98	30 sec
35x	98	10 sec

	70	30 sec
	72	2 min
Final elongation	72	10 min
1x	4	Hold

The PCR products were analysed using agarose gel electrophoresis as described in 2.5.4. The only difference from this description is that a 1.2 % agarose gel was used. For the gel 1.2 g of agarose were dissolved in 100 ml TAE buffer in a piston.

2.7 Statistical analysis

Microsoft Excel 2015 and SPSS.20 software were used to conduct the statistical analysis of the data.

Measured values of the optical density and fluorescence were corrected for the mean of the blank. These fluorescence values were also normed to the mean blank, by dividing them by the mean blank.

The Welch test was used to confirm variance homogeneity. Since the data showed variance homogeneity we used the Tukey-HSD post-hoc test to determine significant differences between growth of the bacterial strains in different culture media and between the fluorescence signal of *L. monocytogenes* + pIMK2(eGFP), *L. monocytogenes* 6179 WT and *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$. *P* values of < 0.05 were considered to be statistically significant.

3. Results

3.1 The most suitable culture medium

Six different culture media were tested for their autofluorescence, namely LB, RPMI, TSB-Y, TSB-Y diluted 1:10, BHI-Y and PBS. This was necessary to determine the medium with the least fluorescence background signal for the fluorescence measurement of the bacterial strains. Three of the media were over the detection range, so only RPMI, TSB-Y diluted 1:10 and PBS remained for further testing. The fluorescence signal of the different media is shown in Tab. 9.

Table 9: fluorescence signals of the culture media; values shown are mean values of six technical replicates

Culture medium	Fluorescence intensity [RFU]	Standard deviation
LB	over detection limit	unidentifiable
RPMI	36114.8	895.168
TSB-Y	over detection limit	unidentifiable
TSB-Y 1:10	34134.3	1553.715
BHI-Y	over detection limit	unidentifiable
PBS	9913.7	249.114

Another crucial requirement for the medium used in the co-cultivation experiments is that all bacterial strains, namely *L. monocytogenes* 6179 WT, *L. monocytogenes* + pIMK2(eGFP), *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$, *L. psicium*, *P. fragi*, *P. fluorescens* and *A. harbinensis* are able to grow in the culture medium. All strains were cultivated in RPMI, TSB-Y diluted 1:10 and PBS respectively and the whole experiment was performed three times. To determine the growth of the cultures the optical density at 610 nm was measured after 0 h, 4 h and 24 h of growth. The mean values from all three replicates of the experiment performed in six technical replicates respectively are shown in Fig. 6.

A Tukey-HSD post-hoc test was performed to determine the significance of the growth differences in the three culture media. After 0 h the optical density was equal in all of the culture media. After 4 h of growth the optical density in PBS was still the same for most of the strains, only *P. fragi*, *P. fluorescens* and *A. harbinensis* were able to grow. All of the *Listeria* strains

grew significantly better in TSB-Y diluted 1:10 in contrast to PBS and RPMI after 4 h, only *L. piscium* grew significantly better in RPMI.

After 24 h of growth all strains except *A. harbinensis* grew significantly better in TSB-Y diluted 1:10 and RPMI than in PBS. The optical density values of the cultures in PBS didn't increase compared to 4 h, meaning that no growth occurred in this time. All strains except *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ had the highest optical density in RPMI, but only *L. monocytogenes* 6179 WT, *L. monocytogenes* + pIMK2(eGFP), *L. psicium*, *P. fragi* and *P. fluorescens* grew significantly better in RPMI than in TSB-Y diluted 1:10.

According to these experiments RPMI was determined to be the best culture medium for the co-cultivation experiments. To reduce further background fluorescence RPMI without phenol red was chosen in the following experiments.

