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Factors of importance for virulence and persistence of Listeria monocytogenes

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Factors influencing persistence and virulence of *Listeria monocytogenes*

Ph. D Thesis

by

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Preface

The work presented in this thesis is the result of a Ph.D. study following the Ph.D. program at the Technical University of Denmark. The Ph.D. study is a part of a project entitled "Microbial Opportunistic Pathogens – a severe problem to human health", which is financed by The Danish Research Agency.

The Ph.D. student has been enrolled at the Technical University of Denmark from 1st October 2004 to 30th September 2007. The work has been carried out at:

- Technical University of Denmark, National Institute of Aquatic Resources, Department of Seafood Research, DK-2800 Kgs. Lyngby, Denmark
- University of Copenhagen, Faculty of Life Sciences, Department for Veterinary Pathobiology, DK-1870 Frederiksberg C, Denmark
- Technical University of Denmark, The Food Institute, Department of Microbiology and Risk Assessment, DK-2860 Søborg, Denmark
- University of Georgia, College of Public Health, Department of Environmental Health Science, Athens, Georgia 30602, USA (5 months stay)

Supervisors were Professor Lone Gram (National Institute of Aquatic Resources), senior research scientist Birte Fonnesbech Vogel (National Institute of Aquatic Resources) and Professor Hanne Ingmer (Faculty of Life Sciences).

The thesis is based on the following three papers:

Paper 1:

Anne Jensen, Marianne H. Larsen, Hanne Ingmer, Birte F. Vogel, Lone Gram (2007). Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of *Listeria monocytogenes* strains. *Journal of Food Protection*. **70**(3):592-599.

Paper 2:

Anne Jensen, Line E. Thomsen, Rikke L. Jørgensen, Marianne H. Larsen, Bent B. Roldgaard, Bjarke B. Christensen, Birte F. Vogel, Lone Gram, Hanne Ingmer (2007). Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models. *International Journal of Food Microbiology*. Submitted.

Paper 3:

Anne Jensen, Denita Williams, Elizabeth A. Irvin, Lone Gram, Mary Alice Smith (2007). A processing plant persistent strain of *Listeria monocytogenes* crosses the feto-placental barrier in a pregnant guinea pig model. *Journal of Food Protection*. Submitted.

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Summary

Factors influencing persistence and virulence of *Listeria monocytogenes*

Listeria monocytogenes is an important human pathogenic food borne bacterium of great health and economic importance. The infectious disease, listeriosis, caused by *L. monocytogenes* is only seen in a low number of cases in Denmark and worldwide, when compared to other food borne human pathogenic bacteria. But when infected, the hospitalization and mortality rate is very high. In Denmark approx. 40 cases are seen every year, and the mortality rate is as high as 20-30%. The infection is primarily seen in immunocompromised humans and in pregnant women, where the fetus is the primary target.

The presence of *L. monocytogenes* can be seen in several types of ready-to-eat food products (soft cheeses made from unpasteurized milk, delicatessen meats, cold-smoked salmon and similar fish products). *L. monocytogenes* is able to grow, despite the presence of preservation methods (NaCl, refrigeration temperature, vacuum-packaging), which allow the bacteria to grow to high numbers. Since the food product is not heat-treated by the consumer before consumption, a high number of *L. monocytogenes* can be ingested, which could result in listeriosis. The main reason for the presence of *L. monocytogenes* in these products is contamination of the food product during production. Several food processing plants have their own “in-house” flora of special DNA-sub-types of *L. monocytogenes* which have colonized the processing plant. It has recently been demonstrated that one group of genetically similar *L. monocytogenes* strains (RAPD type 9) dominate and persist in several independent fish processing plants. The reason for this persistence is not known, but an understanding can lead to improved strategies for elimination of the bacterium from the processing plants. As the persistent strains are likely to contaminate food products, it is important to determine their virulence potential to evaluate the health risk they posses.

This study represented in this thesis had two objectives. The first objective was to investigate factors in the food processing environment or in the persistent DNA-sub-types, which could facilitate and enhance the persistence of *L. monocytogenes*. The second objective was to investigate the virulence potential of these persistent DNA-sub-types of *L. monocytogenes*.

Several hypotheses for persistence have been suggested by different research groups. It has been suggested that a changed growth rate, either higher or lower could explain the ability of specific DNA-sub-types to persist. The growth pattern of *L. monocytogenes* strains having different origins: food processing persistent RAPD type 9 strains, human clinical strains, strains isolated from food products, and strains isolated sporadically in the processing environment, was determined under different conditions such as low and high temperatures and in medium with and without the presence of 5% NaCl. No differences were observed between the growths rates of the strains in the different growth media, indicating that different growth rates are not a reason for persistence of the RAPD type 9 strains.

Differences in the adhesion ability to surfaces have also been suggested as an explanation for persistence of certain DNA-sub-types. An adhesion assay was performed in microtiter plates, where adhered bacteria were visualised by crystal violet staining. Adhesion to the plastic surface was seen by all the strains, when they where grown in a standard laboratory medium. The food processing persistent RAPD type 9 strains did not adhere to a higher level than other strains. Addition of 2-5% NaCl, to mimic the level of NaCl in the food matrix present in the fish processing industry, to the growth medium, did enhance the adhesion dramatically and aggregation of the cells was also seen. The enhanced adhesion and aggregation was not a unique phenomenon for the tested RAPD type 9 strains, since several of the other strains showed the same adhesion and aggregation pattern. Therefore, the presence of NaCl in the food matrix in the food processing industry will facilitate the adhesion and aggregation of *L. monocytogenes* to the production surfaces, but this factor alone is not the reason for persistence.

The virulence potential of the food processing persistent RAPD type 9 strains was compared to clinical strains, strains isolated from foods and reference strains in an intestinal epithelial cell line (Caco-2), in a nematode model (*Caenorhabditis elegans*), in a fruit fly model (*Drosophila melanogaster*), in non-pregnant and pregnant guinea pigs. A correlation of the results was seen between invasion ability into Caco-2 cells, time to death of *C. elegans* and fecal shedding in guinea pigs. The food processing persistent RAPD type 9 strains showed to posses a lower virulence potential compared to the clinical strains. Surprisingly, the virulence potential of a RAPD type 9 strain against the fetuses of the pregnant guinea pigs showed to be slightly higher than that of a clinical strain.

Since addition of NaCl to the growth medium showed to increase adhesion of the strains to a plastic surface, it was investigated if addition of NaCl had an influence on the virulence potential. The presence of NaCl did not influence the virulence potential in adhesion to and invasion into Caco-2 cells, time to death of *D. melanogaster* or the colonization and fecal shedding in non-pregnant guinea pigs.

Resumé (in Danish)

Faktorer af betydning for persistens og virulens af *Listeria monocytogenes*

Listeria monocytogenes er en vigtig human pathogen fødevarebåren bakterie som har både stor helbredsmæssig og økonomisk betydning. I Danmark og i resten af verden er der kun rapporteret et lavt antal tilfælde af den infektiøse sygdom, listeriose, forårsaget af *L. monocytogenes*, sammenlignet med andre fødevarebårne human patogene bakterier, men hospitalsindlæggelses- og dødsraten er meget høj. I Danmark er der omkring 40 tilfælde om året, og dødsraten er ca. 20-30%. Infektionen ses primært hos immunsvækkede personer og hos gravide kvinder, hvor fostret er det primære mål.

Tilstedeværelsen af *L. monocytogenes* ses i flere typer fødevarer i kategorien ”spiseklare produkter” (bløde råmælksoste, skiveskåret pålægsvarer, kold-røget laks og lignende fiskeprodukter). *L. monocytogenes* kan vokse ved, de i disse produkter, anvendte konserveringsparametre, og kan derfor vokse op til et højt niveau. Da produkterne ikke bliver varmebehandlet af forbrugeren inden indtagelse, kan et højt antal levende bakterier blive indtaget og dermed forårsage listeriose. Den primære årsag til tilstedeværelsen af *L. monocytogenes* i denne type produkter er kontaminering af produktet under produktionen, og det er vist at flere fødevareproducerende fabrikker huser deres egen ”husflora” af specifikke DNA-undertyper af *L. monocytogenes*, der har koloniseret fabrikken. For nyligt er det vist, at én gruppe af genetisk sammenlignelige *L. monocytogenes* stammer (RAPD type 9) dominerer og persisterer i flere fiskeprodukt producerende fabrikker. Årsagen til denne persistens er ikke kendt, men en forståelse af fænomenet kan føre til forbedrede stategier for udrydelsen af bakterien fra fabrikkerne. Yderligere, kan persisterende stammer være sandsynlige kontaminanter af fødevarer og derfor er det vigtigt at bestemme deres virulenspotentiale for at evaluere den risiko de udgør.

Dette Ph.D.-studie havde to formål. Det første formål var at bestemme faktorer i fødevareprocesmiljøet eller hos de persisterende DNA-undertyper, som kunne fremme og øge persistensen af *L. monocytogenes.* Det andet formål var at undersøge virulenspotentialet af disse persisterende DNA-undertyper.

Et antal hypoteser til forklaring af persistens er blevet opstillet af forskellige forskningsgupper. Det er blevet foreslået, at en ændret væksthastighed, enten højere eller lavere, kunne forklare evnen til at specifikke DNA-undertyper persisterer. Vækstmønstret af *L. monocytognenes* stammer med forskellige oprindelse: fødevareproces persisterende RAPD type 9 stammer, humane kliniske stammer, stammer isoleret fra fødevarer, og stammer isoleret sporadisk i procesmiljøet, blev undersøgt under forskellige vækstforhold som lav og høj temperatur og i medie med eller uden tilstedeværelsen af 5% NaCl. Ingen forskelle blev observeret mellem stammernes vækstmønstre i de forskellige vækstmedier, hvilket tyder på, at forskellige vækstmønstre ikke er årsagen til persistens af RAPD type 9 stammerne.

Forskelle i adhæsionsevnen til overflader har også været foreslået som en forklaring på persistens af specifikke DNA-undertyper. Der er blevet udført en adhæsionsbestemmelse i mikrotiterbakker, hvor adhærerede bakterier blev farvet med krystal violet. Alle stammer var i stand til at fasthæfte til plastoverfladen, når de blev dyrket i et standard laboratoriemedie. De fødevareproces persisterende RAPD type 9 stammer udviste ikke højere adhæsionsevne end de andre stammer. Tilførsel af 2-5% NaCl, for at efterligne niveauet af NaCl i fødevarematrixen, der er tilstede i fiskeforarbejdningsindustrien, øgede adhæsionsevnen dramatisk og aggregering af cellerne blev også set. Den øgede adhæsionsevne og aggregering var ikke et unikt fænomen for RAPD type 9 stammerne, da flere af de andre stammer udviste samme adhæsions- og aggregeringsmønster. Derfor kan tilstedeværelsen af NaCl i fødevarematrixen i fødevareindustrien kunne fremme adhæsion og aggregering af *L. monocytognenes* til produktionsoverflader, men denne faktor alene er ikke årsag til persistens.

Virulenspotentialet af fødevareproces persisterende RAPD type 9 stammer blev sammenlignet med kliniske stammer, stammer isoleret fra fødevarer og referencestammer i en epithel cellelinje (Caco-2), i en ormemodel (*Caenorhabditis elegans*), i en bananfluemodel (*Drosophila melanogaster*) og i ikke-gravide og gravide marsvin. Der blev set en korrelation mellem resultaterne i evnen til invasion i Caco-2 cellerne, levetid af *C. elegans* og udskillelse gennem fæces hos marsvin, hvor RAPD type 9 stammerne udviste et lavere virulenspotentiale sammenlignet med de kliniske stammer. Virulenspotentialet overfor fostrene i de gravide marsvin var, overraskende, det samme for en RAPD type 9 stamme end for en klinisk stamme.

Eftersom tilførsel af NaCl til vækstmediet viste at forøge adhæsionen af stammerne til en plastoverflade, blev det undersøgt om tilførsel af NaCl også havde en effekt på virulenspotentialet. Tilstedeværelsen af NaCl havde ikke nogen indflydelse på virulenspotentialet i adhæsion til og invasion i Caco-2 celler, tid til død i *D. melanogaster* eller kolonisering og udskillelse gennem fæces i ikke-gravide marsvin.

1 Introduction

Listeria monocytogenes is a facultative intracellular gram-positive bacterium and is the causative agent for the food borne illness listeriosis. The illness will in milder cases lead to gastroenteritidis but in more severe cases cause sepsis or meningitis and in a pregnant woman cause stillbirth or premature birth of the fetus. Immunocompromised people (cancer, organ transplants, HIV) are susceptible to the infection, and will often get sepsis or meningitis, whereas a pregnant mother only will get flu-like symptoms. The disease is rare with an incidence at 0.34 per 100,000 people annually (FDA/FSIS*,* 2003a). Only 1% of the disease cases caused by human pathogens in USA are caused by *L. monocytogenes*, but the mortality rate, 16%, is very high (CDC*,* 2006). The infective dose is between 10^6-10^9 CFU depending of the health situation of the host, the food matrix and the virulence potential of the bacterium.

The bacterium can be isolated in low levels in several types of ready-to-eat (RTE) food products such as soft cheeses, luncheon meats and sea food products (Gombas *et al.,* 2003; Wulff *et al.,* 2006; Latorre *et al.,* 2007). RTE food products are stored at refrigeration temperature, are vacuum-packed and contain a moderate level of NaCl (3-6%). All these parameters are used to inhibit the growth of pathogenic and spoilage microorganisms, but *L. monocytogenes* is able to grow under these conditions. As RTE food products often have long shelf life's, and since the food products are eaten without heating by the consumer, *L. monocytogenes* can be present in high numbers at time of consumption.

Besides being of health importance, *L. monocytogenes* is also of economic importance. The presence of *L. monocytogenes* in food products can cause recall of products, followed by a great economical loss for a small processing plant. During time, close down of processing plants have been seen, but the exact numbers are not known.

The presence of *L. monocytogenes* in RTE food products is mainly due to contamination during processing and therefore not raw material contamination being carried through. Several RTE food processing plants have shown to contain persistent *L. monocytogenes* strains that are able to colonize plant environments and can potentially contaminate the final product (Mafu *et al.,* 1990; Rørvik *et al.,* 1995; Lawrence *et al.,* 1995; Nesbakken *et al.,* 1996; Miettinen *et al.,* 1999b; Norton *et al.,* 2001; Thimothe *et al.,* 2004; Wulff *et al.,* 2006). In the Danish fish processing industry, one group of genetically similar *L. monocytogenes* strains (RAPD type 9) was recently shown to reside in several independent fish processing plants (Wulff *et al.*, 2006)

In this thesis, a persistent sub-type is defined as a specific sub-type of strains that is isolated repeatedly in the same processing plant, and even at the same places inside the factory, during a longer period of time.

The reason for persistence of such sub-types is not known, but several hypotheses have been proposed by research groups. Persistent sub-types might have

- A higher prevalence in the out door environment.
- A decreased or an increased growth rate
- An enhanced ability to adhere to surfaces
- An enhanced tolerance against drying and desiccation
- An enhanced tolerance against cleaning and disinfection agents

It is not known if persistent sub-types are more or less virulent than strains of *L. monocytogenes* that have caused human infection. From a risk analysis perspective, it is important to assess the virulence potential of strains that are likely contaminants of food products, such as the food processing persistent strains. Food processing persisting sub-types of *L. monocytogenes* have been isolated from the finished products (Norton *et al.,* 2001; Vogel *et al.,* 2001a; Wulff *et al.,* 2006; Nakamura *et al.,* 2006), but whether persistent sub-types are causing human clinical cases of listeriosis is still discussed (Martinez *et al.,* 2003; Sauders *et al.,* 2004).

The purposes of the present Ph.D.-study have been to address some of the hypotheses proposed to explain persistence and to compare the virulence potential of strains belonging to the food processing persistent sub-type (RAPD type 9) to strains that have caused human listeriosis. The two hypotheses relating to growth rate and adherence to surfaces have been investigated, and the influence of temperature and NaCl on these phenotypes have been addressed (Jensen *et al.,* 2007a).

The virulence potential was determined using a broad variety of model systems spanning from simple *in vitro* models and non-mammalian models to the complex mammalian models (Jensen *et al.,* 2007b; Jensen *et al.,* 2007c).

2 *Listeria monocytogenes* – a food borne pathogenic bacteria

2.1 Taxonomy and characteristics of *Listeria monocytogenes*

Listeria monocytogenes is a food-borne gram-positive bacterium closely related to *Bacillus* and *Staphylococcus*. The bacterium is a member of the genus *Listeria* which also contains *L. innocua*, *L. grayi*, *L. seeligeri*, *L. welshimeri* and *L. ivanovii* (Sallen *et al.,* 1996). *L. ivanovii* have occasionally been associated with human illness (Snapir *et al.,* 2006), and *L. innocua* and *L. seeligeri* have once been reported to cause a case of bacteraemia and meningitis, respectively (Rocourt *et al.,* 1986; Perrin *et al.,* 2003). In humans *L. monocytogenes* causes listeriosis, which manifests itself in two different forms; the invasive and the non-invasive form. The non-invasive form causes gastroenteritis. The invasive form causes a life-threatening disease in persons belonging to specific risk groups. This risk groups are elderly, immunocompromised people (organ transplants, cancer, alcoholic, HIV), where the clinical signs are fever, diarrhoea, meningitis and sepsis. In pregnant women, the unborn fetus is at risk, and the clinical sign are abortion or still birth. Fetuses in the last trimester of the pregnancy are most susceptible for the infection. *L. monocytogenes* was discovered when Murray *et al.* (1926) that isolated *Bacterium monocytogenes* from rabbits and guinea pigs after a septic disease. The first reported case of human listeriosis was in Denmark in 1929 (Nyfeldt, 1929), and in 1940 Pirie (1940) suggested to change the genus name from *Bacterium* to *Listeria*. For several years, no noteworthy attention was given to listeriosis, but in 1970s and 1980s the number of reported cases of listeriosis increased, and more attention was given to the bacteria (Vazquez-Boland *et al.,* 2001). In 1983, Schlech *et al.* identified food as the vehicle of transmission, when contaminated coleslaw was shown to cause a listeriosis outbreak.

The infective dose of the bacterium has been estimated in several studies (FDA/FSIS, 2003b; FAO/WHO, 2004), where dose-response in mice or epidemiological investigations of human cases have been used. The infective dose has been estimated to 10^6 -10^9 CFU, but because of the variability in the host susceptibility, food matrix effects and strain variation it is not possible to determine a specific infectious dose for *L. monocytogenes* (FDA/FSIS, 2003b). Recently, Williams *et al.* (Williams *et al.,* 2007) showed, in an oral-exposed pregnant guinea pig model, that 10^7 CFU was the dose where 50% of the pregnancies was affected. However, it is generally acknowledged that low levels of the bacterium are unlikely to cause disease.