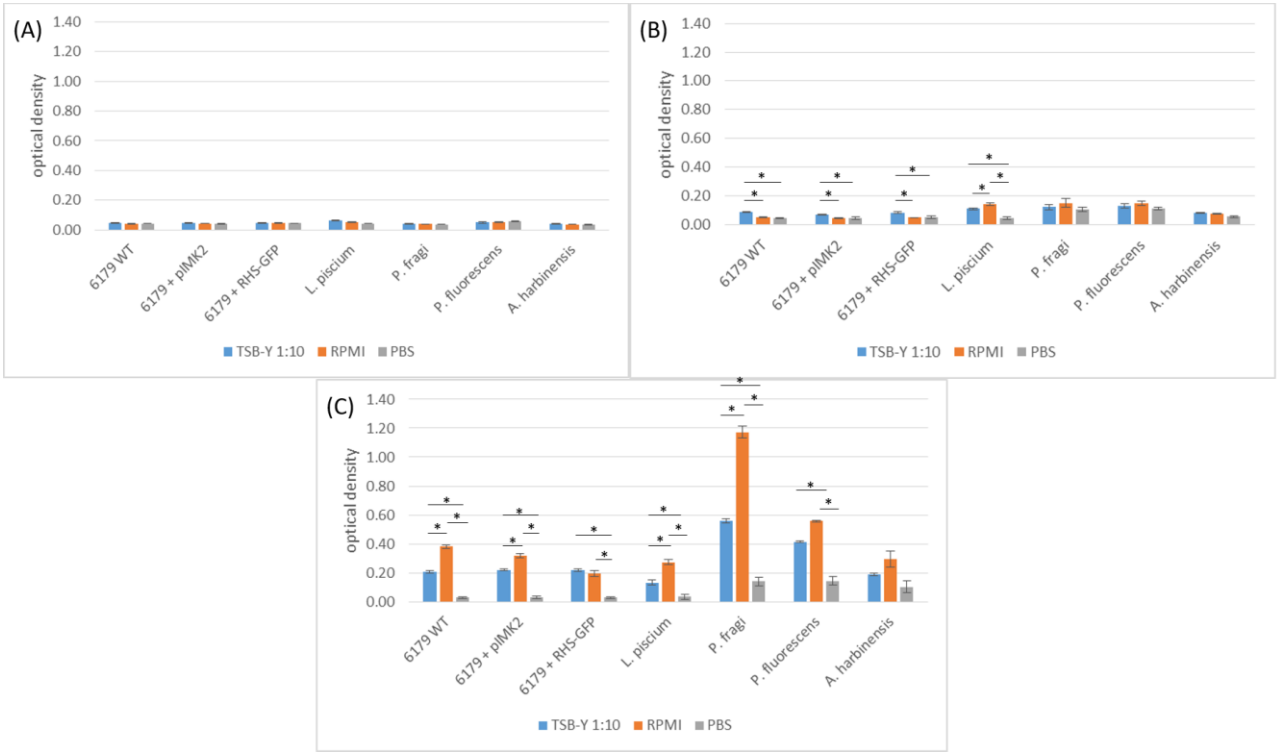

Figure 6: Optical density after 0 h of growth (A), 4 h of growth (B) and 24 h of growth (C); values are mean values of three biological experiments in six technical replicates

3.2 Time points for the fluorescence measurement

To determine the best time point for fluorescence measurements pure cultures of *L. monocytogenes* 6179 WT, *L. monocytogenes* + pIMK2(eGFP) and *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ were measured at different time points. The fluorescence was measured in the unit relative fluorescence units (rfu) and in parallel the OD_{610} was determined, to control the growth of all cultures.

The fluorescence signal normed to the mean blank is shown Fig. 7. Fig. 8 shows the growth corrected for the mean blank.

Fluorescence and OD_{610} were measured at 0 h, 0.5 h, 1 h, 2 h, 4 h and 24 h of growth. The time points 0.5 h, 1 h and 2 h were only measured in one biological replicate, whereas the time points 0 h, 4 h and 24 h were measured in two biological replicates.

For the time points 0 h, 0.5 h, 1 h, 2 h and 4 h the fluorescence normed to the mean blank was negative for all strains (data not shown). After 24 h the fluorescence normed to the mean blank was positive. Thus differences in the expression of the RHS protein will be determined 24 h after the preparation of the 96-well plates.

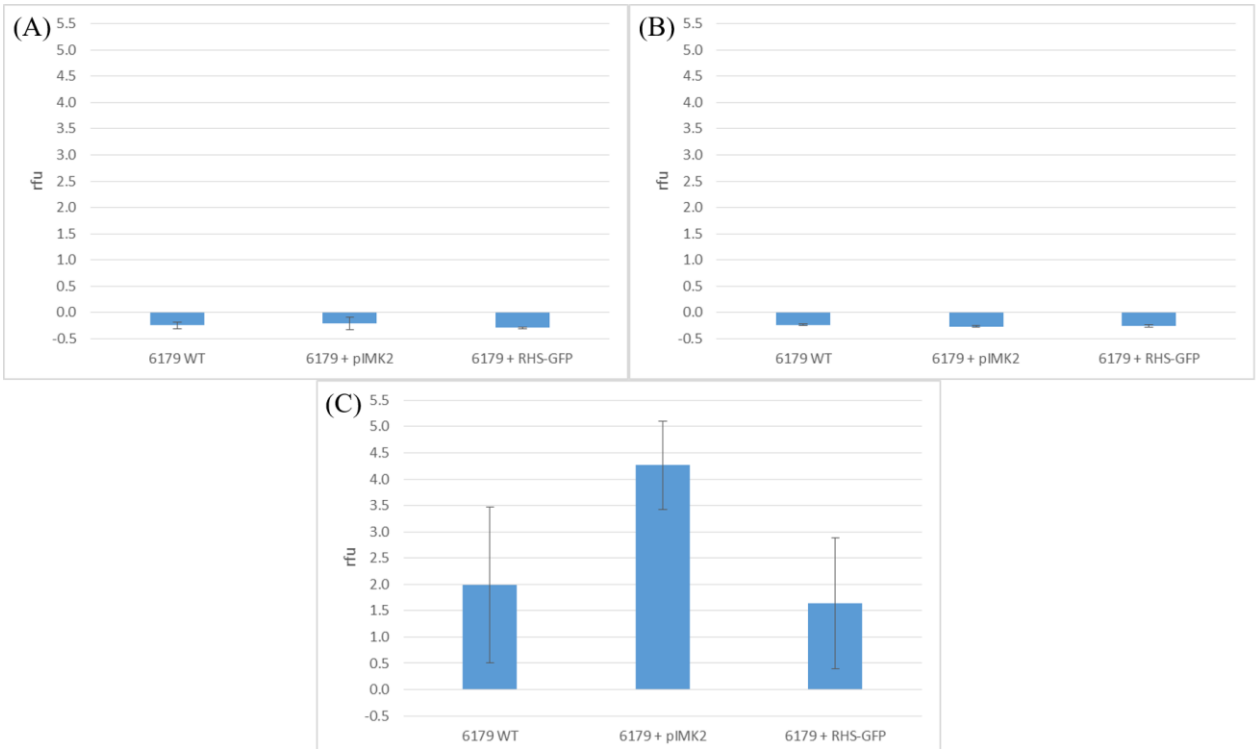

Figure 7: Fluorescence after 0 h (A), 4 h (B) and 24 h (C) of growth; values are mean values of two biological experiments in six technical replicates

L. monocytogenes 6179 harbouring the plasmid pIMK2, which has a *eGFP* gene and a Kanamycin resistance, is meant to overexpress eGFP and therefore should serve as positive control in the experiments to determine under which conditions the RHS protein of *L. monocytogenes* is expressed.

However, first experiments showed that the fluorescence signal of *L. monocytogenes* + pIMK2(eGFP) is not higher than the fluorescence signal of *L. monocytogenes* 6179 WT after 24 h of growth when cultivated in RPMI without phenol red. *L. monocytogenes* 6179 WT is the negative control as its genome doesn't contain the *eGFP* gene and thus doesn't fluoresce.