Listeria is a facultative anaerobic, hemolytic short rod with the size 0.4 µm by 1 to 1.5 µm that does not form a capsule, does not form spores, and is motile by a few peritrichous flagella when cultured at 20-25°C (Seeliger *et al.,* 1986). The motility of *L. monocytogenes* is temperature regulated and the production of flagellin is markedly downregulated at 37°C (Peel *et al.,* 1988; Dons *et al.,* 1992). A high tolerance to moderate and high levels of NaCl is also characteristic for *L. monocytogenes*, and it is able to grow in medium containing up to 12% NaCl (Cole *et al.,* 1990). Furthermore, the growth temperature interval is between 1.7°C to ~55°C depending on the media (Junttila *et al.,* 1988; Dramsi *et al.,* 2003). As the bacterium is facultative anaerobe (Seeliger *et al.*, 1986) it is not inhibited by vacuum-packaging (Hudson *et al.,* 1994). *L. monocytogenes* is very tolerant to low pH since the bacterium is able to grow at pH 4.4 (George *et al.,* 1988). Different preservation agents have been tested against *L. monocytogenes*, and addition of acetate and diacetat can inhibit the growth (Vogel *et al.,* 2006). Also phenolic compounds which are the active compounds in smoke show inhibitory effect to *L. monocytogenes* (Membré *et al.,* 1997; Hansen *et al.,* 2007). *L. monocytogenes* can be isolated from vacuum-packed, refrigerated ready-to-eat foods products, because of the ability to survive and grow at the preservation parameters used for those products.

2.2 Serotyping and lineage

Strains of *L. monocytogenes* are separated into 13 serotypes because of their somatic (O) and flagellar (H) antigens (Seeliger *et al.,* 1979). The 13 serotypes are as followed: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 5, 7. A bias in the distribution of the serotypes causing human listeriosis is seen as 64% of the strains belongs to serotype 4b followed by serotype 1/2a and 1/2b with 15% and 10%, respectively (McLauchlin, 1990). Some indications for association between virulence differences and serotype 1/2b and 4b have been seen (McLauchlin, 1990). Two obvious hypotheses are raised to explain the predominance of the three serotypes in human, clinical cases of listeriosis: 1) Humans are more exposed to these serotypes as they predominate in the nature, 2) these serotype have a higher virulence potential than other serotypes. The serotypes isolated from food products are mainly 1/2a (54%) followed by 4b (20%) (Gilot *et al.,* 1996) and humans are therefore not more exposed to 4b. Strains belonging to serotype 4b may posses characteristics important for enhanced virulence i.e. serotype 4b occurred more often in pregnancy-associated than in non-pregnancy-associated cases of listeriosis.

Strains of *L. monocytogenes* are divided into three groups (lineage 1, 2 and 3) by the nucleotide variation in three virulence genes (*actA*, *inlA*, *hly*) (Rasmussen *et al.,* 1995; Zhang *et al.,* 2003) and by ribotyping it is seen that the genetic lineages separate the serotypes into different clusters (Nadon *et al.,* 2001). Lineage 1 contains serotypes 1/2b, 3b, 3c, 4b, 4d and 4e; lineage 2 includes serotypes 1/2a, 1/2c and 3a and lineage 3 contains 4a and 4c. Use of other sub-typing methods like multilocus enzyme electrophoresis (MEE) (Piffaretti *et al.,* 1989) or pulse field gel electrophoresis (PFGE) (Brosch *et al.,* 1994), results in only two different groups, where Brosch *et al*. (1994) were able to sub-divide the two main groups into two sub-groups each. This relationship between serotypes and lineage mean that strains belonging to lineage 1 are causing more human, clinical cases than lineage 2 and lineage 3 strains (Zhang *et al.*, 2003).

The presence of epidemic clones, that have caused several outbreaks of listeriosis, have been identified, when different sub-typing methods have been used (Jeffers *et al.,* 2001; Gray *et al.,* 2004; Chen *et al.,* 2007). The same ribotype was shown to have caused outbreaks in France (1976), Nova Scotia, Canada (1981), Switzerland (1983) and California (1985), another ribotype caused two outbreaks in Massachusetts and a third ribotype was responsible for two USA multistate outbreaks (Jeffers *et al.,* 2001). These ribotypes do all belong to lineage 1. Therefore it is hypothesized that some sub-types of *L. monocytogenes* are more virulent than others, and an understanding of the variability of virulence of strains isolated from human, animals, food processing environments and outdoor environments is important for the risk assessment (Gray *et al.*, 2004).

2.3 Natural niches

L. monocytogenes is ubiquitous in the outdoor environment and the isolation sites is vegetation, soil, water and sediment and the frequency of positive samples is between 0% and 6% (Macgowan *et al.,* 1994; Hansen *et al.,* 2006). The level of *L. monocytogenes* increases in the presence of human or animal activity (Hansen *et al.*, 2006) and there may be a seasonal variation with the highest prevalence in the spring (Arvanitidou *et al.,* 1997). Such a pattern has not been found in other studies (Hansen *et al.*, 2006).

Animals can act as reservoirs for *L. monocytogenes* since the level of *L. monocytogenes* positive intestinal content samples is between 1-7% (Skovgaard *et al.,* 1989; Iida *et al.,* 1998). Humans can be healthy carriers of *L. monocytogenes*, and the bacteria are excreted through feces. Approximately 4% of the samples from healthy adults contain *L. monocytogenes*, during one year of sampling, and in younger people the level is 1.3% (Iida *et al.,* 1998; Grif *et al.,* 2003). No systematic clustering was seen in the pattern of fecal shedding, and half of the positive results were single events, where no positive samples were detected the previous or the following day. A connection was seen between the intake of *L. monocy-*

togenes positive cold-smoked mackerel and two consecutive days of *L. monocytogenes* fecal shedding (Grif *et al.,* 2003).

2.4 Contamination of food

Due to the ubiquitous occurrence in nature, *L. monocytogenes* is potentially present on almost all raw materials used for food production. Because of the tolerance to elevated levels of NaCl, the ability to grow at refrigeration temperatures and without presence of O_2, which all are used as preservation parameters, *L. monocytogenes* can also be isolated from many food products especially Ready-To-Eat (RTE) food products. RTE foods are often consumed without heating before intake. Furthermore, RTE foods do often have a long shelf life, which allow the bacteria to grow to high levels, and the often used preservation parameters are vacuum packaging and addition of elevated levels of NaCl. If the food product is contaminated, none of the preservation parameters are effective in inhibiting growth of *L. monocytogenes*.

As seen in Table 2.1, several food products may support growth of *L. monocytogenes*. These include cold-smoked fish products e.g. cold-smoked salmon or cold-smoked trout, delicatessen meat products, mayonnaise salads and soft cheeses made from un-pasteurized milk.

The prevalence and the maximum level of *L. monocytogenes* vary between the different types of products, and even in the same product category. In smoked seafood products, the prevalence of *L. monocytogenes* can be very high (0%-79%). The broad interval in the prevalence of positive samples is due to a different number of product samples and also that product samples originates from different processing plants. One study, with a prevalence of 79% positive *L. monocytogenes*, has tested 61 samples (Eklund *et al.,* 1995), but others have tested 2644 samples and had a prevalence of 5% positive *L. monocytogenes* (Gombas *et al.,* 2003). Some processing plants produce products with a prevalence of 41% positive samples, whereas other factories produce products with a prevalence of 0% (Brett *et al.,* 1998).

Table 2.1: The prevalence and maximum cell count of *Listeria monocytogenes* in tested food products. The products have been collected either at the production site in retail packs or were obtained from the supermarkets either as a packed product (self service) or as a handed-packed product (serving stands). The samples have been kept at 4°C, and the level of *L. monocytogenes* has been measured at the last day of the shelf life period as noted by the producer.

Product category	Prevalence	Maximum cell count (CFU/g)[A]	Reference
Fresh soft cheeses	0.2%	$10\text{-}10^2$	Gombas *et al.*(2003)
	0.8%	ND	Latorre *et al.*(2007)
Vegetables	2.8%	10^2	Odumeru *et al.*(1997)
	0.9%	$10^2\text{-}10^3$	Gombas *et al.*(2003)
Blue-veined cheeses	2.6%	$10\text{-}10^2$	Gombas *et al.* (2003)
Mold-ripened cheeses	1.6%	1-10	Gombas *et al.* (2003)
Seafood salads	16%	ND	Hartemink and Georgsson (1991)
	27.3%	1-10	Uttendaele *et al.* (1999)
	4.7%	$10^2\text{-}10^3$	Gombas *et al.* (2003)
Smoked seafood	3%	ND	Hartemink and Georgsson (1991)
	78.7%	10^2	Eklund *et al.* (1995)
	11%	ND	Rørvik *et al.* (1995)
	11.5%	ND	Loncarevic *et al.* (1996)
	11%	1.1×10^2	Cortesi *et al.* (1997)
	40-60%	10^3	Jørgensen *et al.* (1998)
	11.5%	ND	Norton *et al.* (2001)
	9-47%	ND	Vogel *et al.* (2001a)
	10%	ND	Dauphin *et al.* (2001)
	4.3%	$10^3\text{-}10^4$	Gombas *et al.* (2003)
	12%	100	Nakamura *et al.* (2004)
	0-3%	ND	Thimothe *et al.* (2004)
	18%	ND	Wulff *et al.* (2006)
	10.6%	ND	Latorre *et al.* (2007)
	0-41%	2.7×10^2	Beaufort *et al.* (2007)
Gravad fish	20.7%	ND	Loncarevic *et al.* (1996)
	25%	10^3	Jørgensen *et al.* (1998)
Luncheon meats	6.70%	1-10	Uttendaele *et al.* (1999)
	1.17%	$10^3\text{-}10^4$	Gombas *et al.* (2003)
	3.7%	ND	Latorre *et al.* (2007)
Deli salads	17%	1-10	Uttendaele *et al.* (1999)
	1.94%	$10^3\text{-}10^4$	Gombas *et al.* (2003)

[A] ND: Not determined

Also, the cell count may increase to levels of 10^2-10^4 CFU/g which exceed 100 CFU/g which is the maximum level allowed (EC, 2005) (EC-regulation is described briefly in chapter 2.5). It should be emphasised that the prevalence of *L. monocytogenes* in seafood products has decreased during the years, probably because of the enhanced focus on the problems with *L. monocytogenes* in the fish industry and an enormous effort (cleaning and disinfection procedures, procedures to prevent cross-contamination) by the fish processing industry. Recently, a survey of four fish smokehouses found 0% *L. monocytogenes* positive product samples in two plants, and 6-13% in two other plants (Wulff *et al.*, 2006).

Although potentially present on raw materials, the processing plant environment seems to be the most likely source of *L. monocytogenes* contamination of cold-smoked fish (Eklund *et al.,* 1995; Autio *et al.,* 1999; Norton *et al.,* 2001; Wulff *et al.,* 2006), meat products (Samelis *et al.,* 1999; Giovannacci *et al.,* 1999; Keto-Timonen *et al.,* 2007) and dairy products (Chambel *et al.,* 2007). However, product contamination can be caused by contaminated raw material. Among Hispanic people in e.g. USA, it is a tradition to make home-made Mexican-style cheese from sometimes inadequately pasteurized milk originating from small local farms, which can potentially be contaminated with *L. monocytogenes* (CDC, 2001) (Table 2.2). Another example is the production of coleslaw, contaminated raw cabbage contained *L. monocytogenes* (Schlech *et al.,* 1983). On the cabbage farm cases of ovine listeriosis was seen and this was the source of the cabbage contamination (Table 2.2).

In Table 2.2 it is shown that several fish, delicatessen meat and dairy products have caused outbreaks or sporadic cases of listeriosis in humans. The symptoms are very often the same: fever, abdominal pain, diarrhoea and influenza-like symptoms. The incubation time vary between either 1-2 days or several days up to weeks depending on the dose of ingested bacteria and the health situation of the host. Often the diarrhoea is seen when the incubation time is short and the infection dose is high (Junttila *et al.,* 1989; Riedo *et al.,* 1994; Miettinen *et al.,* 1999a; Farber *et al.,* 2000), while more severe symptoms like sepsis and meningitis are seen with a longer incubation time and partly, a lower infection dose (Bannister, 1987).

The level of *L. monocytogenes* in food that have caused illness is between 10^2-10^9 CFU/g, but for several of the cases, it has not been possible to determine the level of *L. monocytogenes*. The infectious dose for *L. monocytogenes* is approx 10^6-10^9 CFU depending on the host susceptibility, virulence of the strain and the food matrix, and not many food products contain that high numbers of *L. monocytogenes* (Table 2.1).

Fish products have one of the highest risk-per-serving ratios for *L. monocytogenes* (6.2×10^{-9} - 2.1×10^{-8}) when compared to e.g. delicatessen type salads (5.6×10^{-13}) (FDA/FSIS, 2003a; FAO/WHO, 2004), but interestingly, cold-smoked salmon have never been reported to be involved in sporadic cases or outbreaks of *L. monocytogenes*, even though other fish products have seen to cause smaller outbreaks of listeriosis.

Table 2.2: Food-borne outbreaks and sporadic cases of listeriosis.

Source	Symptoms	No. patients	No. death	Incubation time	Level of *L. monocytogenes* (CFU/ml) or (CFU/g)	Serotype	Reference
Shellfish and raw fish	Influenza like symptoms, diarrhoea	19	5	NA[A]	NA	1b	Lennon *et al.* (1984)
Shrimps	Fever, diarrhoea	10	1	19-23 h	NA	4b	Riedo *et al.* (1994)
Gravad rainbow trout	Septicemia, meningitis, premature birth	6	1	NA	6.2×10^2	4b	Ericsson *et al.* (1997)
Smoked mussels	NA	3	0	NA	NA	1/2b	Brett *et al.* (1998)
Cold-smoked rainbow trout	Febrile gastroenteritis	5	0	24 h	1.9×10^5	1/2a	Miettinen *et al.* (1999a)
Imitation crab meat	Diarrhoea, fever, vomiting	2	0	12-15 h	$> 10^6$	1/2b	Farber *et al.* (2000)
Coleslaw (cabbage)	Meningitis, premature birth, ill infant	41	18[B]	NA	NA	4b	Schlech *et al.* (1983)
Raw vegetables	Fever, Bacteremia, meningitis	20	3[B]	NA	NA	4b	Ho *et al.* (1986)
Salted mushrooms	Fever, diarrhoea	1	0	24 h	3×10^6	4b	Junttila and Brander (1989)
Turkey frank	Sepsis	1	0	NA	NA	1/2a	CDC (1989)
Pork tongue in jelly	NA	279	NA	NA	NA	4b	Jacquet *et al.* (1995)
Rilletts	Fever, diarrhoea	38	9[D]	48 h	10^4	4b	Goulet *et al.* (1998)
Hot dog	NA	50	8[E]	NA	NA	4b	CDC (1998), CDC (1999)
Deli turkey meat	NA	29	7[F]	NA	NA	NA	CDC (2000)
Jellied pork tongue or other meat products	Bacteremia, central nervous system listeriosis	32	10[G]	NA	NA	4b	Valk *et al.* (2001)
Rillettes	Bacteremia, central nervous system listeriosis	10	3	NA	< 10	4b	Valk *et al.* (2001)
Deli turkey meat	NA	49	3[H]	NA	NA	NA	CDC (2002)
Pasteurized milk	Meningitis, septicemia	49	14[I]	NA	NA	4b	Fleming *et al.* (1985)
Mexican style cheese	Fever	86	29[J]	NA	NA	4b	Linnan *et al.* (1988), CDC (1985)
Soft cheese	Meningitis	1	0	< 1 week	NA	4b	Bannister (1987)
Swiss soft cheese	Bacteremia, meningitis	57	18	NA	NA	4b	Büla *et al.* (1995)
Chocolate Milk	Diarrhoea, fever	45	0	20 h	10^9	1/2b	Dalton *et al.* (1997)
Butter	NA	25	6	NA	NA	3a	Lyytikäinen *et al.* (2000)
Mexican-style cheese	Fever	12	5[K]	NA	NA	NA	CDC (2001)
Cheese	NA	NA	NA	NA	NA	1/2b	Vit *et al.* (2007)

[A] NA: Not available
[B] Inclusive spontaneous abortions (5), stillbirths (4), live births (7)
[C] Two additional patients died but listeriosis was not the cause
[D] Inclusive fetal deaths (9)
[E] Inclusive spontaneous abortions (2)
[F] Inclusive spontaneous abortions/still births (3)
[G] Inclusive spontaneous abortion (1), premature births (4)
[H] Inclusive stillbirths or miscarriages (3)
[I] Inclusive still birth (2)
[J] Inclusive neonatal deaths (8)
[K] Inclusive still births (5)

2.5 EU-regulation on *Listeria monocytogenes* in ready-to-eat food products

Per January 1st 2006, the European Union introduced a new regulation on microbiological criteria for foods (EC, 2005). This includes limits for *L. monocytogenes* in ready-to-eat (RTE) food products (Table 2.3). The new regulation divides RTE into two different categories; products that are able to support the growth of *L. monocytogenes* and product that are unable to support the growth. The criteria reflect a risk based approach since products with very low levels are highly unlikely to cause disease.

Table 2.3: Microbiological criteria for *Listeria monocytogenes* in ready-to-eat food products. RTE foods are divided into two different categories; products that are able to support the growth of *L. monocytogenes* and products not able to support the growth. Reference: EC (2005).

Ready-to-eat foods	Critical limit	Comment
Support growth	100 CFU/g	• It must be documented that 100 CFU/g is not exceeded within the storage period
Support growth	None in 25 g	• When produced[A]
Unable to support growth - pH ≤ 4.4 or $a_w \geq 0.92$ - pH ≤ 5.0 or $a_w \geq 0.94$ - Shelf life less than 5 days	100 CFU/g	• It must be documented that the product have the described characteristics • It must be documented that 100 CFU/g is not exceeded within the storage period

[A] This criterion applies to products where the producer is not able to demonstrate that the product will exceed the limit of 100 CFU/g throughout the shelf-life

Products that are able to support the growth are further divided into two sub-groups. If the manufacturer can document that 100 CFU/g is not exceeded at the end of the storage period, then 100 CFU/g at the end of the storage period is the critical limit. If the manufacture not is able to document that the product will not exceed the limit of 100 CFU/g at the end of the storage period, then the critical limit is no detection of *L. monocytogenes* in 25 g product, just after production. If the manufacturer can document that the product is unable to support growth, then it must be documented that 100 CFU/g is not exceeded within the storage period. Products that are able to support the growth could be "gravad fish", and products that are unable to support growth could be marinated herring.

3 *Listeria monocytogenes* and persistence in food processing plants

Many types of bacteria are capable of “colonizing” food processing plants and may reside for many years. This has been used by man-kind, even unknowingly, for instance in the production of fermented foods like cheese, milk products, wine and beer. The production of these products has been dependent of persistent strains of lactic acid bacteria or yeast.

However in recent years focus has been directed to persistence of bacteria causing spoilage or disease like *Salmonella enterica* in fish feed factories (Nesse *et al.,* 2003), *Enterobacter sakazakii* in infant formula processing facilities (Mullane *et al.,* 2007) or *Listeria monocytogenes* in fish processing factories (Wulff *et al.*, 2006).

Persistence is defined as a specific sub-type of a strain that is isolated repeatedly in the same factory, and even the same places inside the factory, during a longer period of time (Keto-Timonen *et al.*, 2007). The term non-persistent, is avoided in this thesis and instead the use of sporadic isolated strains are used, since it is not known if a sporadic isolated strain could be persistent in other types of environments (Jensen *et al.*, 2007a).

L. monocytogenes is able to reside in food processing plants, including poultry production plants (Lawrence *et al.,* 1995; Ojeniyi *et al.,* 1996; Ojeniyi *et al.,* 2000), meat processing plants (Nesbakken *et al.,* 1996; Giovannacci *et al.,* 1999), ice cream plants (Miettinen *et al.,* 1999b), shrimp peeling plants (Destro *et al.,* 1996; Gudmundsdóttir *et al.,* 2006), dairies (Chambel *et al.*, 2007) and plants in which ”gravad” and smoked fish products (Rørvik *et al.,* 1995; Autio *et al.,* 1999; Vogel *et al.,* 2001a; Wulff *et al.,* 2006) are produced.

3.1 Sub-typing of *Listeria monocytogenes*

To identify the source and route of contamination, processing environmental samples have been collected either by swabbing or by air sampling. Samples are collected before production, during production and after production, before cleaning and disinfection or after these procedures. After identification of isolated *L. monocytogenes*, all the bacteria are subtyped and it is, in principle, possible to trace the source of product contamination.

Molecular sub-typing methods are important in epidemiological investigations, to detect outbreaks and verify epidemiological associations, but also for contamination routes and tracing of *L. monocytogenes* in processing plants. A number of methods have been used throughout the years (Table 3.1). Characteristic for the molecular sub-typing methods, when compared to phenotypic test like e.g. serotyping or phage typing, is the high discriminatory power (Ojeniyi *et al.*, 1996).

Beside Vogel *et al.* (2004), Aarnisalo *et al.* (2003) have calculated the discriminatory index for some of the methods listed in Table 3.1 and the values are almost similar. The most efficient method to discriminate strains of *L. monocytogenes* is AFLP, but also PFGE and RAPD are very discriminative. When compared to PFGE and AFLP, RAPD typing is rapid and inexpensive (Vogel *et al.*, 2001a), and therefore several studies have used RAPD for the primarily sub-typing of *L. monocytogenes* strains (Vogel *et al.,* 2001a; Vogel *et al.,* 2001b; Vogel *et al.,* 2004; Hansen *et al.,* 2006; Wulff *et al.,* 2006; Jensen *et al.,* 2007a)

Table 3.1: Molecular sub-typing methods used for contamination routes and epidemiological investigations for *Listeria monocytogenes*.

Method	Full name	Discriminatory index[A]	Principle of the method	Reference
AFLP	Amplified Fragment Length Polymorphism	0.974	DNA is cut with rare-cutting and frequent-cutting enzymes, and double-stranded adaptors are ligated to the ends of the DNA-fragments to act as primer binding sites for PCR. Only when primers are fully complimentary to their targets the PCR amplification will occur. The PCR products are separated by gel electrophoresis and the patterns are compared.	Vos *et al.* (1995), Vogel *et al.* (2001a), Vogel *et al.* (2004), Wulff *et al.* (2006), Keto-Timonen *et al.* (2007)
PCR-RFLP	PCR- Restriction Fragment Length polymorphism	0.392	The genes are amplified by PCR, cut with a restriction enzyme and the restriction fragment profiles are compared between different strains	Wiedmann *et al.* (1997) Vogel *et al.* (2004), Lukinmaa *et al.* (2004)
PFGE	Pulsed-Field Gel Electrophoresis	0.969	PFGE employs large fragments of DNA that are generated by digestion of genomic DNA. The fragments are separated by electrophoresis where the electric field keeps changing orientation.	Destro *et al.* (1996), Ojeniyi *et al.* (2000), Vogel *et al.* (2001a), Dauphin *et al.* (2001), Wiedmann (2002), Vogel *et al.* (2004), Gudmundsdóttir *et al.* (2006)
RAPD	Random Amplified Polymorphic DNA	0.954	RAPD make use of PCR to amplify genomic DNA segment with single primers with nucleotides in a random order. The amplified products are separated by electrophoresis and the pattern of the DNA-products is unique for each RAPD sub-type	Lawrence and Gilmour (1995), Vogel *et al.* (2001a), Vogel *et al.* (2001b), Wiedmann (2002) Vogel *et al.* (2004), Wulff *et al.* (2006), Chambel *et al.* (2007)
RFLP	Restriction Fragment Length polymorphism	ND	Genomic DNA is digested into smaller pieces by a restriction enzyme (very often *Hae*III) and the products are separated by gel electrophoresis	Nesbakken *et al.* (1996)
Ribotyping	Ribotyping	0.875	Ribotyping is partly similar to PFGE, where genomic DNA is digested into smaller pieces by a restriction enzyme (very often *Eco*RI). After separation by gel electrophoresis a southern blot is performed and only DNA fragment containing genes encoding the ribosomal RNA is detected, and these patterns are compared.	Ojeniyi *et al.* (1996), Gendel and Ulaszek (2000), Holah *et al.* (2002), Wiedmann (2002), Vogel *et al.* (2004), Thimothe *et al.* (2004)

[A] The discriminatory index is calculated by Vogel *et al.* (2004). ND: Not determined.

3.2 Distribution of *Listeria monocytogenes* in the fish processing environment

As mentioned, *L. monocytogenes* is able to persist in the processing environment of fish production. The distribution of positive *L. monocytogenes* samples has been determined by several research groups in different slaughter- and smokehouses and typical places for *L. monocytogenes* isolation are knifes, conveyer belts, floors, drains and aprons (Norton *et al.,* 2001; Hoffman *et al.,* 2003; Thimothe *et al.,* 2004). The prevalence of *L. monocytogenes* positive samples from the raw fish entering the slaughterhouse is very low (Rørvik *et al.,* 1995; Hansen *et al.,* 2006; Wulff *et al.,* 2006), but the fish during production and the indoor environment have a high prevalence of *L. monocytogenes* positive samples (Table 3.2).

Table 3.2: Prevalence of *Listeria monocytogenes* positive samples in fish processing plants. Samples are taken in the indoor environment and from fish during processing. Some of the sample locations where chosen to represent the most likely to harbour *L. monocytogenes*.

Slaughterhouse		Smokehouse		Reference
Prevalence of *L. monocytogenes*		Prevalence of *L. monocytogenes*		
Before cleaning	After cleaning and disinfection	Before cleaning	After cleaning and disinfection	
		52%	0%	Eklund *et al.* (1995)
		28%		Rørvik *et al.* (1995)
		30%	0%	Autio *et al.* (1999)
		13%		Norton *et al.* (2001)
		25%		Vogel *et al.* (2001a)
		6%		Vogel *et al.* (2001a)
		80%		Dauphin *et al.* (2001)
		48%		Hoffman *et al.* (2003)
		1%		Hoffman *et al.* (2003)
		28%		Thimothe *et al.* (2004)
		5%		Thimothe *et al.* (2004)
		16%		Thimothe *et al.* (2004)
		0%		Thimothe *et al.* (2004)
		15%		Gudmundsdottir *et al.* (2005)
		16%		Nakamura *et al.* (2006)
14%		68%		Hansen *et al.* (2006)
25%	27%	16%	9%	Wulff *et al.* (2006)
67%	10%	32%	16%	Wulff *et al.* (2006)
24%	57%	32%	28%	Wulff *et al.* (2006)
14%	5%	20%	16%	Wulff *et al.* (2006)

Cleaning and disinfection of the production plant should lower the prevalence of *L. monocytogenes* even though the numbers in Table 3.2 does not support that. Often the sporadic isolated sub-types are sensitive to cleaning and disinfection procedures, but the strains belonging to persistent sub-types will still be present after the procedures (Vogel *et al.,* 2001a; Wulff *et al.,* 2006). The relative low number of taken samples could, and the fact that samples were taken where *L. monocytogenes* most likely harboured could influence the prevalence of *L. monocytogenes* positive samples.

The contamination pattern of specific sub-types of *L. monocytogenes* can be determined, by using molecular sub-typing methods. One could ask the question if the sub-types are the same seen in the three areas of fish processing; the raw material, the indoor environment during processing and in the final product. There are few examples were the same sub-type has been found in raw material and final product indicating that sometimes raw material contamination are carried through (Norton *et al.,* 2001; Vogel *et al.,* 2001a; Wulff *et al.,* 2006; Nakamura *et al.,* 2006). Only very few of the sub-types isolated in the indoor processing environment can be detected in the final product, but a persisting sub-type can be found in the final product (Norton *et al.,* 2001; Vogel *et al.,* 2001a; Wulff *et al.,* 2006; Nakamura *et al.,* 2006). The raw fish do only contain the food processing persistent strains in low levels (Hoffman *et al.*, 2003; Thimothe *et al.*, 2004; Wulff *et al.*, 2006).

Almost every plant has an "in-house" sub-type of *L. monocytogenes* (Mafu *et al.,* 1990; Rørvik *et al.,* 1995; Lawrence *et al.,* 1995; Nesbakken *et al.,* 1996; Miettinen *et al.,* 1999b; Norton *et al.,* 2001; Thimothe *et al.,* 2004; Wulff *et al.,* 2006) that may persist inside the facility for prolonged periods. The reason for persistence of those sub-types of *L. monocytogenes* is still unknown, but several research groups are investigating this issue.

In the Danish fish processing industry (fish slaughter- and smokehouses), Wulff *et al.* (2006) and Vogel *et al.* (2001b) have shown that one specific RAPD sub-type of *L. monocytogenes* have been isolated during a period of eight years. Strains belonging to RAPD type 9 have been isolated from a smokehouse in 1995, from the same smokehouse in 2003 and are very prevalent in other smoke- and slaughter-houses through the period 1995-2004 (Wulff *et al.*, 2006) (Table 3.3). Some of these processing plants have an inter-trade relationship, but some have never been in contact with each other.

Table 3.3: Distribution of *Listeria monocytogenes* RAPD types in smokehouses and slaughterhouses[A] . Reference: Wulff *et al.* (2006).

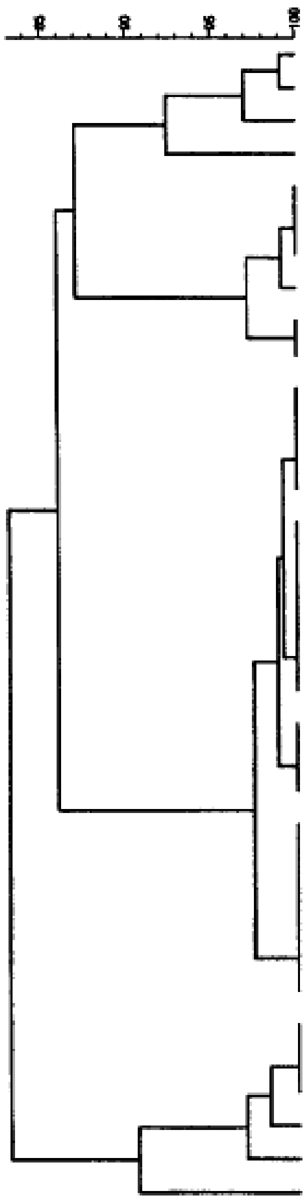
RAPD type		No. of *L. monocytogenes* strains of RAPD type at processing plant:								Total no. of strains
		Fish slaughterhouse				Smokehouse				
No.	Symbol	A	B	C	D	1	2	3	4	
5	▽	**3**				**1**				**4**
9	■	**30**		**18**		**3**	**25**		**10**	**86**
30	▱	5								5
31	○	2								2
32	⬢	1								1
33	◆	1								1
7	⬟		**1**			**4**	**1**			**6**
34	▼		5							5
35	■		1							1
36	◤			7						7
37	✚			1						1
38	▲				5					5
39	●				1					1
40	▰				1					1
41	◣					1				1
42	⬟					1				1
44	◆					4				4
45	●					8				8
57	▲					**5**		**11**	**12**	**28**
46	◣						1			1
47	▰						1			1
48	▽						1			1
49	✿						1			1
52	⬢						1			1
53	○						2			2
54	■						2			2
55	◆						5			5
6	●						**1**	**17**		**18**
58	■							2		2
59	○							1		1
60	▰							1		1
61	◣							1		1
62	●							1		1
63	▼							1		1
64	●								4	4
65	▽								1	1
66	◆								1	1
X	X			3		5	2	11	4	25
Total		46	7	29	7	28	43	46	32	238

[A] Strains were isolated during production and after cleaning and disinfection one to three months apart at each fish processing plant. The numbers of RAPD type found in more than one fish plant are in boldface. X, the number of *L. monocytogenes* positive samples detected only by PCR and from which no strains were recovered

It is clear that every slaughter- and smokehouse have their own flora of *L. monocytogenes*, where some of the RAPD sub-types are isolated sporadically and some are isolated with a high prevalence. Also, some RAPD type (RAPD type 9 and 57) are isolated in more than one plant and also in a high prevalence in the different plants.

By using the more discriminating method, AFLP typing, it was possible to differentiate RAPD type 9 strains into four different AFLP-types (Figure 3.1), that even differentiation were very similar thus confirming the close genetic relationship between the strains.

% Similarity



Strain designation	AFLP type	RAPD type	Yr and country of isolation	Factory
N42-3	11a	7	2002, Denmark	2
M 45-1	11b	7	2002, Denmark	A
AP8-1	11c	7	2003, Denmark	B
La22[1]	12a	12	1995, Denmark	V
N86-1	6i	6	2003, Denmark	2
No37-1	6i	6	2001, Denmark	3
N278-1	6i	6	2003, Denmark	2
20.08.2003 17	6g	6	2003, Denmark	3
3R110-1	6c	6	2003, Denmark	1
No3-1	6c	6	2003, Denmark	3
A202-255-15a	9a	9	2002, Faroe Islands	C
La150 [1]	9a	9	1995, Denmark	I
N250-1	9a	9	2004, Denmark	A
OB5-1[2]	9a	9	2003, Denmark	5
N97-1	9e	9	2003, Denmark	2
► **H13-1**	9e	9	2003, Denmark	4
H116-1	9e	9	2003, Denmark	4
► **N53-1**	9e	9	2002, Denmark	2
► La111[1]	9e	9	1996, Denmark	G
3R32-1	9e	9	2003, Denmark	1
ERB 991[3]	9f	9	2004, Greenland	E
N510-1	9f	9	2004, Denmark	2
La105[1]	9f	9	1996, Denmark	K
ERLS 1569[3]	9g	9	2004, Greenland	E
H122-1	9g	9	2003, Denmark	4
M40-1	9g	9	2001, Denmark	A
► **M103-1**	9g	9	2003, Denmark	A
M165-1	9g	9	2004, Denmark	A
LA42[1]	9g	9	1996, Denmark	4
No40-1	17a	57	2003, Denmark	3
No80-1	17a	57	2003, Denmark	3
H49-1	17a	57	2003, Denmark	4
3R23-1	17b	57	2003, Denmark	1
16.092003 52	17c	57	2003, Denmark	4
3R52-1	8a	45	2003, Denmark	1

Figure 3.1: Dendogram for 35 *Listeria monocytogenes* strains isolated from the Danish fish processing industry. Their genetic relationship is analyzed by RAPD and AFLP sub-typing. Strains used in this PhD-study are H13-1, N53-1, La111 and M103-1 (-►). Reference: Wulff *et al.* (2006).

In this Ph.D.-study, a *L. monocytogenes* strain collection has been used, where four of the strains belong to RAPD type 9. Three of the four RAPD type 9 strains belong to the same AFLP type 9e (H13-1, N53-1 and La111), whereas the last strain (M103-1) belongs to AFLP type 9g. The four strains behaved similarly in all performed experiments e.g. adhesion to a plastic surface (Jensen *et al.*, 2007a), adhesion, invasion and intracellular growth in the intestinal cell line Caco-2 (Jensen *et al.*, 2007b), pathogenicity against the nematode *Caenorhabditis elegans* (Jensen *et al.*, 2007b) and fecal shedding in both non-pregnant and pregnant guinea pigs (Jensen *et al.,* 2007b; Jensen *et al.,* 2007c).

3.3 Why do specific sub-types of *Listeria monocytogenes* persist?

The persistent sub-types have often been isolated repeatedly for a number of years. One could therefore assume that the persistent strains are able to adapt to physical parameters prevalent in food processing environment or to tolerate processing parameters.

It is expected that the sub-types that persist in the food processing environment share traits that enable them to persist. Several hypotheses have been proposed to explain persistence. Some of the hypotheses can be rejected, but most of them seem, partly, to have an influence on the persistence, but more work is needed to investigate this issue further.

The nature of persistence may be due to physical adaptation or enhanced tolerance to processing factors (Holah *et al.,* 2002; Wulff *et al.*, 2006).

1. A changed growth pattern. A higher growth rate could result in an out competing of the sporadic isolated strains. In contrast, a reduced growth rate could slow down metabolic processes which could result in protection against the stresses the bacteria are exposed to.
2. Enhanced adhesion and aggregation to the surfaces in processing plants.
3. Enhanced tolerance to drying and desiccation
4. Enhanced tolerance to cleaning and disinfection agents.

Besides physical adoption or tolerance to processing parameters persistent sub-types could be present in the out-door environment in high numbers, but the persistence of the RAPD type 9 is not caused by a massive presence in the outside environment, since RAPD type 9 strains are only found sporadically in the outside environment (Hansen *et al.*, 2006).