A Tukey-HSD post-hoc test was performed, to see if the fluorescence signal differs significantly between *L. monocytogenes* + pIMK2(eGFP) and *L. monocytogenes* 6179 WT at any time point of the measurement, but the test confirmed that there is no significant difference in fluorescence signals.

Thus *L. monocytogenes* + pIMK2(eGFP) cannot be used as a positive control, as its fluorescence signal should be higher than the signal of *L. monocytogenes* 6179 WT.

Growth control showed that the optical density of all of the strains increased from 4 h to 24 h of growth, meaning that all strains were growing in this time frame with *L. monocytogenes* + pIMK2(eGFP) growing the best.

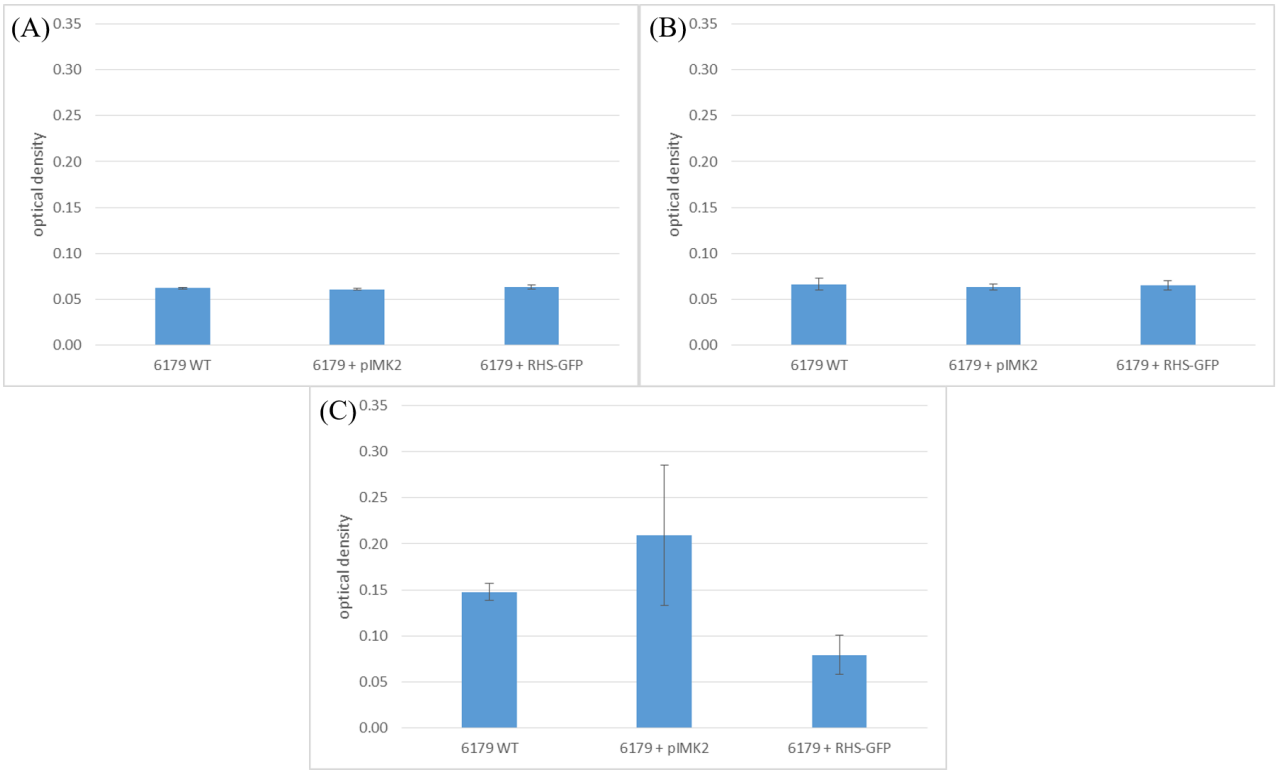

Figure 8: Optical density after 0 h (A), 4 h (B) and 24 h (C) of growth; values are mean values of two biological experiments in six technical replicates

3.3 Preliminary results of the co-cultivation

We co-cultivated *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ with *L. piscium*, *P. fragi*, *P. fluorescens* and *A. harbinensis*. *L. piscium* and *P. fluorescens* showed a fluorescence signal in the pure culture. All co-cultures, except *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ with *A. harbinensis*, had a lower fluorescence signal than the pure cultures of *L. piscium*, *P. fragi*, *P. fluorescens* and *A. harbinensis*. However the optical density of the co-culture of *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ with *A. harbinensis* is higher than the pure culture of *A. harbinensis.* The fluorescence signal was not higher in any co-culture compared to the *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ pure culture.

But as *L. monocytogenes* + pIMK2(eGFP) is not suitable as a positive control these results have to be interpreted with great care.