3.3.1 A changed growth pattern of persistent strains

It is hypothesised that different growth rate, either lower or higher, of persistent strains could be a reason for persistence (Holah *et al.*, 2002; Wulff *et al.*, 2006). The growth pattern of a variety of strains with different origin and serotype have been determined (Barbosa *et al.,* 1994; Begot *et al.,* 1997; Vialette *et al.,* 2003; Lianou *et al.,* 2006; Jensen *et al.,* 2007a), but the conclusions differ. Strains identified as food-processing persistent (RAPD type 9) were compared to strains of other origins and growth pattern at low (5°C) and high (37°C) temperature, with or without NaCl did not differ between persistent and sporadic isolated strains (Jensen *et al.*, 2007a) (Figure 3.2).

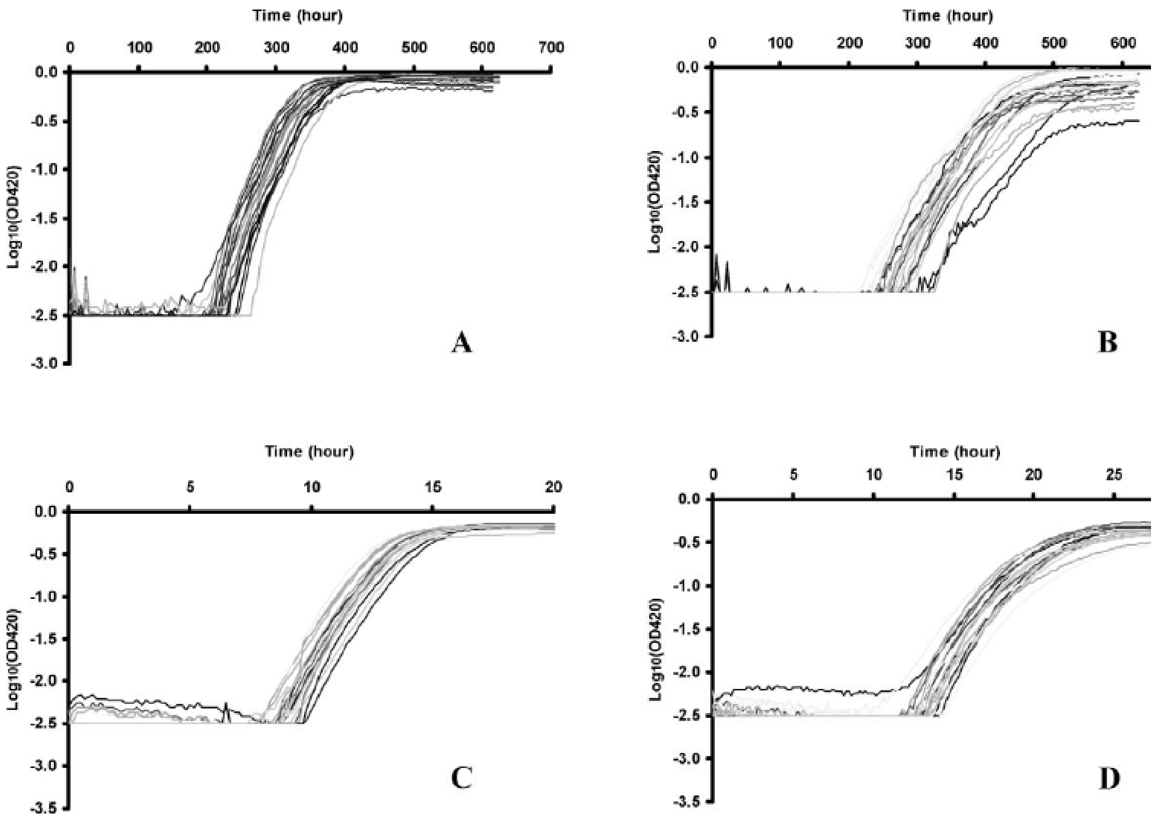


Figure 3.2: Growth of *Listeria monocytogenes* strains in LB (1% glucose) at 5°C (A), LB (1% glucose + 5% NaCl) at 5°C (B), LB (1% glucose) at 37°C (C), and LB (1% glucose + 5% NaCl) at 37°C (D). Growth was measured by optical density in a Bioscreen. All measurements below OD_{420} of 0.003 are considered below the detection level of the instrument and are therefore not included in the figure. Reference: Jensen *et al.* (2007a).

Other studies (Barbosa *et al.,* 1994; Begot *et al.,* 1997; Vialette *et al.,* 2003; Lianou *et al.,* 2006) indicate, that when *L. monocytogenes* are exposed to moderate levels of NaCl a slight differentiation in growth rate is seen between the strains, but no consistent pattern regarding to origin, serotype and lineage is seen. Hence, at present it does not appear likely that a higher or lower growth rate explains persistence.

3.3.2 Enhanced adhesion to surfaces

L. monocytogenes adheres to stainless steel, plastic or rubber surfaces (Kim *et al.,* 1995; Norwood *et al.,* 1999; Djordjevic *et al.,* 2002; Chavant *et al.,* 2002; Bereksi *et al.,* 2002; Borucki *et al.,* 2003; Jensen *et al.,* 2007a). The term "biofilm" will not be used in this thesis since *L. monocytogenes* not is a massive biofilm former such as *Pseudomonas aeruginosa* (Webb *et al.,* 2003), but only forms a thin layer of adhered cells. The term aggregation is used when *L. monocytogenes* are adhered as a clump of cells.

Different methods to measure adhesion to surfaces have been developed. The crystal violet method is very common and is able to test the adhesion of several samples in a short time. The method was developed by O'Toole and Kolter (1998) for biofilm formation of *Pseudomonas flourescens*, and was introduced by Djordjevic *et al.* (2002) to measure adhesion of *L. monocytogenes* to a plastic surface. Also, adhesion to small coupons made of different types of materials (e.g. plastic, stainless steel) followed by either staining of the adhered bacteria (Kim *et al.,* 1995; Lunden *et al.,* 2000; Kalmokoff *et al.,* 2001; Norwood D.E. *et al.,* 2001) or by removal and quantification of adhered bacteria (Norwood *et al.,* 1999; Moltz *et al.,* 2005), are often used. The adhesion of *L. monocytogenes* to surfaces varies both as a consequence of strain differences or the growth medium (Djordjevic *et al.,* 2002; Moltz *et al.,* 2005; Jensen *et al.,* 2007a) (Figure 3.3).

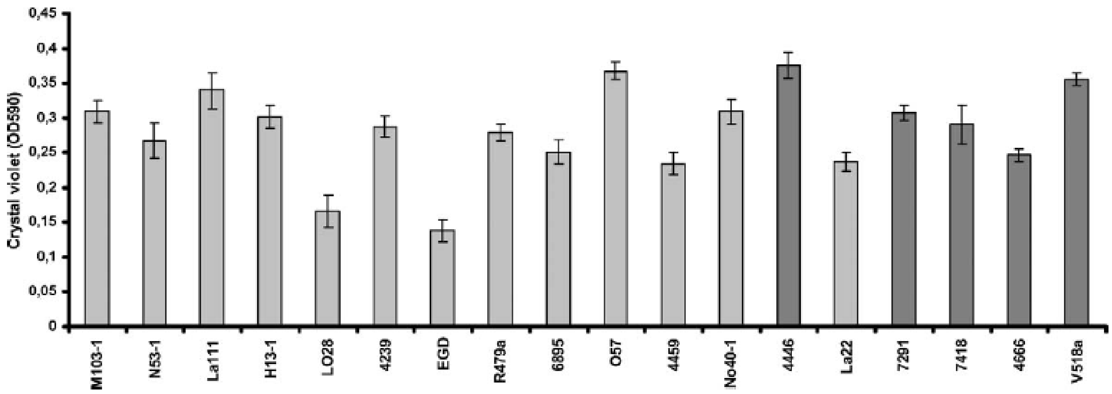


Figure 3.3: Adhesion to microtiter wells of *Listeria monocytogenes* grown in TSB with 1% glucose for 48 h at 37°C. Adhesion was measured by crystal violet adhesion assay. Lineage 1 strains are in dark grey, and lineage 2 strains are in light grey. Columns are average of eight replicate determinations, and error bars indicate standard deviations. Reference: Jensen *et al.* (2007a).

The same conclusion is not reached when comparing adhesion of persistent *L. monocytogenes* strains to sporadic isolated strains. Borucki *et al.* (2003) and Norwood and Gilmour (1999) concluded that persistent strains adhere better to stainless steel surfaces than sporadic isolated strains, but opposite, no relationship between environmental persistence and adhesion was found by Djordjevic *et al.* (2002) and Jensen *et al.* (2007a). Two different types of surfaces (stainless steel and plastic) have been used for the studies, but both of them are hydrophilic (Harvey *et al.,* 2007). Persistent strains from food processing equipment adhered to a higher level than sporadic isolated strains when contact time was 1-2 hours, but after 72 h, no difference in adhesion was seen (Lunden *et al.*, 2000). The question whether strains of serotype 1/2 may have an enhanced ability to colonizing food processing environment has been raised since both Lawrence and Gilmour (1995) and Harvey and Gilmour (1994) found food processing persistent strains to be serogroup 1/2. The food processing persistent RAPD type 9 strains are serotype 1/2a (Wulff *et al.,* 2006; Jensen *et al.,* 2007a), but they did not show significantly higher ability to adhere to a plastic surface, when compared to strains (sporadic isolated processing strains and human, clinical strains) with other serotypes. In contrast, lineage 1 strains adhered better than lineage 2 and lineage 3 strains (Djordjevic *et al.,* 2002), where this systematic differences was not found by Jensen *et al.* (2007a). Therefore, at present it is still not clear whether food processing persistent strains have an enhanced ability to adhere and aggregate as compared to strains sporadically isolated from the processing equipment, but the persistence of RAPD type 9 strains is not caused by enhanced adhesion.

3.3.2.1 Factors influencing adhesion and aggregation of *Listeria monocytogenes*

Different factors in the processing of food products may have an ability to enhance the adhesion and aggregation of *L. monocytogenes*. These factors could be e.g. the presence of NaCl in the food matrix (Jensen *et al.*, 2007a), the use of alcohol as disinfecting agent (Gravesen *et al.,* 2005), lower temperature inside the processing plant (Jensen *et al.*, 2007a) or the presence of other bacteria which *L. monocytogenes* can form co-culture with (Sasahara *et al.,* 1993; Carpentier *et al.,* 2004).

Influence of NaCl on *L. monocytogenes* adhesion

Many ready-to-eat food products associated with listeriosis contain moderate levels of NaCl, where it is used as a flavouring agent and as preservation to inhibit growth of unwanted bacteria. The content of NaCl in smoked fish products is 3-6% and the food matrix present in the production environment therefore contain a moderate level of NaCl. Addition of 2-5% NaCl influences the adhesion and aggregation pattern of some strains of *L. monocytogenes* (Figure 3.4) (Jensen *et al.*, 2007a).

We showed that several of the strains including the food processing persistent RAPD type 9 strains showed increased adhesion as the level of NaCl increased and aggregation was seen at 2-5% NaCl (Jensen *et al.,* 2007a). Further, some strains started to aggregate at 3-4% NaCl. The enhanced adhesion and aggregation in the presence of increasing levels of NaCl was not unique for persistent strains.

Figure 3.4: Adhesion of *Listeria monocytogenes* strain RAPD type 9 (A), a reference strain (B) and a human, clinical strains (C) to a plastic surface (microtiter plate). Photos were taken after strains were grown at 37°C in following media: TSB (1% glucose) (column 1), TSB (1% glucose and 2% NaCl) (column 2), TSB (1% glucose and 3% NaCl) (column 3), TSB (1% glucose and 4% NaCl) (column 4), TSB (1% glucose and 5% NaCl) (column 5). Reference: Jensen *et al.* (2007a).

The biofilm formation of *Staphylococcus aureus* increases when grown in medium containing 0-5% NaCl (Rachid *et al.,* 2000; Knobloch *et al.,* 2001; Hof*,* 2003; Rode *et al.,* 2007), and at 5% NaCl variation is seen, since some *S. aureus* strains increase and some decrease their adhesion (Hof*,* 2003).

Hence, the presence of moderate levels of NaCl in the food matrix present on surfaces in the food processing industry is increasing the adhesion of *L. monocytogenes* to surfaces and could affect the persistence of *L. monocytogenes*, but not as a single factor.

Influence of ethanol on *L. monocytogenes* adhesion

Ethanol and isopropanol are often used as disinfection and cleaning agents. The use of sublethal concentrations of these two disinfection agents can enhance the adhesion of *L. monocytogenes* when grown at low temperature (Gravesen *et al.,* 2005). Presumably an enhanced exopolysaccharid production is one of the major reasons for induced adhesion (Knudsen *et al.,* 2005). Also *S. aureus* and *S. epidermidis* attach better to a surface if ethanol are added (Knobloch *et al.,* 2001; Rode *et al.,* 2007). These results indicate that sublethal concentration of ethanol may contribute to persistence of *L. monocytogenes* and other food borne pathogens.

Influence of temperature on *L. monocytogenes* adhesion

The degree of bacterial attachment or aggregation to a surface is hypothesised to be influenced by the surrounding temperature. The attachment of *L. monocytogenes* is low at low temperatures, 10°C, 20°C and 30°C, when compared to the attachment at 37°C (Gravesen *et al.,* 2005), as was also demonstrated in this thesis even when NaCl was added (Jensen *et al.*, 2007a). The adhesion at low temperatures increased dramatically when ethanol or isopropanol was added (Gravesen *et al.,* 2005). Therefore, the temperature as a single factor does not influence the adhesion of *L. monocytogenes*, but in combination with other factors like ethanol there might be a higher degree of adhesion.

Influence of co-culture on *L. monocytogenes* adhesion

The number of *L. monocytogenes* attached to a surface may be influenced by other colonizing bacteria (Sasahara *et al.*, 1993). Both *Pseudomonas* spp. and *Staphylococcus* spp. produce an pronounced amount of exopolysaccharid, which can act as a grid and traps *L. monocytogenes* cells (Sasahara *et al.*, 1993). Especially the presence of *Pseudomonas fragi*, *P. flourescens* and *Staphylococcus scuri* are enhancing the ability of *L. monocytogenes* to adhere to a surface (Sasahara *et al.,* 1993; Carpentier *et al.,* 2004). Whether the presence of other bacteria has an enhanced effect on the adhesion of *L. monocytogenes* or the number of adhered *L. monocytogenes* is not known.

3.3.3 Enhanced tolerance to drying and desiccation

Long periods of inactivity in some food processing plants can be seen during a production year. All surfaces are drying, which makes it difficult for food processing plant contaminating bacteria to survive. In spite of this, it is possible to isolate strains of *L. monocytogenes*, in this environment. One could hypothesize, that persistent strains, that have been isolated repeatedly from the same processing plant are more tolerant to the dry environment.

Only one study investigating the survival of *L. monocytogenes* in drying models has been reported. The survival of surface attached *L. monocytogenes* strains in drying models have been tested, and over a period of 10 months no differences between the survivals of strains in a drying model was seen (Vogel *et al.,* 2007). Surprisingly, the strains characterized as food processing persistent (RAPD type 9) were not more tolerant to the dry conditions. The presence of organic materials may have a protective effect of the survival. The protective effect is probably caused by the enhanced osmolarity when the water is evaporating. The cells produces osmolytes, glycine betaine and carnitine (Ko *et al.,* 1994; Smith, 1996), which also helps to resist the dry conditions (Bonaterra *et al.,* 2005). Trehalose and to some extend glycine betaine is accumulated by *Escherichia coli* (Welsh *et al.,* 1999), *Enterobacter sakazakii* (Breeuwer *et al.,* 2003) and *Pantoea agglomerans* (Bonaterra *et al.*, 2005) during desiccation. Also, *L. monocytogenes* is able to survive at a low relative humidity, and are following able to attach to and be recovered from a variety of ready-to-eat food products (De Roin *et al.,* 2003).

Even though *L. monocytogenes* have shown to be present on the dry surfaces in the food processing industry for a long time, the desiccation tolerance phenomenon has never been investigated further.

3.3.4 Enhanced tolerance to cleaning and disinfection agents

Differences in the tolerance of *L. monocytogenes* strains to disinfectants have been suggested to influence the survival of the bacteria in food processing plants and may contribute to persistence (Earnshaw *et al.,* 1998; Aase *et al.,* 2000; Holah *et al.,* 2002; Lemaitre *et al.,* 2998).

Most isolated strains are susceptible to cleaning and disinfection agents (Aase *et al.,* 2000; Mereghetti *et al.,* 2000; Heir *et al.,* 2004; Soumet *et al.,* 2005). A higher number of persistent strains or strains from the meat industry are tolerant to disinfectants (Aase *et al.,* 2000; Heir *et al.,* 2004) whereas Earnshaw and Lawrence {499/d} saw that persistent strains were as sensitive as sporadic strains when exposed to disinfectants. Ongoing work is trying to determine if food processing persistent RAPD type 9 strains are more tolerant to disinfectants than the strains isolated sporadically (Kastbjerg *et al.,* 2007), but preliminary results shows no difference between persistent strains and sporadic isolated strains.

At present, the amount of published work on this area is too small to determine whether persistence of sub-types of *L. monocytogenes* is due an enhanced tolerance against cleaning and disinfection agents, but the preliminary results indicates that the RAPD type 9 strains are not especially tolerant.

3.3.5 The influence of NaCl on the *Listeria monocytogenes* cells

The enhanced adhesion of *L. monocytogenes* when 5% NaCl is added to the growth medium could either be caused by physical-chemical attraction between the cells because of the presence of Na^+ and Cl^--ions, or it could be a stress response resulting in a changed surface protein expression leading to a different composition of proteins on the surface of the cells. Both hypotheses have been tested in this thesis. The changed surface charge was tested by “microbial affinity to solvents” (MATS) (unpublished work) and the changed expression of genes encoding surface proteins were investigated by DNA microarray (unpublished work). “Scanning electron microscopy” (SEM) (unpublished work) was used to visualize the enhanced adhesion and aggregation that was seen in the crystal violet assay.

MATS is used to determine whether the surface of a bacterium is hydrophilic or hydrophobic. The method was developed by Bellon-Fontaine *et al.* (1996), and has been used to describe the surface properties of *Lactococcus* spp., *Bacillus* spores, *Staphylococcus* spp. and *L. monocytogenes* at different temperatures, salinities and in acidic environments (Briandet *et al.,* 1999; Chavant *et al.,* 2002; Bereksi *et al.,* 2002; Faille *et al.,* 2002; Lerebour *et al.,* 2004; Ly *et al.,* 2006).

The purpose of the experiment was to test whether NaCl changed the surface charge of the *L. monocytogenes* strains. We grew *L. monocytogenes* as planktonic or adhered bacteria in media with or without the presence of NaCl (Table 3.4).

The distribution of bacterial cells in mixtures of water and solvent is indicative of surface properties. The method can be used to give indications of the surface properties of bacteria, and should be used to support another method, because of partly inconsistent results. The discussed results should therefore be seen as indications.

The percentage of affinity to non-polar solvents (hexadecane and decane), for both planktonic and adhered bacteria, did not exceed 44%, indicating that the surface of all the strains is hydrophilic. Addition of 5% NaCl to the growth medium, at planktonic and adhesive, resulted in the largest increase in percentage affinity for the polar solvent indicating an increase in hydrophilicity of the strains. Strains growing as planktonic cells both with and without NaCl had the highest percentage increase in the non-polar solvents indicating that strains are more hydrophobic when grown as planktonic than as surface attached cells. Ly *et al.* (2006) were able to differentiate between *Lactococcus lactis* strains and *Lactococcus cremoris* strains, however we could not detect any significant difference between the *L. monocytogenes* strains. The hydrophilicity of *L. monocytogenes* increases the presence of increasing amounts of NaCl (0-10%) (Bereksi *et al.*, 2002). This is in agreement with our results where the presence of 5% NaCl increased the hydrophilicity of the strains and this can explain the increased adhesion and aggregation of the strains to a hydrophilic microtiter plate surface (Jensen *et al.*, 2007a). In the food processing industry, plastic and stainless steel surfaces are hydrophilic (Harvey *et al.,* 2007) and *L. monocytogenes* can therefore adhere to these surfaces and an enhanced adhesion could be seen when the food matrix contains moderate levels of NaCl.

Table 3.4: Affinities for *Listeria monocytogenes* strains for two non-polar solvents (decane, hexadecane) and two polar solvents (ethylacetate, chloroform) used in the MATS analysis. The growth conditions are planktonic in TSB (1% glucose), planktonic in TSB (1% glucose + 5% NaCl), adhesion in TSB (1% glucose), adhesion in TSB (1% glucose + 5% NaCl). The assay was done in three independent trials. Unpublished data

Strain	Medium	% affinity for:			
		Decane	Hexadecane	Ethylacetate	Chloroform
N53-1	Plankt	20 ± 5	20 ± 4	40 ± 1	49 ± 18
(RAPD type 9 strain)	Plankt +NaCl	28 ± 18	23 ± 11	51 ± 13	81 ± 11
	Adhes	33 ± 11	23 ± 0	43 ± 3	53 ± 9
	Adhes +NaCl	41 ± 11	35 ± 4	55 ± 3	78 ± 14
La111	Plankt	14 ± 2	21 ± 2	43 ± 5	56 ± 16
(RAPD type 9 strain)	Plankt +NaCl	34 ± 16	27 ± 13	68 ± 13	67 ± 10
	Adhes	33 ± 10	30 ± 4	47 ± 5	74 ± 15
	Adhes +NaCl	36 ± 11	29 ± 3	56 ± 2	69 ± 12
EGD	Plankt	19 ± 10	14 ± 10	34 ± 7	25 ± 8
(reference strain)	Plankt +NaCl	32 ± 10	13 ± 13	20 ± 10	48 ± 8
	Adhes	33 ± 7	26 ± 5	30 ± 10	55 ± 15
	Adhes +NaCl	26 ± 12	32 ± 6	33 ± 9	85 ± 12
Scott A	Plankt	25 ± 12	16 ± 6	43 ± 10	69 ± 16
(human clinical strain)	Plankt +NaCl	35 ±11	25 ± 10	76 ± 9	81 ± 14
	Adhes	32 ± 4	24 ± 5	44 ± 6	57 ± 15
	Adhes +NaCl	35 ± 3	37 ± 4	76 ± 6	78 ± 11
7418	Plankt	20 ± 8	17 ± 9	33 ± 5	62 ± 22
(food strain)	Plankt +NaCl	32 ± 8	29 ± 6	52 ± 13	83 ± 13
	Adhes	29 ± 8	25 ± 2	38 ± 5	63 ± 12
	Adhes +NaCl	44 ± 10	32 ± 10	42 ± 6	89 ± 8
4446	Plankt	24 ± 18	19 ± 8	37 ± 5	63 ± 19
(human clinical strain)	Plankt +NaCl	29 ± 14	23 ± 9	64 ± 4	76 ± 12
	Adhes	27 ± 4	22 ± 3	41 ± 9	61 ± 13
	Adhes +NaCl	36 ± 4	29 ± 5	54 ± 4	79 ± 3

As a concluding remark, the enhanced adhesion and aggregation seen for the strains when increasing amount of NaCl is added to the growth medium could be due to an increased hydrophilicity of the cells.

DNA microarray analysis was used to investigate whether the expression of genes coding for surface proteins or other proteins that could influence the surface proteins was changed when NaCl was added. Exposure to high levels of NaCl can result in two types of stress; either the salt shock stress or the salt acclimation stress (Duche *et al.,* 2002a). The salt shock stress is in the first period of time (0-30 minutes) after the NaCl have been added, whereas the salt acclimation stress is the period after the salt shock stress, where the cells have adjusted to the level of NaCl.

We have tested conditions similar to the acclimation stress because bacteria adhering to surfaces, both during processing of cold-smoked salmon and in the crystal violet assay have been exposed to elevated levels of NaCl for a longer period of time. In brief, strains were grown under agitation in TSB with or without addition of 5% NaCl. At an OD_{450} of 0.7

cultures were diluted to an OD_{450} of 0.1 in the growth medium and grown up to OD_{450} of 0.7 again. RNA was extracted and a spectrometric control of the quality was done at OD_{260} and OD_{280}.

Several genes changed their expression in the RAPD type 9 strain growing in the presence of 5% NaCl when compared to growth without the presence of NaCl (Table 3.5). Of the total of 2913 genes, 63 had a P-value below 0.05, when comparing expression with and without addition of NaCl. The false discovery rate the experiment was calculated to 0.04%, on basis of Volcano plots, indicating that one gene out of the 2913 genes is a false positive. The 10 genes with the lowest P-values or genes present in the same operon as one with a low P-value were chosen (Table 3.5).

Table 3.5: Genes from a *Listeria monocytogenes* RAPD type 9 strain, which are up- or down-regulated when the bacteria are grown in media containing 5% NaCl for approx. 30 hours. The DNA microarray work was done in collaboration with associate professor Hanne Jarmers group at Center for Biological Sequence Analysis, Technical University of Denmark. Unpublished data.

Gene	Fold change	P-value	Protein[A]	Functional class[B]
Lmo0006	-0.86	0.0486	GyrB, DNA gyrase subunit B	DNA replication, recombination and repair
Lmo0007	-0.57	0.018	GyrA, DNA gyrase subunit A	DNA replication, recombination and repair
Lmo0189	-1.21	0.0126	HP, *Bacillus subtilis* Veg	N.C[C]
Lmo0386	0.44	0.0136	IolD protein, acetolactate synthase	Amino acid transport and metabolism
Lmo0654	0.62	0.00815	HP, rRNA processing	N.C.[C]
Lmo1293	1.01	0.00705	GlpD, glycerol-3-P-dehydrogenase	Energy production and conversion
Lmo2010	0.7	0.035	HP, similar to two-component response regulator	Signal transduction mechanisms
Lmo2011	0.85	0.00498	HP, similar to two-component sensor histidine kinase	Signal transduction mechanisms
Lmo2589	-1.27	0.0174	HP, transcription regulator TetR/AcrR	Transcription
Lmo2695	1.32	0.00868	HP, dihydroxyacetone kinase	Carbohydrate transport and metabolism

[A] HP: Hypothetical protein
[B] The functional class when genes names where compared to *Listeria monocytogenes* EGD-e genes by using: (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&Cmd=ShowDetailView&TermToSearch=204)
[C] N.C.: Not classified

None of the listed proteins were classified as outer membrane proteins, but submitting the protein sequence of Lmo0189 (hypothetical protein, similar to *Bacillus subtilis* Veg) to the "Protein function prediction" (http://dragon.bio.purdue.edu/pfp/) (2007b) the protein was predicted to be "involved in peptidoglycan synthesis". In *B. subtilis*, Veg is assumed to be a typical cytoplasmic protein (Fukushima *et al.,* 2003). The localization of Veg during sporulation in *B. subtilis* is in the core region of the spores (Fukushima *et al.*, 2003). The *veg* gene is suggested to have two functions in *B. subtilis*; an unknown function during the vegetative growth phase and a germination related function.

None of the genes listed in Table 3.5 and the respective proteins have been identified as being involved in NaCl-stress of *L. monocytogenes* before (Duche *et al.,* 2002a; Duche *et al.,* 2002b; Gardan *et al.,* 2003; Chatterjee *et al.,* 2006), but GlpD was found expressed at a higher level during stationary growth compared to exponential growth (Folio *et al.,* 2004). A strategy to investigate the influence of these up- or down-regulated genes on the adhesion and aggregation phenomenon is to make knock-out mutations in each of the genes and test the adhesion and aggregation to a plastic surface for each of the mutants.

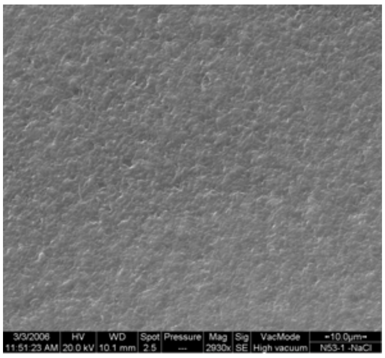
A 1-dimensional gel electrophoresis of the surface proteins of food processing persistent RAPD type 9 strains and other *L. monocytogenes* strains was done, to determine any difference in the surface protein expression. No significant difference in surface protein expression was measured between strains grown as adhered bacteria with or without the presence of 5% NaCl (unpublished work). This is in agreement with the reached results from the DNA microarray analysis.

Hence, a changed expression of surface proteins did not appear to be a major reason for enhanced adhesion aggregation when adding 5% NaCl to the growth medium.

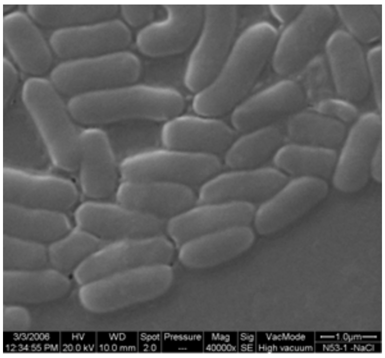
SEM could be a way to visualize, on cell-level, the enhanced adhesion and aggregation that was seen in the crystal violet assay, when 5 % NaCl was added to the growth medium. *L. monocytogenes* exposed to stress full conditions such as 7-9% NaCl (Isom *et al.,* 1995) or low pH (pH 5) together with high levels of NaCl (10%) (Bereksi *et al.*, 2002) grew as filamentous cells. We have used SEM to attempt to visualize any differences in adhesion, aggregation and single-cell morphology of a RAPD type 9 strain grown on a surface with or without the presence of NaCl (Figure 3.5)

Figure 3.5: Scanning electron microscopy of a food processing persistent RAPD type 9 strain of *Listeria monocytogenes.* The strain was grown on a surface under to different conditions (TSB (1% glucose) or TSB (1% glucose + 5% NaCl)) and the magnification was either ×3000 or ×40000. The SEM work was done in collaboration with associate professor Jose Bresciani and associate research professor Michael Hansen at Department for Ecology, Faculty of Life Science, University of Copenhagen. Unpublished data.

RAPD type 9 strain TSB (1% glucose)

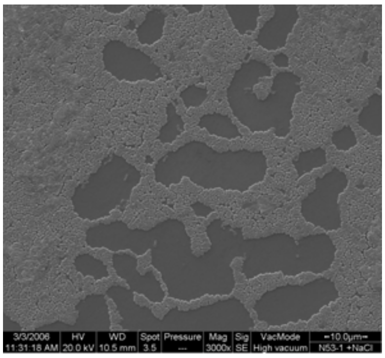


Magnification ×3000

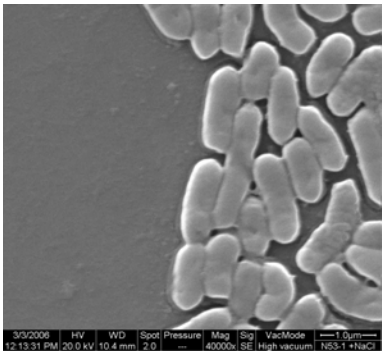


Magnification ×40000

RAPD type 9 strain TSB (1% glucose + 5% NaCl)



Magnification ×3000



Magnification ×40000

Surprisingly, the layer of adhered bacteria to the plastic surface appeared more massive after growth in media without 5% NaCl, when compared to the layer of adhered bacteria grown in the presence of 5% NaCl. The morphology of the single cells is very similar for the cells grown under the two different conditions, except that cells grown in the presence of 5% NaCl appear more irregular on the surfaces. We did not see any filamentation of the cells, but this is probably due to the relatively moderate level of NaCl added to the growth media. Further studies with SEM with a higher number of strains need to be done to determine if NaCl changes the surface topography of the cells.

3.4 Conclusion

Based on the work done in this thesis, we cannot point out one reason explaining the persistence of *L. monocytogenes* RAPD type 9. However, several factors were shown, when present at the same time, to enhance adhesion and aggregation, but is was not unique for RAPD type 9 strains. Especially addition of moderate levels of NaCl enhanced the adhesion, and work done by Birte Vogel has demonstrated that the presence of an organic matrix surrounding the bacteria when exposed to desiccation also appeared to increase the tolerance to desiccation.

4 Listeriosis

Listeriosis is an infectious disease where *Listeria monocytogenes* spreads intracellularly during the infection. *L. monocytogenes* is also capable of crossing the brain-blood and the feto-placental barrier (Lecuit, 2005). *L. monocytogenes* can therefore infect the fetus in a pregnant woman, and cause sepsis or meningitis in elderly and immunocompromised people or people with underlying diseases (Lennon *et al.,* 1984; Lyon *et al.,* 1987; Junttila *et al.,* 1989; Bula *et al.,* 1995; Ericsson *et al.,* 1997; Valk *et al.,* 2001). Occasionally healthy people get infected too (Miettinen *et al.,* 1999a; Farber *et al.,* 2000; Lyytikainen *et al.,* 2000).

4.1 Clinical forms and incidence of listeriosis

Listeriosis is the disease caused by *L. monocytogenes*, and it can be seen in different forms. The main clinical forms of 782 cases during 1991-1992 in 27 countries covering Europe, North and South America and Asia have been reported; Maternal and neonatal infections accounts for 34%. Of the non-neonatal cases (66%) symptoms divide between bacteriemia/sepsis (56%), central nervous system infections (36%) or atypical forms (8%) (Rocourt *et al.,* 1997). The fetus from a pregnant woman is very susceptible to listeriosis, because *L. monocytogenes* infects the placenta and fetus. Listeriosis in pregnant women will result in flu-like symptoms (Frederiksen *et al.,* 1992), but very often the infection will result in spontaneous abortion, still birth, premature birth to either an infected or a healthy child (Schlech *et al.,* 1983; Frederiksen *et al.,* 1992; Valk *et al.,* 2001; Mylonakis *et al.,* 2002).

There are approx. 40 cases of listeriosis every year in Denmark and the mortality rate is approx 21%, but in 2004 and 2005, the number of cases has increased slightly to 42 and 46 cases (Smith *et al.,* 2006). Using DNA-sub-typing methods such as ribotyping or pulse field gel electrophoresis it has been shown that the increase is caused by several DNA types and it is therefore believed to be sporadic cases. The incidence in Denmark is 0.85 per 100,000 inhabitants, which is much higher than the incidence in USA, where it is only 0.27 per 100,000 inhabitants (Table 4.1). These differences may be due to different surveillance systems or different food consumption patterns (Valk *et al.,* 2003).

In USA the incidence of listeriosis is very low compared to other diseases caused by food borne human pathogen bacteria, however the risk of death and the number of cases that are hospitalized is several times higher than for the other bacteria (Table 4.1).

Table 4.1: Number of cases, hospitalization, death and incidence caused by human pathogens in USA in 2004. The numbers represents 15% of the U.S. population (44.470.395 persons). Reference: CDC (2006)

Pathogen	No. cases	No. of cases at hospital	Death	Incidence per 100,000
Salmonella	6498 (42%)	26%	38 (0.6%)	14.61
Cryptosporidium	637 (4%)	27%	5 (0.8%)	1.43
Campylobacter	5684 (37%)	15%	9 (0.2%)	12.78
STEC[A] O157	402 (3%)	42%	4 (1%)	0.9
Shigella	2248 (15%)	18%	3 (0.1%)	5.06
Yersinia	176 (1%)	27%	1 (0.6%)	0.4
Vibrio	123 (1%)	32%	5 (4%)	0.28
Listeria	119 (1%)	97%	19 (16%)	0.27
STEC non-O157	110 (1%)	21%	-	0.25

[A] STEC: Shiga toxin producing *Escherichia coli*

4.2 Overview of virulence factors in *Listeria monocytogenes*

The ability of *L. monocytogenes* to grow intracellularly is a very important characteristic, because it enables the bacterium to escape from the immune defense during infection of the host. An important issue is the ability of *L. monocytogenes* to cross three important barriers in the human host, namely the intestinal-blood, the blood-brain and the feto-placental barrier. Not many bacteria are able to grow intracellularly in the human host, but *L. monocytogenes* has this ability because of an artillery of different virulence factors, which are expressed during the intracellular growth (Figure 4.1). Virulence factors are elements that enable a microorganism to colonize a host where the organism proliferates and causes tissue damage or systemic inflammation (Chen *et al.,* 2005). Virulence factors are described as secreted proteins (toxins, enzymes) and cell-surface structures such as capsular polysaccharides, lipopolysaccharides and outer membrane proteins, which directly contribute to the disease processes.

Several virulence factors are involved in the intracellular life cycle, which proceeds as followed: The bacterium crosses the eukaryotic cell membrane (InlA, InlB), and is thereafter surrounded by vacuole, from which it escapes by producing enzymes (LLO, PlcA) that destroys the membrane. Following proliferation, the cells can move intracellular and spread to neighboring cells by using the produced actin tail (ActA). After invasion of the neighboring cells, the bacterium is surrounded by a double-membrane consisting of membrane from the previous eukaryotic cell and from the newly invaded cell. Enzymes are again produced to escape from this double-membrane (LLO, PlcB).