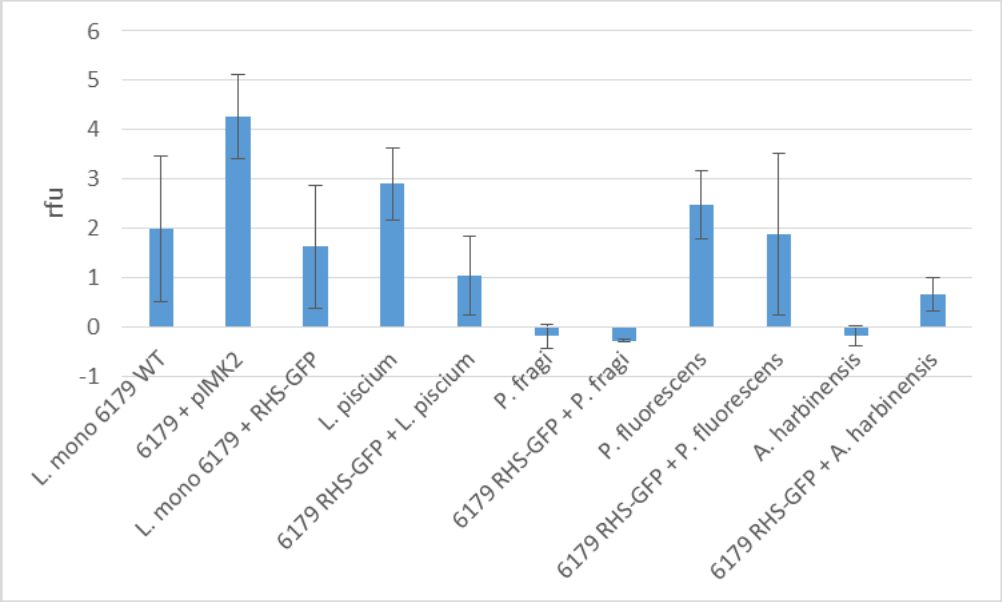

Figure 9: Fluorescence after 24 h of growth; values are mean values of two biological experiments in six technical replicates

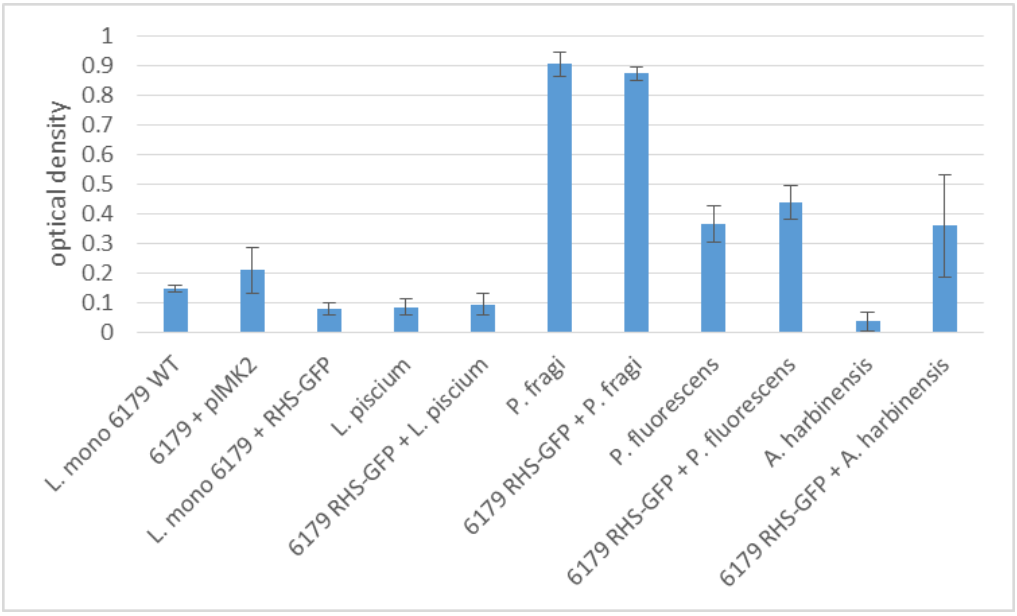

Figure 10: Optical density after 24 h of growth; values are mean values of two biological experiments in six technical replicates

3.4 Cloning into *Listeria monocytogenes* 6179 wildtype

As *L. monocytogenes* + pIMK2(eGFP) cannot be used as a positive control there was need for another one. *L. monocytogenes* + pIMK2(eGFP) harbours the *eGFP* gene on the plasmid pIMK2 and the expression of genes on plasmids is not as stable as the expression of genes on the bacterial chromosome. So the aim was to clone the *eGFP* gene chromosomally into *L. monocytogenes* 6179 WT using the bacteriophage pPL2 harbouring the *eGFP* gene.

Therefore primer flanking the integration site of pPL2, the tRNA-Arg-TCT site, were designed and then tested for the best annealing temperature. This was performed using gradient PCR and agarose gel electrophoresis. The primers worked well for all tested temperatures so the highest temperature was chosen for further PCRs, because the higher the annealing temperature the more specific the binding of the primers.

The results of the gradient PCR were used for the realization of the colony PCR. pPL2 harbours a resistance to chloramphenicol so *L. monocytogenes* 6179 WT was selected on cultivation medium supplemented with chloramphenicol after the electroporation with pPL2. The resulting colonies were then used for the colony PCR to check if integration of pPL2 really occurred and the PCR products were analysed by agarose gel electrophoresis.

The gel electrophoresis showed that the PCR products of the electroporated colonies were as large as the product of *L. monocytogenes* 6179 WT, which served as negative control. If integration of pPL2 had occurred, the PCR product would be larger than the product of the negative control, because pPL2 introduces into the tRNA-Arg-TCT site and thus makes the locus larger. The gel electrophoresis showed, that integration of pPL2 did not occur in any of the tested cultures. A second, lower band can be seen on the gel for almost every of the samples, which results from the primers. The gel of the colony PCR is shown in Fig. 11.