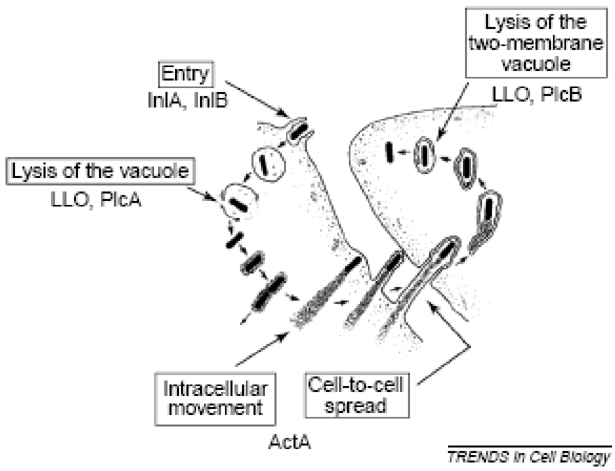


Figure 4.1: The infection process of a host cell by *Listeria monocytogenes*. Each infection step is indicated together with the virulence factors involved. Reference: Cossart *et al.* (2003).

The expressions of the proteins involved in these steps are showed in Figure 4.2. They are all controlled by the positive regulatory factor A (PrfA), which can bind to a palindrome sequence found in the promoter region of genes of the virulence factors (Menguad *et al.,* 1989). These six proteins are grouped as the “classical” virulence factors, because they are all known to be essential for bacterial infection and their only function is in virulence. The proteins are unique for the *Listeria* species, and they are all organized in the central virulence cluster (Figure 4.2). The two internalins InlA and InlB are encoded by the *inlAB* operon (Gaillard *et al.,* 1991), and are positioned in an other gene cluster than the other virulence genes.

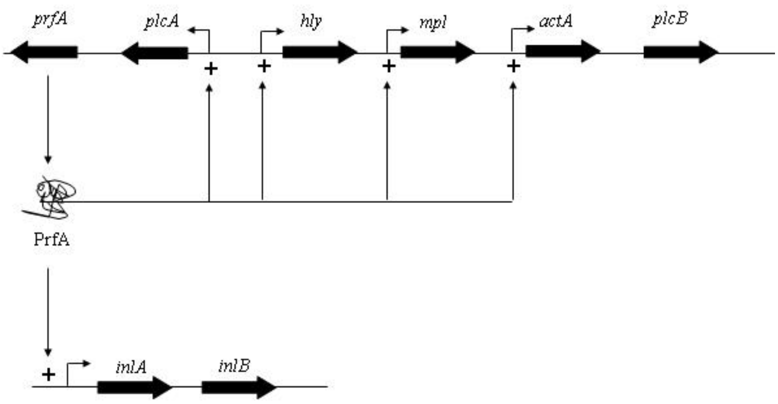


Figure 4.2: Organization of the central virulence gene cluster of *Listeria monocytogenes*. PrfA is the transcriptional activator. *plcA* and *plcB* encodes for phosphatidylinositol-specific phospholipase C (PlcA) and phosphatidylcholine-specific phospholipase C (PlcB) respectively. *hly* encodes for Listeriolysin O (LLO) and *mpl* encodes for a metalloprotease necessary for activation of PlcB. The protein involved in actin polymerization (ActA) is encoded by *actA*. At another position of the genome are *inlA* and *inlB* located, these two encodes for internalins (InlA and InlB) used for invasion. Modified from Vazquez-Boland *et al.* (2001)

Several other proteins are suggested to be involved in virulence besides the classical virulence genes located in the central virulence cluster. These are all located outside the virulence cluster, but some of them are however regulated by the transcriptional activator PrfA. These genes are sometimes called accessory virulence factors because they are also involved in other processes than virulence in *L. monocytogenes*.

The expression of *prfA* is controlled in different ways either by PrfA itself or by the alternative sigma factor σ^B (Leimeister-Wachter *et al.,* 1990; Leimeister-Wachter *et al.,* 1992; Freitag *et al.,* 1993; Freitag *et al.,* 1994). Also the temperature has an influence on the production of virulence factors because the secondary structure of untranslated *prfA*-mRNA is temperature dependent (Leimeister-Wachter *et al.,* 1992; Johansson *et al.,* 2002). At 30°C, the Shine-Dalgarno sequence is blocked and the ribosomes are not able to bind and translate the sequence. Due to the positive feedback mechanism, only a small amount of *prfA* is therefore transcribed (Leimeister-Wachter *et al.*, 1992). At 37°C, the secondary structure have changed, which results in translation of *prfA*-mRNA followed by synthesis of PrfA and results in a higher amount of transcribed *prfA*. PrfA is the primary regulator of the expression of the virulence factors present in the virulence gene cluster, but other proteins act as virulence gene regulators too. VirR, is a response regulator critical for *L. monocytogenes* virulence (Mandin *et al.,* 2005). The genes regulated by VirR encode ABC-transporters, proteins involved in resistance to human defensins in *Staphylococcus aureus* and cell wall modification proteins (Mandin *et al.*, 2005). The alternative sigma factor σ^B is an overall regulator of the expression of several genes as a response of several types of stresses, and as mentioned, it also regulates the expression PrfA and thereby the expression of virulence factors.

The classical method to investigate genes of importance for virulence has been creation of knock-out mutants followed by determination of their virulence potential and identification of the non-functional gene (Mengaud *et al.,* 1991; Gaillard *et al.,* 1991). After the genome sequences of *L. monocytogenes* and the non-pathogenic *Listeria innocua* have been published (Glaser *et al.,* 2001), comparative genomics have been used with great success. Genes not present in *L. innocua*, but on the other hand present in *L. monocytogenes* and encoding interesting sequences such as a membrane binding region have been investigated further by this method. Especially accessory virulence factors of importance for adhesion to and invasion into eukaryotic cells are identified by comparative genomics e.g. Auto (Cabanes *et al.,* 2004).

4.2.1 Adhesion to eukaryotic cells

Adhesion of *L. monocytogenes* to a eukaryotic cell is the first step in the intracellular life cycle of *L. monocytogenes*. Adhesion can occur between proteins, carbohydrates or other components of the cell membranes of both the bacterial or eukaryotic cell. Several factors are involved in the adhesion of *L. monocytogenes* to eukaryotic cells but the primary adhesion is mediated by surface proteins (Table 4.2). It should be noted to Table 4.2 and Table 4.3, that a variety of cell lines with different animal origin have been used to study proteins of importance for adhesion and invasion of *L. monocytogenes* to eukaryotic cell lines. It is difficult to make final conclusions because of the inconsistency between the chosen cell lines.

Table 4.2: *Listeria monocytogenes* surface proteins of importance for adhesion between bacterium and eukaryotic cells. Other types of tissue than the ones mentioned in the table can be in involved in adhesion with *Listeria monocytogenes*.

Protein	Gene	Receptor	Tissue	Reference
InlA	*inlA*	E-cadherin	Intestines	Milohanic *et al.* (2001), Gaillard *et al.* (1991)
InlB	*inlB*	Met, gC1q-R [A], heparin sulfate proteoglycans	Liver, cervix, kidney	Braun *et al.* (2000), Dramsi *et al.* (1995), Gaillard *et al.* (1991), Jonquieres *et al.* (2001), Shen *et al.* (2000)
P104/LAP		HSP60 on Caco-2 cells	Intestines (ileum, cecum, colon), bladder, kidney, larynx, skin	Jaradat *et al.* (2003a), Pandiripally *et al.* (1999), Wampler *et al.* (2004)
P60	*iap*	Not identified	Fibroblast cells, intestines	Bubert *et al.* (1992), Park *et al.* (2000)
Ami	*ami*	Not identified	Liver	Milohanic *et al.* (2001)
ActA	*actA*	Heparan sulfate proteoglycans	Macrophage (mouse), ovary cells (hamster)	Alvarez-Dominhuez *et al.* (1997)
FbpA	*fbp*	Immobilized fibronectin	Intestines, liver	Dramsi *et al.* (2004), Gilot *et al.* (2000)

[A] InlB do not activate Met or gC1q-R originating from guinea pigs (Khelef *et al.,* 2006)

InlA and InlB are primarily used for invasion, but the two proteins bind effectively to their respective receptors on the surface of the eukaryotic cell, and will thereby contribute to the adhesion. The autolysin Ami surface protein plays a minor role in adhesion to eukaryotic cell lines, since the effect of lost adhesion ability only was seen in *ami*-mutants also lacking *inlA* and/or *inlB* (Milohanic *et al.,* 2001). This is most likely due to the effect InlA and InlB have on adhesion and they are probably able to overcome the defect in Ami cell adhesion function (Milohanic *et al.*, 2001).

Also adhesion is influenced by genes not identified. New loci have been described to be involved in the adhesion of *L. monocytogenes* to eukaryotic cells (Milohanic *et al.,* 2000), but the genes and the mechanisms that are used is still unknown.

The virulence of *L. monocytogenes* strains varies and this could be caused by differences in the ability to adhere and attach to eukaryotic cells. However, when testing the adhesion of *L. monocytogenes* strains to guinea pig epithelial intestinal cells, all strains (environment, human, animal) were able to attach the cells (Meyer *et al.,* 1992; Bunduki *et al.,* 1993; Chiu *et al.,* 2006). In this thesis, we found that adhesion to Caco-2 cells varied between *L. monocytogenes* strains, but there was no correlation between the degree of adhesion and the origin, serotype or invasion into cell lines of the strains (Figure 4.3) (Jensen *et al.*, 2007a)

The adhesion affinities can be changed due to changes in the environmental conditions e.g. pH, temperature or osmolarity. An enhanced aggregation and adhesion to a plastic surface was seen when 5% NaCl was added to the growth medium of several *L. monocytogenes* strains (Jensen *et al.*, 2007a). However, addition of 5% NaCl to the growth medium of several *L. monocytogenes* strains did not significantly influence the adhesion of the strains to Caco-2 cells (Jensen *et al.*, 2007b) (Figure 4.3).

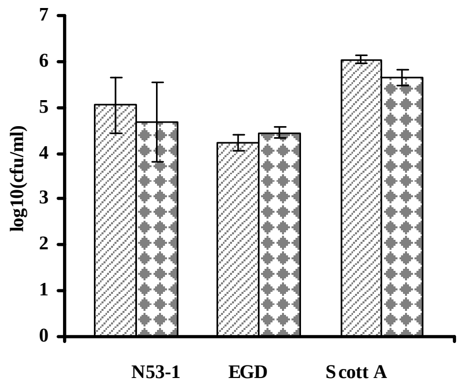


Figure 4.3: Adhesion of *L. monocytogenes* strains RAPD type 9 (N53-1), a reference strain (EGD) and a human, clinical strain (Scott A) to Caco-2 cells. Strains were grown in TSB (1% glucose) ( ) or TSB (1% glucose + 5% NaCl) ( ) before the assays. Error bars are based on standard deviations from two independent experiments in duplicate. Reference: Jensen *et al.* (2007b).

4.2.2 Invasion into eukaryotic cells

The second step in the infection is the invasion where *L. monocytogenes* is crossing the eukaryotic cell membrane. The two most important *L. monocytogenes* surface proteins for invasion are InlA and InlB, and the two proteins are important for invasion into different eukaryotic cell types. Different cell types of different animal origins are also used for proteins of importance for invasion. It is therefore difficult to make a clear conclusion from the different experiments.

The first identified surface protein involved in virulence was InlA, which is encoded by *inlA* (Gaillard *et al.*, 1991). InlA is a 744 amino acid surface protein that contains several regions with different physical properties (Figure 4.4). In the N-terminal end, the signal sequence is followed by a Leucine Rich Region (LRR) containing 15 repeats of 22 amino acids and three successive repeats, where the same amino acids is found in 27 of the 49 positions of the repeats. At the C-terminal end a LPXTG sequence is present, which acts as an anchor region of surface proteins of gram-positive bacteria (Fischetti *et al.,* 1990).

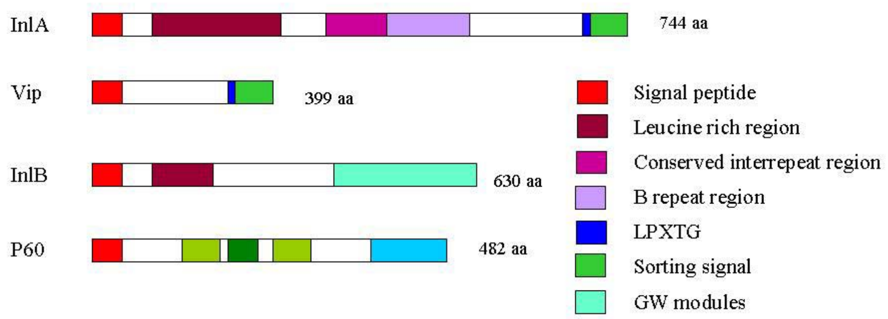


Figure 4.4: Surface proteins of *Listeria monocytogenes* representing four different groups of invasion proteins. Different colours describe different domains characteristics for the families. Modified after Cabanes *et al.* (2002).

E-cadherin (Epithel cadherin) is the InlA receptor molecule (Mengaud *et al.,* 1996). It is a protein expressed on the surface of enterocytes (Hermiston *et al.,* 1995), but also on the surface of other epithelial tissues such as skin and liver. E-cadherin is primarily positioned at tight junction between the cells, but is also distributed sporadically elsewhere on the surface. *L. monocytogenes* does not interact with the tight junction, but when cells in the epithelium layer dies, they are expelled and detached from the epithelium by extrusion (Pentecost *et al.,* 2006). The surrounding cells are reorganized and E-cadherin is transiently exposed to the luminal surface of the eukaryotic cells, which enables *L. monocytogenes* to attach.

Table 4.3: *Listeria monocytogenes* surface proteins of importance for invasion of the bacterium into the eukaryotic cell. Other types of tissue than the ones mentioned in the table can be in involved in invasion of *L. monocytogenes*.

Protein	Gene	Receptor	Tissue	Reference
InlA	*inlA*	E-cadherin[A]	Most epithelial cells (intestinal cell, liver cell)	Dramsi *et al.* (1995), Gaillard *et al.* (1991), Mengaud *et al.* (1995)
InlB	*InlB*	Met, gC1q-R [B], heparin sulfate proteoglycans	Liver, spleen	Braun *et al.* (2000), Dramsi *et al.* (1995), Gaillard *et al.* (1991), Jonquieres *et al.* (2001), Shen *et al.* (2000)
Vip	*vip*	Gp96[C]	Fibroblast, intestines[D]	Cabanes *et al.* (2005)
Auto	*aut*	Not identified	Intestines, nodes, liver, spleen	Cabanes *et al.* (2004)

[A] E-cadherin from humans are similar to E-cadherin from guinea pigs and rabbits, but different than E-cadherin from mice and rats.
[B] InlB do not activate Met or gC1q-R originating from guinea pigs (Khelef *et al.*, 2006)
[C] The human endoplasmatic reticulum chaperone Gp96
[D] Vip have no effect in guinea pig epithelial cells or in monkey kidney cells (Cabanes *et al.,* 2005)

Mice have been used as infection models for *L. monocytogenes* for many years. However, in 1999 Lecuit *et al.* compared E-cadherin from mouse, rat, chicken, human, guinea pig and rabbit and discovered, surprisingly that E-cadherin from mouse and rat cluster in another group than chicken, human, guinea pig and rabbit E-cadherin (Lecuit *et al.,* 1999). In humans, the amino acid at position 16 is glutamic acid, where in mice it is proline. Because of this difference in the amino acid composition, there is a very low binding affinity between mouse or rat E-cadherin and InlA from *L. monocytogenes*. After this discovery, guinea pigs have been preferred as experimental animals when investigating oral exposure with *L. monocytogenes*. For this reason, a transgenic mouse was designed, expressing human E-cadherin on their intestinal cell (Lecuit *et al.,* 2001). These mice do not express human E-cadherin on other cell surfaces than the enterocytes, but binding InlA and E-cadherin have shown to be of importance also in the liver (Table 4.3). Therefore the transgenic mouse can only be used to oral infection studies of mice and not the colonization of organs further in the body of mice.

Jonquiéres *et al.* (1998) discovered that *L. monocytogenes* LO28, which is a commonly used reference strain, harbors a nonsense mutation in *inlA*. The mutation is a deletion of an adenine at position 1637, and the frame shift mutation leads to the creation of a nonsense codon, TAA at position 1729, resulting in an open reading frame encoding a 63 KDa protein, which is lacking the cell wall anchor. Because of this, the InlA cannot be attached to the cell wall, and it is therefore secreted into the supernatant. This phenomenon, named "Premature Stop Codon (PMSC)", in *L. monocytogenes* LO28 has been found in other *L. monocytogenes* strains (Jonquieres *et al.,* 1998; Olier *et al.,* 2003; Rousseaux *et al.,* 2004; Nightingale *et al.,* 2005; Felicio *et al.,* 2007; Handa-Miya *et al.,* 2007). Studies have identified at least 12 different naturally occurring *inlA* mutations leading to the production and secretion of truncated

InlA, and strains from USA, France, Portugal and Japan have been tested This could indicate that the presence of PMSC in *inlA* is a globally distributed phenomenon. The prevalence of PMSC in *L. monocytogenes* from the out door environment has not been determined.

Invasion into a human cell line is used as a preliminary descriptor of the *L. monocytogenes* virulence potential. The invasive potential of *L. monocytogenes* strains vary (Larsen *et al.,* 2002; Werbrouck *et al.,* 2006; Jensen *et al.,* 2007a; Jensen *et al.,* 2007b; Jensen *et al.,* 2007c). Werbrouck *et al.* (2006) found that clinical strains had lower invasion capacity into Caco-2 cells than non-clinical strains. We did not find the same clear differentiation, when the invasiveness of food processing persistent RAPD type 9 strains, sporadic isolated food processing strains, food and clinical strains in Caco-2 cells was compared (Jensen *et al.,* 2007a) (Figure 4.5).

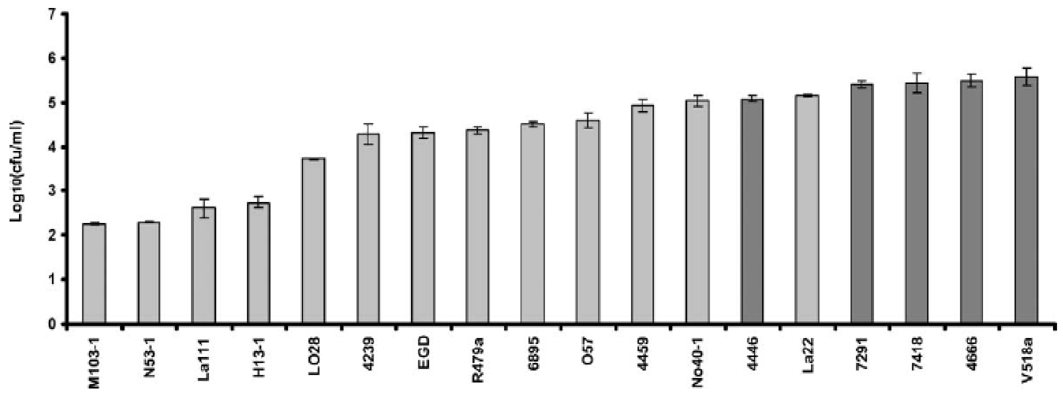


Figure 4.5: Invasion of strains of *Listeria monocytogenes* in Caco-2 cells. Strains M103-1, N53-1, La111 and H13-1 all belong to the same food processing persistent RAPD type 9. The other strains have different origins (human, clinical cases, food, food processing environment) and RAPD types. Lineage 1 strains are in dark grey, and lineage 2 strains are in light grey. Reference: Jensen *et al.* (2007a).

Four strains belonging to the same food processing persistent RAPD type (RAPD type 9) showed significant lower invasive potential than the rest of the tested strains (different origin and RAPD types) even LO28, which have been shown to be a very poor invader (Jonquieres *et al.,* 1998). It should be noticed, that strain V518a belongs to another group of persistent strains (RAPD type 57), and this strain is having a very high invasion potential. Therefore, not all persistent strains have a low invasion potential.

Differences in *inlA* and *inlB* expression can explain poor invasion (Werbrouck *et al.,* 2006), but also mutations in *inlA* may cause low invasion ability (Jonquieres *et al.,* 1998; Jensen *et al.,* 2007c). We have sequenced the 3´region of *inlA* from seven different strains of *L. monocytogenes*, and did identify two single point mutations (Figure 4.6).

```
Aminoacid                               539                          572
                                         |                            |
InlA (ref)      PAKPVKEGHTFVGWFDAQTGGTKWNFSTDKMPTNDINLYAQFSINSYTATFDNDGVTT
EGD             PAKPVKEGHTFVGWFDAQTGGTKWNFSTDKMPTNDINLYAQFSINSYTATFDNDGVTT
N53-1           PAKPVKEGYTFIGWFDAKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
La111           ----------------AKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
H13-1           -------------WFDAKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
M103-1          PAKPVKEGYTFIGWFDAKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
7418            PAKPVKEGYTFVGWFDAQTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATFDNDGVTT
4446            ---------------DAQTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATFDNDGVTT
                                *:******************:*************:*******
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Figure 4.6: InlA from different *Listeria monocytogenes* strains were aligned with the ClustalW program. InlA from following strains were aligned: InlA (reference, accession no. NC_003210), a reference strain (EGD), RAPD type 9 strains (N53-1, La111, H13-1, M103-1), a food isolate (7418) and a human clinical strain (4446). The two mutations are boxed in black, and their position in the InlA protein is indicated. Glutamine (Q) is changed to lysine (K), and phenylalanine (F) is changed to leucine (L). Stars indicates identity, semi-colon indicates similarity. Reference: Jensen *et al.* (2007b).

The two amino acids changes were seen at position 539 and 572. In both cases the four RAPD type 9 strains showed the same change in amino acid as compared to the other tested strains. At position 539 glutamine is changed to lysine, and at position 572 phenylalanine is changed to leucine (Figure 4.6) (Jensen *et al.*, 2007b). We have hypothesized that these two changes may cause conformation changes in the synthesized InlA, leading to lower affinity to E-cadherin on the surface of Caco-2 cells and therefore lower invasion ability.

In the future, we will sequence the complete *inlA* from all the strains, since we did not identify any PMSC in the 3`region of *inlA* and some of the other PMSC have been identified in the 5`region of *inlA* (Olier *et al.,* 2002; Rousseaux *et al.,* 2004).

Serotype correlates with the presence of PMSC, since all strains belonging to serotype 1/2b, 1/2c and 4b encode a full-length *inlA*, whereas 83% of the serotype 1/2a strains encode a full-length *inlA* (Jacquet *et al.,* 2004). We did only find single point mutations in strains belonging to 1/2a, and they all belonged to the food processing persistent RAPD type 9 (Jensen *et al.*, 2007b).

Another surface protein required for invasion into eukaryotic cells is InlB, which promotes bacterial internalization into a variety of cell lines (Gaillard *et al.,* 1991; Dramsi *et al.,* 1995; Shen *et al.,* 2000; Braun *et al.,* 2000; Jonquieres *et al.,* 2001). InlB does not bind to E-cadherin on the intestinal cells (Dramsi *et al.*, 1995), and InlB is therefore not necessary for entry into Caco-2 cells. Three different proteins have shown to act as receptor molecules for InlB. The first is the hepatocyte surface protein, Met, where the only other known ligand is the Hepatocyte Growth Factor (HGF) (Shen *et al.*, 2000). The second is heparin sulfate proteoglycans (HSPG) (Jonquieres *et al.*, 2001) and the third is gC1qR (Braun *et al.*, 2000).

InlB is very important in liver and spleen colonization in mice, but does not play a role in crossing the intestinal barrier in mice {238}. InlB plays no role in rabbit and guinea pig infections (Khelef *et al.*, 2006), even though guinea pigs and rabbits do express Met and gC1q-R. Similar to E-cadherin in mice, there may be a few amino acid differences in the sequence of the receptor proteins, resulting in a poor binding between Met or gC1q-R and InlB. Partial sequencing of the InlB binding site of Met from human, mouse, guinea pig and rabbit, showed single amino acids differences between human, mouse Met and guinea pig, rabbit Met (Khelef *et al.*, 2006). This could cause the non-activation of Met by guinea pig InlB.

Recently, two new surface proteins (Vip and Auto) from *L. monocytogenes* have shown to influence the invasion (Cabanes *et al.*, 2005); (Cabanes *et al.,* 2004) (Table 4.3). Only the receptor for Vip has been identified (Table 4.3). Since both studies are new, further work has not been performed on these new virulence factors.

4.2.3 Intracellular growth in eukaryotic cells

The next step in the infection process is the intracellular growth of the invaded bacteria. First of all, *L. monocytogenes* must be able to resist the defense of the host e.g. antimicrobial peptides, H_2O_2 and other compounds excreted from the immune defense to survive and grow intracellularly. This resistance could be e.g. export pumps or presence of oxidase enzyme.

No specific virulence genes are important for the intracellular growth, but a high growth rate may correspond to a higher virulence potential of the strain. Differences in the intracellular growth rate have been seen between a wild type reference strain and a mutant strain (Larsen *et al.,* 2006). We were not able to see any differences between the intracellular growth rates when the food processing persistent RAPD type 9 strains were compared to human, clinical strains or a food strain (Figure 4.7).

The differences in virulence potential that we subsequently found in the different models can therefore not be due to different intracellular growth rate in intestinal cells (Jensen *et al.,* 2007b; Jensen *et al.,* 2007c).

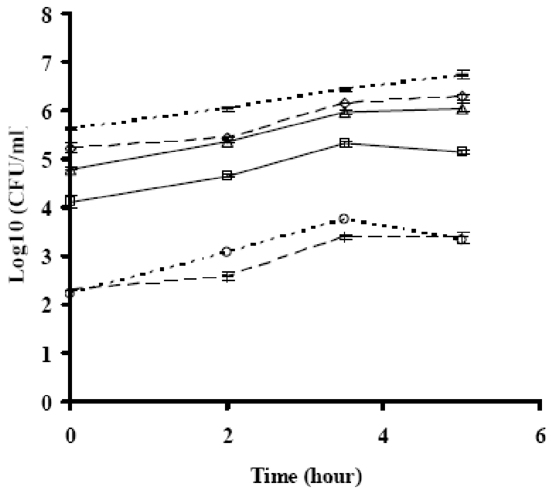

Figure 4.7: Invasion and intracellular growth of *Listeria monocytogenes* strains in Caco-2 cells. Strains were grown in TSB (1% glucose) before beginning of the assay. Strains are as followed: Food strain (−−◊−−), RAPD type 9 strain (−−×−−), human, clinical strain (—Δ—), human clinical strain (--——---), reference strain (—□—) and RAPD type 9 strain (---○---). Hours is the number of hours after addition of gentamicin. Error bars are based on standard deviations from duplicate measurements. The figure is representative of two independent experiments. Reference: Jensen *et al.* (2007b).

4.2.4 Escape from the membranes and cell-to-cell spread

L. monocytogenes bacteria are surrounded by vacuole membranes at two different stages of the infection process. Just after entry, the bacteria are trapped in a single-layer membrane. By getting access to the cytosol of the host cells, the bacteria are able to replicate and spread to neighbouring cells. Later in the infectious process, the bacteria are surrounded by a double-membrane vacuole. Escaping from both the single layer and double layer vacuole is essential for an effective infection, and failure to escape from the membranes result in an infection that is eliminated fast from the tissues (Le Monnier *et al.,* 2007).

The functions of the surface active virulence factors are still under investigation. Two phospholipases are involved; the phophatidylinositol-specific phospholipase C (PlcA) is encoded by *plcA* (Mengaud *et al.*, 1991) and phosphatidylcholin phospholipase C (PlcB) is encoded by *plcB* (Vazquez-Boland *et al.,* 1992). Maturation of PlcB is dependent of a zinc metalloprotease (*mpl*) which also is present in the PrfA-regulated virulence gene cluster (Poyart *et al.,* 1993). Also, Listeriolysin O (LLO) is involved in the escape from the membranes.

It has been believed that PlcA and LLO were of importance in the lysis of the single layer vacuole membrane, and that PlcB and LLO destroy the double layer membrane (Mengaud *et al.*, 1991; Vazquez-Boland *et al.*, 1992). Recently, it was shown that the two phospholipases PlcB and PlcA are important for the escape from the single-layer membrane, but are not sufficient for the break-down of the outer-membrane in the double-layer vacuole (Alberti-Segui *et al.,* 2007), and that LLO is a key factor in the dissolution of the double membrane.

SvpA is a protein facilitating the bacterial escape from the phagosomes of macrophages (Borezee *et al.,* 2001). SvpA is a surface-exposed protein, which may also protect intraphagosomal bacteria from being killed by the host cell and might also have other synergistic functions with other virulence factors produced by *L. monocytogenes*.

The actin polymerization, encoded by *actA*, that enables in spread to neighbouring cells, is another very important step in the invasion of a host (Kocks *et al.,* 1992). ActA is required by the bacteria for nucleation of actin filaments and thereby formation of a tail, using components of cytoskeleton of the host cell, that enables the *L. monocytogenes* bacterium to move intracellularly and spread to neighbouring cells (Brundage *et al.,* 1993). ActA is very important in guinea pig placenta and fetus infection, since an ActA mutant strain is unable to spread and proliferate in the placenta, and thereby the fetus will not be infected (Bakardjiev *et al.,* 2005).

4.2.5 *Listeria monocytogenes* infection during pregnancy

There is a high risk for spontaneous abortion or stillbirth when a pregnant mother is infected by *L. monocytogenes*. Recently, it was suggested that the placenta acts as a niche for *L. monocytogenes* growth during the maternal infection, while the maternal organs are cleared (Bakardjiev *et al.,* 2006). The placenta re-infects the mother and the mother cannot be cleared before the trafficking of *L. monocytogenes* from placenta to maternal organs is interrupted. This will lead to rejection of the placenta and fetus from the body of the mother.

The placenta is a dynamic organ which consists of both maternal and fetal cells that are connected in an ingenious system. The structure and function changes throughout the pregnancy, but the primary function is to act as a barrier between a mother and her fetus. The physiological barrier separating fetal and maternal blood primarily consists of a single layer of fetally derived trophoblastic cells (the outer cell layer of the villi) (Figure 4.8). Nutrients and cells from the immune defense are able to cross the barrier, whereas blood cells do not cross the barrier. Very few organisms are able to cross the placenta barrier, and *L. monocytogenes* is the only food-borne human pathogenic bacteria with this ability. Other organisms are viruses (Koi *et al.,* 2001), parasites such as *Toxoplama gondii* (Kravetz *et al.,* 2005), *Plasmodium falciparum* (Scherf *et al.,* 2001) and very rare bacteria e.g. *Chlamydophila abortus* (Johnson *et al.,* 1985).

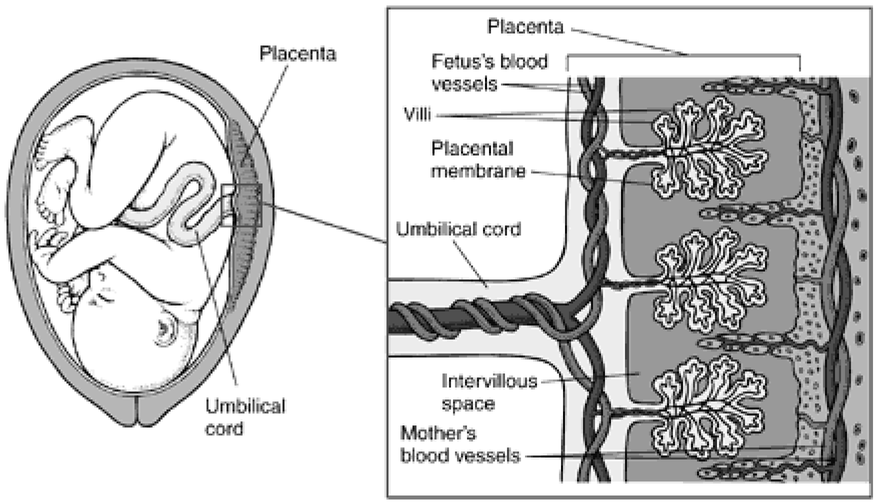

Figure 4.8: Composition of the placenta. Some of the fetus's blood vessels are contained in tiny hair-like projections (villi) of the placenta that extend into the wall of the uterus. The mother's blood passes through the space surrounding the villi (intervillous space). Only a thin membrane (placental membrane) separates the mother's blood in the intervillous space from the fetus's blood in the villi. Nutrients and also *L. monocytogenes* in the mother's blood can cross this membrane into blood vessels in the villi and pass through the umbilical cord to the fetus. Reference: http://www.merck.com/mmhe/sec22/ch259/ch259a.html

Little is known about how *L. monocytogenes* is able to cross the blood-placenta barrier, but a lot of work has been done to investigate the intracellular life of *L. monocytogenes* when passing the barrier. The most obvious factors of importance, when crossing the placenta barrier, are the virulence factors InlA, InlB, ActA and LLO, since they are involved in the invasion of cells, cell-to-cell spread and lyses of the cell membranes. Jacquet *et al.* (2004) suggest that InlA—E-cadherin interaction plays a role in crossing of the placental barrier. This is supported by several *in vitro* models like established trophoblastic cell lines (BeWo cells), primary trophoblast cultures (placentas were obtained immediately after a caesarean section delivery at the end an uncomplicated full-term pregnancy) or placental villous explants (from the same placentas from the cesarean section) (Bakardjiev *et al.,* 2004; Lecuit *et al.,* 2004). However, in an *in vivo* model such as a pregnant guinea pig, the spread of *L. monocytogenes* to the placenta was independent of InlA (Bakardjiev *et al.,* 2004). The same was seen in pregnant mice, where neither InlA nor InlB appeared to be of importance when invading the placenta and the fetus from infected pregnant mice (Le Monnier *et al.,* 2007). Possible explanations for the differences in the two model systems could be that in *in vivo* infections, the spread occurs via cell-to-cell spread from the infected blood cells to the placenta trophoblast, but in an *in vitro* model the invasion step, where InlA and InlB is of importance (Bakardjiev *et al.,* 2004). Both direct invasion of the endothelial cells and cell-to-cell spread from infected blood cells to endothelial cells has a role in the case of infection of the central nervous system (Drevets, 1999). Another explanation could be that invasion into trophoblast cells *in vivo* is mediated by other internalins. The role of all the internalins with homology to InlA in feto-placental invasion has not been identified yet. Even though the placenta itself and trophoblast cells from guinea pigs and humans are very similar (Leiser *et al.,* 1994) the conflicting results could also be due to small differences between these organisms.

Evidence for the importance of cell-to-cell spread required for crossing the feto-placental barrier in mice (Le Monnier *et al.,* 2007), but not for placental infection (Bakardjiev *et al.,* 2005) have been seen. Crossing the feto-placental barrier involves two layers of different cells. First the step from the maternal blood stream through the trophoblastic cells, and next through the layer of cells of the fetal blood vessel. An *actA*-deletion mutant is able to grow in the placenta, but with a slower growth rate compared to the wild type, and a significantly lower growth rate was seen in the fetus (Le Monnier *et al.,* 2007). A very high number of the *actA*-mutant bacteria was seen inside the trophoblastic cells as a result of the missing ability to spread to the neighboring cells (Bakardjiev *et al.,* 2005).

Listeriolysin O is important when *L. monocytogenes* is infecting placenta and fetus in mice and guinea pigs. A *hly*-deletion mutant behaved like *L. innocua*, meaning that they were rapidly eliminated from the maternal organs and were not able to infect the fetus (Bakardjiev *et al.,* 2005; Le Monnier *et al.,* 2007). The placenta could be infected, but the mutant was not able to grow in the placenta since it is not able to escape the phagosomes of the trophoblastic cells.

Thus, genes of importance for crossing the feto-placental barrier and infection of the fetus is still being investigated, but in *in vivo* models, *hly*, *actA* appear to be very important. In contrast, *inlA* and *inlB* are not of importance *in vivo*, probably because *L. monocytogenes* already is intracellular in the maternal blood cells and will thereby infect placenta and fetus by cell-to-cell spread.

Cautions should be made when comparing *in vitro* and *in vivo* placental-fetus infection studies. Also using different mammalian host organisms such as human, guinea pig or mice, both for *in vitro* and *in vivo* model systems, could give different results because of differences in receptor molecules or other interactions between bacteria and host.

However, further studies are needed for investigating the function of *inlA* in placental infection. We showed that a food processing persistent RAPD type 9 strains is slightly better to spread to placenta and fetus of pregnant guinea pigs, than a strain isolated from a primate stillbirth (Jensen *et al.*, 2007c). A human, clinical strain normally used as a reference strain was not able to infect placenta and fetus. The InlA from the RAPD type 9 strains contain two other amino acids than InlA from other strains, which is hypothesized to cause an misfolded InlA (Jensen *et al.*, 2007b). This incorrect folded InlA could result in improper infection of the maternal host, but could also cause very effective infection of placenta and fetus through E-cadherin or another receptor. However, other virulence factors like *actA* could also play a role in the increased infection ability of the RAPD type 9 strain. Therefore further studies with invasion into placenta cells (BeWo) and cell-to-cell spread in other cell lines are necessary.

5 Virulence models