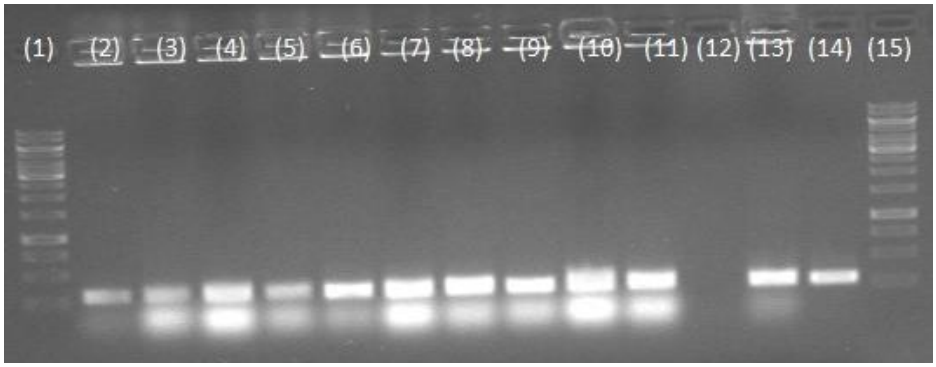

Figure 11: result of the gel electrophoresis with the products of the colony PCR: from left to right are shown the 1kB ladder (1); one single colony of the electroporated *L. monocytogenes* 6179 each from TSA plates supplemented with 25 µg/ml of chloramphenicol (2-4); single colonies from TSA plates supplemented with 10 µg/ml of chloramphenicol (5 -11); negative control with water (12); negative control with a *L. monocytogenes* 6179 WT colony (13), negative control with isolated DNA from *L. monocytogenes* 6179 WT (14) and the 1kB ladder (15)

The first colony PCR showed that the integration of pPL2 hasn't occurred. However, tested cultures, although negative, could still harbour the bacteriophage pPL2, which hasn't integrated yet. Hence a second colony PCR was performed this time aiming for the *eGFP* gene, as it is part of the vector pPL2.

The products of the second colony PCR were also analysed by gel electrophoresis. If the negative colonies would harbour not integrated pPL2 and thus the *eGFP* gene, there would be a visible band on the gel. Gel electrophoresis showed that none of the previously negative cultures harbours pPL2, so all of them are definitely negative and cloning hasn't succeeded. The gel of the second colony PCR is shown in Fig. 12.

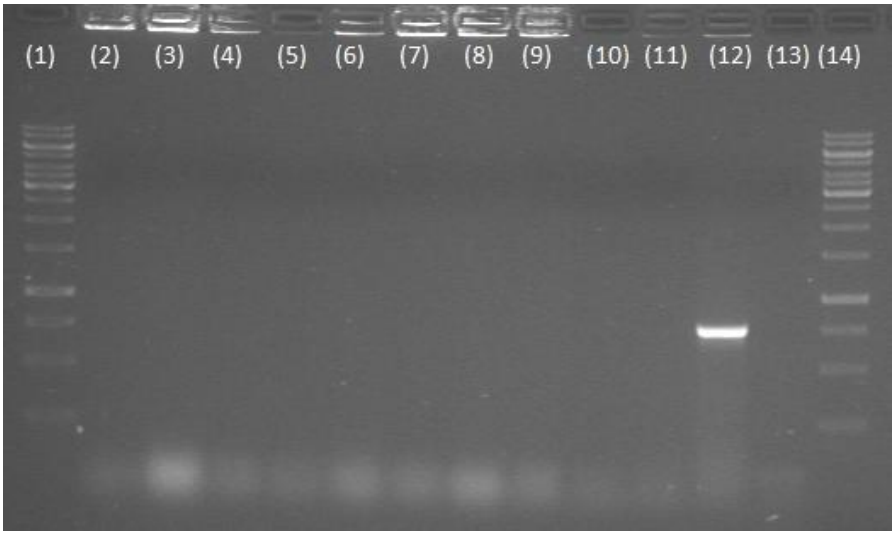

Figure 12: result of the gel electrophoresis with the products of the second colony PCR: from left to right are shown the 1kB ladder (1); one single colony of the electroporated *L. monocytogenes* 6179 each from TSA plates supplemented with 25 µg/ml of chloramphenicol (2-4); single colonies from TSA plates supplemented with 10 µg/ml of chloramphenicol (5-11); positive control with a *L. monocytogenes* + pIMK2(eGFP) colony (12); negative control with water (13) and the 1kB ladder (14)

4. Discussion

Listeriosis is a rare but potentially serious infection caused by the food-borne, bacterial pathogen *L. monocytogenes*. It manifests in form of gastroenteritis in healthy individuals, but can cause severe illnesses, like sepsis, meningitis or encephalitis, in immunocompromised individuals with a high mortality, reaching up to 20-30 %. Groups at higher listeriosis risk are pregnant women, elderly people, immunocompromised people, unborn babies, and neonates. During pregnancy infections with *L. monocytogenes* can result in abortions, stillbirths or preterm birth (Allerberger and Wagner 2010, Cossart 2011).

Transmission of *L. monocytogenes* occurs through consumption of contaminated food. The main foods implicated are milk, unpasteurized soft cheese, ready-to-eat prepared meats, undercooked poultry and fish, unwashed raw vegetables and contaminated fruits. Contamination of food occurs because the capacity of *L. monocytogenes* to adapt to a variety of niches and growth conditions in the food processing environment is very distinctive (Cossart 2011, Noordhout et al. 2014).

L. monocytogenes is difficult to control along the entire food chain from production to storage and consumption, as it is able to grow under conditions normally used for food conservation, like low or high temperatures, extreme pH values or low moisture content. But *L. monocytogenes* also encounters and survives other food-related stress conditions along the food chain, including oxidative and osmotic stress, the presence of bacteriocins and other stresses resulting from alternative decontamination and preservation technologies such as high hydrostatic pressure, UV light and pulsed electric fields. These stresses are either intrinsic to the food matrix or extrinsic to preservative agents or imposed onto *Listeria* upon consumption by the host. The same stress may occur on several occasions along the food chain and thus it is important for *L. monocytogenes* that the resistance to different stresses is interconnected. For example, osmotic stress enhances its resistance to heat. The resistance to various stress conditions supports the colonization and persistence of *L. monocytogenes* in the food processing environment and thus increases the risk of contamination (Bucur et al. 2018).

However, there is a high degree of *L. monocytogenes* strain divergence in stress response and environmental adaptation. Among a great diversity of strains from the food processing environment, particularly strains belonging to the sequence type 121, like 6179, can be found more frequently and are persistent more often. Genomes of the ST121 are highly similar to each

other (more than 99.73% average nucleotide identity) and are highly conserved among prophages and plasmids. Differences between the strains are mainly based on the absence or presence of plasmids and the number of prophages. These plasmids and prophages provide important adaptations for survival and persistence in the food processing environment (Pasquali et al. 2018, Rychli et al. 2017, Schmitz-Esser et al. 2015).

L. monocytogenes ST121 genomes display a genomic G+C content of 37.9 %, which is found for most *Listeria* genomes. They harbour between one and four prophages, in 6179 one prophage is inserted downstream of the tRNA Arg-TCT, another prophage downstream of tRNA Arg-CCG and the third phage is inserted downstream of the tRNA Thr-GGT. There is also a prophage integrated into the *comK* gene, which is important for the adaptation of *L. monocytogenes* to the food production environment. The similarity of the tRNA phages is very high for the ST121, but it is considerably lower to other described phages for *L. monocytogenes*, which is striking as prophages are important drivers of short-term genome evolution in *L. monocytogenes* and are the major source of diversity within the genus *Listeria.* Thus the phages might be advantageous for ST121 to survive under stress conditions of the food processing environment. A high level of transcription and differential expression of the tRNA-Arg-TCT and tRNA-Arg-CCG prophage could be shown in *L. monocytogenes* 6179 under benzethonium chloride challenge, which suggests an important role of these phages at least under these conditions.

Genetic elements of ST121 involved in stress tolerance include the stress survival islet 2, which is involved in the alkaline and oxidative stress responses, and the transposon Tn*6188*, which is involved in increased tolerance to various quaternary ammonium compounds. Plasmids confer increased stress tolerance, too. There is high degree of conservation between plasmids, which suggests that strong selective pressure is acting on the ST121 plasmids. This high conservation of ST121 plasmids may thus be the result of niche adaptation.

Between the EGDe *lmo2753* and *lmo2754* homologues there is a 12.1 kbp insertion in ST121 genomes that encodes a protein harbouring 29 RHS domains. Being present in all ST121 genomes, this insertion is almost identical among the ST121 genomes with over 99.99 % nucleotide identity. Proteins harbouring RHS repeats like RhsAB from *D. dadantii* have been shown to be involved in bacterial intercellular competition by inhibiting growth of target cells.

Upstream of the RHS protein is the putative RNA 2'-phosphotransferase KptA, which likely catalyses tRNA cleavage (Harter et al. 2017a, Rychli et al. 2017, Schmitz-Esser et al. 2015).
TA systems encode a stable toxin and a highly labile antitoxin. Under stress conditions the antitoxin is degraded and the toxin is free to target and inhibit diverse biological processes, for example cell membrane integrity, assembly of the translational machinery, DNA replication and ATP synthesis. This inhibition then results in rapid growth arrest of the bacterial cells. It has been shown that TA systems also play a role in the induction of the persister phenotype and in bacterial competition. TA systems exist in various bacterial species, including *L. monocytogenes*, but little is known about TA systems in *L. monocytogenes* yet.
So far 14 different TA systems have been identified in 352 different *L. monocytogenes* genomes, of which two TA systems, *lmo0168* and *lmo0887*, appear to be orphans. *Lmo0113-0114* and *lmo0887-0888*, two TA systems of the class II, have been studied revealing that *lmo0113* is upregulated upon heat stress.
The *mazEF* TA locus (*lmo0887-0888*) is located directly upstream of the alternative sigma factor B (σ^B) operon in *L. monocytogenes*, which has a role in the bacterial stress response. MazF, the toxin, classically influences transcript levels of genes within the σ^B operon, but that is not true for the *mazEF* TA system in *L. monocytogenes*. *Lmo0887-888* does not affect the level of persister formation upon antibiotic treatment, but the expression of σ^B-dependent genes *opuCA* and *lmo0880* under sub-inhibitory norfloxacin conditions. However, the exact role and how the system operates is still unclear (Agüero et al. 2020, Curtis et al. 2017).
The RHS protein is secreted via a TA system class VI and is used in intracellular competition of *D. dadantii* and *Bacillus subtilis* (Benz and Meinhart 2014, Jamet and Nassif 2015, Koskiniemi et al. 2013, Poole et al. 2011). The aim of this bachelor thesis was to determine under which conditions the RHS protein of *L. monocytogenes* is expressed and to characterize its role in bacterial competition, as there is nothing know about it yet. As *L. monocytogenes* often can be found in the food processing environment, strains of the ST121 were used in the experiments and co-cultivated with other species that often appear in the food production environment. Beforehand a *L. monocytogenes* 6179 strain with eGFP-tagged promotor of the *rhs* operon, *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$, was created to measure the expression of the *rhs* locus via detection of the fluorescence signal of eGFP. *L. monocytogenes* 6179 WT was

the negative control, as it does not harbour the *eGFP* locus. *L. monocytogenes* + pIMK2(eGFP) was tested as positive control.

Before the co-cultivation experiments with *L. piscium*, *P. fragi*, *P. fluorescens* and *A. harbinensis* could start the settings for these experiments had to be determined. First six different culture media were tested for autofluorescence and growth of all the strains that were used in the experiments. The experiments showed that only TSB-Y diluted 1:10, RPMI and PBS meet the criteria according to the lowest possible autofluorescence. With regard to growth of the bacterial strains only TSB-Y and RPMI were suitable for the experiments, as the strains could not grow in PBS. Most of the strains showed the best growth in RPMI. To reduce the level of autofluorescence further RPMI without phenol red was chosen for the experiments.

Next the time points for the measurement of the fluorescence signal had to be determined so that the measurement occurred when the expression of the RHS protein is most stable (at least for the positive control). The fluorescence signal was measured after 0 h, 0.5 h, 1 h, 2 h, 4 h and 24 h of cultivation. 24 h of cultivation proved to be the time point when the expression is most stable, as the fluorescence signal was positive and the bacterial cultures were still alive. At 0 h, 0.5 h, 1 h, 2 h and 4 h of cultivation the fluorescence signal normed to the mean blank, which was RPMI without phenol red alone, was negative and very variable among strains and time points. These negative signals could be the result of adaptation to the new environment.

Bacterial populations growing in a changing world must adjust their proteome composition in response to alterations in the environment, such as a change in growth medium. Rapid responses of the proteome correspond to an increase in average growth rate and fitness value of the population (Pavlov and Ehrenberg 2013).

Before cultivation the strains were activated in RPMI without phenol red overnight, so the type of medium didn't change, only the nutrient content of the medium did. The strains were also exposed to environmental stress due to centrifugation and resuspending in the new medium before they were cultivated in the 96-well plates for measurement. As can be seen in the growth rates at 0 h, 0.5 h, 1 h, 2 h and 4 h of cultivation, only *P. fragi* and *P. fluorescens* managed to grow in this time frame, all other strains didn't, probably as a result of the exposure to stress. The negative fluorescence signal could result from consumption of components of the cultivation medium that contribute to the autofluorescence of RPMI. After 24 h all strains have

grown and thus adapted to the new environment. The positive fluorescence signal might be due to a change in the proteome resulting in the expression of fluorescent products.

L. monocytogenes + pIMK2(eGFP) was tested as positive control, as it contains the plasmid pIMK2, which harbours the *eGFP* gene. Monk et al. 2008 described pIMK2 as a vector, which is overexpressed and can be selected for its Kanamycin resistance. However, our experiments showed that the fluorescence signal of *L. monocytogenes* + pIMK2(eGFP) in the cultivation medium RPMI is not significantly higher than the fluorescence signal of *L. monocytogenes* 6179 WT.

Thus *L. monocytogenes* + pIMK2(eGFP) cannot be used as a positive control and a new one has to be created. The aim was to clone the *eGFP* gene into the chromosome of *L. monocytogenes* 6179 WT using the bacteriophage pPL2 as vector. *L. monocytogenes* + pIMK2(eGFP) harbours the *eGFP* locus on a plasmid and harbouring and maintaining a plasmid always comes with a cost.

Chromosomal genes are expressed more stable than genes on plasmids and are a low metabolic burden to the host cell (Ou et al. 2018). Thus integration of the *eGFP* locus into the chromosome might result in a better positive control.

Cloning of pPL2 into *L. monocytogenes* 6179 WT was performed according to Lauer et al. 2002, but was not successful, which was verified by a colony PCR targeting the integration site of pPL2. A second colony PCR targeting the *eGFP* gene showed that pPL2 not only did not integrate in the genome, but neither did the bacterial cells harbour not integrated pPL2.

As a good positive control is essential to perform the co-cultivation experiments the cloning will be repeated according to the instructions of Azizoglu et al. 2014.

Preliminary results of the co-cultivation showed that the fluorescence signal was not higher in any co-culture compared to the *L. monocytogenes* 6179 $P_{RHS\text{-}operon::eGFP}$ pure culture.

To conclude the experiments there is no evidence that the expression of the RHS protein is altered when *L. monocytogenes* 6179 is co-cultivated with bacterial species found in the food processing environment, as *L. monocytogenes* + pIMK2(eGFP) cannot be used as a positive control and thus the preliminary results of the co-cultivations have to interpreted with care.

5. Summary

Listeria (*L.*) *monocytogenes* is a food-borne pathogen and responsible for the infection disease listeriosis, which can be fatal for elderly people, immunocompromised, newborn and unborn individuals. The main foods implicated in listeriosis cases are milk, unpasteurized soft cheeses, ready-to-eat prepared meats, undercooked poultry and fish, unwashed raw vegetables and fruits. *L. monocytogenes* can perfectly survive in the food processing environment leading to food contamination. However, beside *L. monocytogenes* many bacteria are present in the food producing environment including the food spoilers *Lactococcus piscium*, *Pseudomonas fragi*, *Pseudomonas fluorescens* and *Acinetobacter harbinensis*, too. Bacteria have developed different strategies to reduce the growth of the other competitors like the toxin-antitoxin systems. The RHS protein is part of such a toxin-antitoxin system and its role in bacterial competition has been shown for species like *Dickeya dadantii*, but not yet for *L. monocytogenes*. RHS proteins are mainly harboured by *L. monocytogenes* strains of sequence type 121, a sequence type predominately found in the food processing environment.

The aim of this bachelor thesis was to determine the conditions under which the RHS protein of *L. monocytogenes* is expressed. Therefore a strain with an eGFP-tagged promotor of the *rhs* operon was created, so that the expression of RHS could be measured as a fluorescence signal in co-cultivation experiments. First the optimal parameters for the experiment had to be determined. Different cultivation media were tested for growth of the target strains and their autofluorescence. This revealed that RPMI, a cell culture medium, is the most suitable medium. To reduce background fluorescence RPMI without phenol red was finally chosen as medium for the experiments. Measurement of fluorescence was performed at various time points, revealing that the signal is most stable after 24 hours growth of the bacterial cultures.

L. monocytogenes 6179 harbouring the plasmid pIMK2(eGFP) was used as positive control, because it is meant to overexpress eGFP. However, first experiments showed that the fluorescence signal of *L. monocytogenes* + pIMK2(eGFP) is not significantly higher than the signal of *L. monocytogenes* 6179 WT.

Therefore a *L. monocytogenes* strain expressing eGFP chromosomally will be created, as the expression of chromosomal genes is more stable than the expression of genes on plasmids. The vector pPL2 was used for cloning and introduced into competent *L. monocytogenes*, via electroporation. Positive transformants were selected on TSA plates supplemented with

chloramphenicol and a control PCR and gel electrophoresis was performed to verify the transformation. This revealed that the transformation wasn't successful and pPL2 didn't integrate. Cloning will be repeated as a functional positive control is essential for all subsequent experiments.

6. Deutsche Zusammenfassung

Listeria (*L.*) *monozytogenes* ist ein lebensmittelbedingtes Pathogen und verursacht die Krankheit Listeriose, die für ältere, immunkomprimierte, neugeborene und ungeborene Menschen tödlich enden kann. Listeriosefälle werden mit diätetische Risikofaktoren assoziiert und häufig betroffene Lebensmittel sind Milch, nicht-pasteurisierter Weichkäse, ready-to-eat Fleisch, nicht gares Geflügel und Fisch, sowie rohes Gemüse und Obst.

Aber *L. monozytogenes* ist nicht das einzige Bakterium, das man in der Lebensmittelverarbeitung finden kann, und so tauchen auch die Lebensmittelverderber *Lactococcus piscium*, *Pseudomonas fragi*, *Pseudomonas fluorescens* und *Acinetobacter harbinensis* auf. Um mit anderen Bakterien zu konkurrieren gibt es verschiedene Strategien das Wachstum der Konkurrenten zu reduzieren, wie etwa Toxin-Antitoxin Systeme. Das RHS Protein ist auch Teil eines solchen Systems und seine Rolle bei der bakteriellen Konkurrenz konnte schon für *Dickeya dadantii*, aber noch nicht für *L. monozytogenes* gezeigt werden. In *L. monozytogens* zeigte sich, dass vor allem ein spezifischer Sequenztyp ST121, welcher häufig im Lebensmittelumfeld vorkommt, die RHS Proteine besitzt.

Das Ziel dieser Bachelorarbeit war herauszufinden unter welchen Bedingungen das RHS Protein von *L. monozytogenes* exprimiert wird. Dafür wurde ein Stamm mit eGFP-markiertem Promotor des *rhs* Operons geschaffen um die Expression von RHS als Fluoreszenzsignal bei Co-Kultivierungsexperimenten messen zu können. Am Anfang mussten die optimalen Bedingungen für die Experimente bestimmt werden. Verschiedene Nährmedien wurden auf Wachstum der Zielstämme und Autofluoreszenz getestet. Das ergab, dass RPMI, ein Zellkulturmedium, am besten geeignet ist. Um die Hintergrundfluoreszenz weiter zu reduzieren wurde RPMI ohne Phenolrot für die Experimente ausgewählt. Fluoreszenzmessungen wurden zu verschiedenen Zeitpunkten gemacht und dabei stellte sich heraus, dass das Signal nach 24 Stunden Wachstum der Bakterienkulturen am stabilsten ist.

L. monozytogenes 6179, welches das Plasmid pIMK2(eGFP) enthält, wurde als Positivkontrolle verwendet, da es eGFP überexprimieren sollte. Erste Experimente haben aber gezeigt, dass das Fluoreszenzsignal von *L. monozytogenes* + pIMK2(eGFP) nicht signifikant höher ist als das von *L. monozytogenes* 6179 Wildtyp.

Daher planen wir einen *L. monozytogenes* Stamm zu erzeugen, der eGFP chromosomal exprimiert, da die Expression von chromosomalen Genen stabiler ist als die von Genen auf

einem Plasmid. Zum Klonieren wurde der Vektor pPL2 verwendet und in kompetenten *L. monozytogenes* elektroporiert. Positive Transformaten wurden auf TSA-Platten mit Chloramphenicol selektiert. Eine Kontroll-PCR mit anschließender Gelelektrophorese wurde durchgeführt, um die Transformation zu bestätigen. Dabei stellte sich heraus, dass die Transformation nicht erfolgreich war und sich pPL2 somit nicht integriert hatte. Das Klonieren wird wiederholt, da eine gut funktionierende Positivkontrolle für alle weiteren Versuche entscheidend ist.

7. Appendix

List of abbreviations

L.	*Listeria*
TSB-Y	Tryptic Soy Broth with yeast
TSB	Tryptic Soy Broth
L. piscium	*Lactococcus piscium*
P.	*Pseudomonas*
A.	*Acinetobacter*
TSA	Trypto-Casein Soy Agar
LB	Luria Bertani Broth
BHI-Y	Brain Heart Broth with yeast
BHI	Brain Heart Broth
RPMI	Roswell Park Memorial Institute
PBS	Phosphate buffered saline
L. monocytogenes + pIMK2(eGFP)	*Listeria monocytogenes* 6179 harbouring the plasmid pIMK2(eGFP)
L. monocytogenes 6179 WT	*L. monocytogenes* 6179 wildtype
OD_{600}	optical density at 600 nm
rpm	rounds per minute
sec	seconds
min	minutes
Vol.	Volume
V	Volt
FBS	fetal bovine serum
NEA	non-essential amino acids
TA	Toxin-antitoxin
E.	*Escherichia*
PTS	polymorphic toxin systems
sRNA	small regulatory antisense RNA

CDI	contact-dependent growth inhibition
RHS	rearrangement hotspot
D.	*Dickeya*
ST	sequence type
OD_{610}	optical density at 610 nm
rfu	relative fluorescence units
σ^B	alternative sigma factor B

List of figures and tables

List of equipment for common processes

- Water bath W20D (Peter Huber Kältemaschinenbau AG; Germany)
- IKA® Vortex Genius 3 (IKA-Werke GmbH & Co. KG; Germany)
- Safe-Lock Tubes 1.5 mL (Eppendorf AG; Germany)
- Disposable protective gloves made of nitrile rubber (NBR) (Paul Hartmann AG; Germany)
- Pipettes (Eppendorf AG; Germany)
- Safeguard Filter Tips (peqlab a VWR brand; Germany)
- mikrozid® AF liquid (Schülke & Mayr GmbH; Germany)

8. References

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