Since *L. monocytogenes* was discovered in 1926 by Murray *et al.* and was seen as a human pathogen bacterium in 1929 by Nyfeldt, the bacterium has been studied intensively. In the beginning the bacterium was identified as the cause of human listeriosis, however its food borne transmission was not discovered until 1980s (Fleming *et al.,* 1985).

The virulence potential of *L. monocytogenes* or another bacterium can be defined as the ability of the bacterium to infect and even kill a host organism. It is important to understand the virulence potential of *L. monocytogenes* from a risk assessment perspective. For instance, it is important to know how many ingested bacteria are required to cause illness, or whether different sub-types of the bacteria differ in virulence potentials.

It is known that 20-30% of human infections caused by *L. monocytogenes* are lethal, which completely exclude the possibility of using human volunteers, and instead mice and guinea pigs have been used as animal models. These two animals were the original hosts of the infection by *L. monocytogenes* (Murray *et al.,* 1926). The increased focus on the use of experimental animals has made it necessary to develop new models to describe the virulence potential of *L. monocytogenes*. Examples of such models are the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. Interaction with eukaryotic cell lines can not describe the virulence potential, but are describing the single steps in the infection process e.g. adhesion of the bacterium to the eukaryotic cell surface, invasion into, intracellular growth and propagation in the cells. Other types of models include phenotypical tests and PCR-detection of virulence genes, and both methods act as secondary tests and do not describe the interaction between host defence mechanisms and bacteria.

It is difficult to define the best model, because it is depending on what part of the infection process that is of interest. If the interesting part is the function and activity of certain genes, the simple models like PCR-detection, phenotypic tests and cell line models are suitable. The more complex models; flies, worms, mice and guinea pigs should be used when interaction with the host defence system and a more complex host are needed.

The majority of the methods described in the following chapters have been used to describe the virulence potential of *L. monocytogenes* food processing persistent RAPD type 9 strains and compared to the virulence potential of other *L. monocytogenes* strains.

5.1 Phenotypic tests and PCR-detection of virulence genes

A number of phenotypic tests have been used to characterize the production of some of the virulence factors in *L. monocytogenes*. Listeriolysin O (LLO), encoded by *hly*, plays an important role in enabling *L. monocytogenes* to escape from the endosome when invading a host cell. The activity of LLO was previously characterized by measuring the diameter of a hemolytic zone on blood agar, but this method is difficult to interpret, and some strains produces very small zones. A micro plate technique, with erythrocyte suspensions, was developed for the routine determination of hemolytic activity (Rodriguez *et al.,* 1986). Larsen *et al.* (2006) used a modified version of the method for characterization of the response regulator ResD in *L. monocytogenes*. It is possible to describe the differences in hemolytic activity between a wild type strain and constructed mutant strains (Larsen *et al.*, 2006), however the method is not sensitive enough to differentiate between several wild type strains isolated from the indoor environment of fish producing factories, including food processing persistent RAPD type 9 strains and strains involved in human listeriosis (Figure 5.1) (results not published).

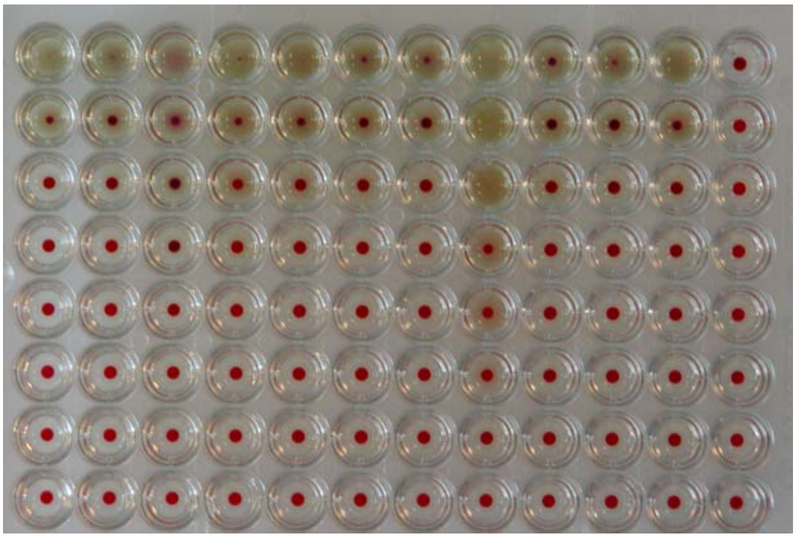

Figure 5.1: Activity of listeriolysin O tested by the microtiter plate assay. The supernatant from an overnight culture of each tested strain (columns) is 2-fold diluted (rows) and added to erythrocytes from cattle blood. The activity is determined by measuring to what dilution of supernatant hemolysis of erythrocytes is seen. Each column represents one strain. Last column is the negative control. Results not published.

We have used the EGDΔ*resD* mutant as in Larsen *et al.* (2006), and this mutant was very hemolytic (column 8). All the other strains had the same ability to lyse the erythrocytes and therefore the same hemolysin activity. The method is thus not sensitive enough to differentiate wild type strains.

When a *L. monocytogenes* cell has invaded a cell, the bacterium is surrounded by a eukaryotic cell membrane. To escape from this membrane, the PlcB phospholipase (*plcB*) enzyme is excreted (Mengaud *et al.*, 1991), and the production and activity of the enzymes is measured by egg yolk media assay (Coffey *et al.,* 1996). Without any addition of external factors like NaCl, temperature or pH, it is almost impossible to detect any clearing zones around the colonies on egg yolk agar, but production of PlcB phospholipase has shown to be optimal at 1.75-2.0% NaCl, pH 7.0-7.3 and 37-40°C (Coffey *et al.*, 1996), and it was possible to differentiate *L. monocytogenes* from other *Listeria* species. *L. monocytogenes* had an induced production of PlcB phospholipase after supplementation of charcoal to the medium (Ermolaeva *et al.,* 2003). It was not possible to differentiate the phospholipase activity of *L. monocytogenes* strains, even with addition of charcoal to the medium (results not shown and results not published).

Phenotypic testing of another virulence factor phosphatidylinositol-specific phospholipase C (PlcA) (*plcA*) is possible, but the test is not as easy to carry out and to visualize as the two tests previously described. The activity is also measured by spot inoculation onto agar but with an overlay of L-α-phosphatidylinositol substrate in which *L. monocytogenes* strains produce turbid halos (Notermans *et al.,* 1991). It is possible to differentiate *L. monocytogenes* and *L. ivanovii* from other *Listeria* species by using this method.

To test whether production of virulence-associated compounds is correlated to the virulence potential of *L. monocytogenes*, Chui *et al.* (2006) compared the production of these compounds to the presence of the genes (by PCR-detection), the hydrophobicity assays and invasion of the Caco-2 cell line. No correlation between behaviors in these models was found.

New methods like northern or western blot or DNA microarray have substituted the use of phenotypical tests. The phenotypical tests are being used as secondary measurements since their sensitivity is not that accurate, and it is not possible do differentiate between strains. Hence, in the present study we were not able to differentiate between the strains with respect to hemolytic activity or PlcB phospholipase activity. This could be due to either the low sensitivity of the methods or that the strains are similar in their hemolytic and phospholipase activity.

The presence of virulence genes in the genome of a bacteria strain can partly be correlated to the level of virulence, although the presence will not indicate if the gene is actually expressed. Also, genes containing single point mutations or genes with mutations in the regulator gene will by PCR-detection give the same results as the wild type genes. Therefore PCR-detection of virulence genes, to determine virulence potential, can only be used in combination with other methods. PCR-amplification and sequencing of three virulence genes (*hly*, *actA* and *inlA*) was shown to divide *L. monocytogenes* into three different lineages (lineage 1, 2 and 3) (Rasmussen *et al.*, 1995; Zhang *et al.*, 2003), and there is a connection between grouping of lineages and serotypes. This is also discussed in chapter 2.2.

5.2 Human cell line assay

L. monocytogenes is an intracellular pathogen bacterium and is characterized by is the ability to internalize cultured eukaryotic cells that are not normally phagocytic, like epithelial cells and intestinal cells, liver cells and placenta cells (Jaradat *et al.,* 2003b; Bakardjiev *et al.,* 2004; Lecuit *et al.,* 2004), and its ability to spread from cell to cell. The invasion requires the expression of surface proteins that are able to interact with the eukaryotic surface proteins and induce internalization.

A broad variety of human, mouse, guinea pig and monkey cell lines are used to describe the steps in the *L. monocytogenes* infection process. Different cell types are chosen, depending on what step of the infection is to be characterized. Adhesion to and crossing the intestinal barrier is often modeled using the human colon cell line Caco-2. Caco-2 cells originate from the colon of a 72 years old Caucasian male {317}. Also Int-407 (human, jejunum/ileum) and HT-29 (human, colon) are used as models for intestinal tissues (Roche *et al.,* 2001; Jaradat *et al.,* 2003b; Larsen *et al.,* 2006). Invasion and intracellular growth is necessary for the bacterium to infect a host. To study the invasion, intracellular growth and cell-to-cell spread a variety of cells are being used; Caco-2 cells (human, colon), HepG-2 cells (human, liver), Vero cells (monkey, kidney), CHO (hamster, ovary), HeLa cells (human, cervix) and BeWo cells (human, placenta) are used (Jaradat *et al.,* 2003b; Bakardjiev *et al.,* 2004; Lecuit *et al.,* 2004). All the mentioned cell types require active invasion of *L. monocytogenes*, whereas in J774.A1 cells (mouse, macrophage like) will actively uptake the *L. monocytogenes* cells. It may be difficult to conclude which factors, both from *L. monocytogenes* and the eukaryotic cells, is of importance for the intracellular life cycle. This is due to the inconsistency in the use of different cell lines from different animal origins,

We have used Caco-2 cells to test the ability of food processing persistent RAPD type 9 strains to adhere to, invade into and grow intracellularly in intestinal cells (Jensen *et al.,* 2007a; Jensen *et al.,* 2007b; Jensen *et al.,* 2007c). The adhesion into and intracellular growth in Caco-2 cells were similar for all the tested strains (Figure 4.3 and Figure 4.7). RAPD type 9 strains invaded Caco-2 cells in a significantly lower level than the other tested strains having different origins (Figure 4.5 and Figure 5.2). Since addition of 5% NaCl to the growth medium showed enhanced adhesion and aggregation, 5% NaCl was added to the bacterial growth medium to test whether this could influence adhesion, invasion and intracellular growth by *L. monocytogenes* (Figure 5.2).

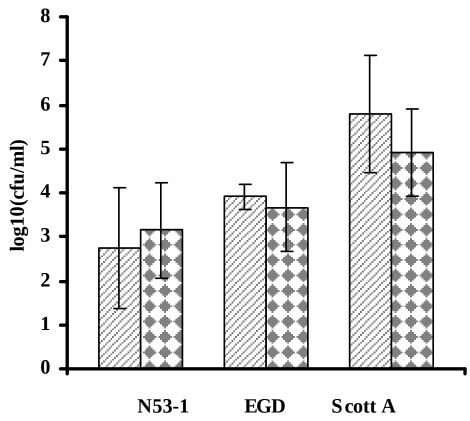

Figure 5.2: Invasion of *Listeria monocytogenes* RAPD type 9 (N53-1), reference strain (EGD) and a human, clinical strain (Scott A) into Caco-2 cells. Strains were grown in TSB (1% glucose) (▨) or TSB (1% glucose + 5% NaCl) (▩) before the assays. Error bars are based on standard deviations from two independent experiments in duplicate. Unpublished figure.

A RAPD type 9 strain invaded into to Caco-2 cells in a lower level than the two other strains and the addition of 5% NaCl did not change the degree of invasion into the Caco-2 cells (Figure 5.2). Neither was adhesion to Caco-2 cells influenced by addition of 5% NaCl to the growth medium (Figure 4.3).

Several surface proteins are present on the surface of the bacterium, and these interact with the eukaryotic surface protein to mediate the active uptake of the bacterium. The eukaryotic cells express specific proteins depending from which type of tissue they originate. Caco-2 cells were found to express E-cadherin to which InlA from *L. monocytogenes* can attach (Gaillard *et al.*, 1991). The interaction between the other surface protein InlB and receptors on the surface of *L. monocytogenes* is still not completely clear. InlB has shown to interact with three different proteins on the surface of the host cell – Met (Shen *et al.*, 2000), gC1q-R (Braun *et al.*, 2000) and glycosaminglycans (GAG) (Jonquieres *et al.*, 2001).

5.3 Non-mammalian model hosts

There is a need for development of infection models that are more complex than the phenotypic tests and the human cell assay, but less ethically controversial than experimental animals like guinea pigs and mice, because of the ethical considerations and research economy. The non-mammalian hosts are often easy to handle experimentally, and factors relevant for host-pathogen interaction can be analyzed. Also, it is relatively easy to genetically manipulate the hosts, and thereby identify host genes important for interaction with pathogen bacteria.

Often non-mammalian hosts have some limitations such as the way of infection, absence of receptor proteins on the surface of tissues important for infection, different immune defense or changed response on the disease. Other disadvantages with the simple methods are the absence of relevant pathways or a requirement for experimental condition, which is not optimal for the physiology of the pathogen bacteria.

5.3.1 *Caenorhabditis elegans* as a model for *Listeria monocytogenes* infection

The nematode *Caenorhabditis elegans* has been used to describe virulence in *Staphylococcus aureus* (Sifri *et al.,* 2003), *Pseudomonas aeruginosa* (Tan *et al.,* 1999) and *L. monocytogenes* (Thomsen *et al.,* 2006; Forrester *et al.,* 2007; Jensen *et al.,* 2007b) and several other both gram-negative and gram-positive bacteria with different infection foci (intestines, whole body, head or anal region) and with differences in the pathogenic effect in *C. elegans* (Gravato-Nobre *et al.,* 2005).

Many advantages by using the *C. elegans* as a model for infection can be listed (Gravato-Nobre *et al.*, 2005). These include low cost, simple maintenance, small laboratory space required, are easy to handle, and they are fed on a lawn of bacteria. Also the short life cycle, the self-fertilizing reproduction and the known genome sequence are benefits to this organism as a virulence model. It is possible to visualize the infection process, because of the transparency of the body of the nematode, when using bacteria that have been genetic modified to express fluorescent reporter genes (Tan *et al.,* 1999; Sifri *et al.,* 2003). Even though a lot of advantages can be listed, some disadvantages also need to be considered when using this model host. The worms cannot grow at 37°C, but this temperature is required for expression of virulence factors of some human pathogenic bacteria e.g. *L. monocytogenes*. Also, worms are able to self-fertilize, which can result in new progeny during the experiment, and this can interfere in a mortality experiment, when counting surviving worms. This problem has been solved by introduction of a temperature-sensitive sterile mutant *pha-1* (Schnabel *et al.,* 1990). This mutant grows normally at 15°C, but 15°C is embryonic lethal.

C. elegans is infected by feeding the worms on a lawn of bacteria grown on an agar plate. The mode of action for killing *C. elegans* by the different pathogenic bacteria differs. The killing of *C. elegans* by *P. aeruginosa* can happen in two different ways; either the bacteria produces small toxin molecule that kills the worms within a few hours (fast killing), or the worms die because of accumulation of bacteria in the intestines, which take several days (slow killing) (Tan *et al.*, 1999). Thomsen *et al.* (2006) introduced *C. elegans* as a virulence model for *L. monocytogenes* and showed different virulence genes are involved in *L. mono-*

cytogenes virulence against *C. elegans* i.e. the key regulator of *Listeria* virulence genes, PrfA, is essential for killing *C. elegans*. Another study (Forrester *et al.*, 2007) has used the same wild type and mutant strains as Thomsen *et al.* (2006), but the results did not correlate. Forrester *et al.* (2007) were not able to detect any differences between *L. monocytogenes*, the *prfA*-mutant and the negative control *E. coli* OP50. However, they did not use the temperature sterile mutant *pha-1*, but instead the wild type *C. elegans* N2, which may explain the disagreement.

The *C. elegans* model can group wild type strains of *L. monocytogenes* into different groups of mortality kinetics (Thomsen *et al.,* 2006; Jensen *et al.,* 2007b) (Figure 5.3)

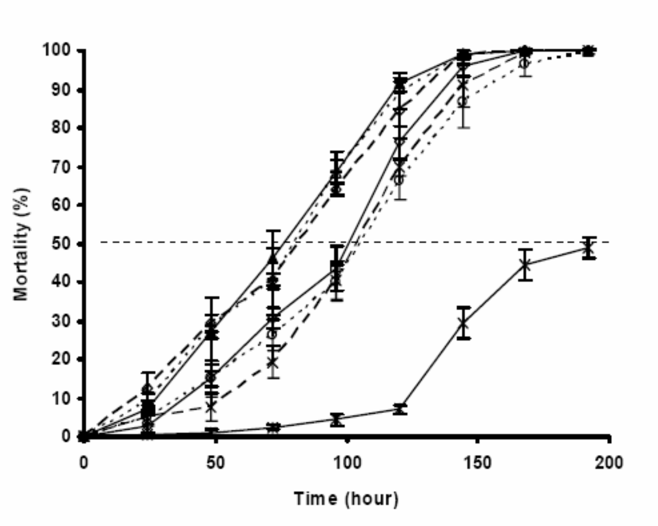

Figure 5.3: Mortality of *Caenorhabditis elegans* fed on different *Listeria monocytogenes* strains. *L. monocytogenes* strains and *E. coli* OP50 were grown in LB before giving to *C. elegans*. Strains are as followed: Food strain (−−◊−−), RAPD type 9 strain (−−×−−), human clinical strain (—Δ—), human, clinical strain (---—---), reference strain (—□—) and RAPD type 9 strain (---○---) and control strain (—*—). Error bars are based on standard deviations from three independent measurements. Reference: Jensen *et al.* (2007b).

The strains were divided into two different groups, when measuring the time for the *L. monocytogenes* strains to cause 50% mortality in *C. elegans* worms. The two RAPD type 9 strains and a reference strain were in the group with slower killing strains, when compared to the group containing two human clinical strains and the food strain. The division of the strains was the same as was seen for invasion assay into Caco-2 cells, for the *Drosophila melanogaster* mortality assay and also in fecal shedding of one RAPD type 9 strain and one human clinical strains (Jensen *et al.*, 2007b).

Thomsen *et al.* (2006) found that *L. monocytogenes* accumulates in the intestines of the *C. elegans* worms, and that an *actA* mutant strain, which is unable to spread between cells, kills the *C. elegans* as a wild type strain. Therefore, the lower ability of the RAPD type 9 strains to invade eukaryotic cells is unlikely to have an influence on the mortality of *C. elegans* worms. In spite of this, we see the same strain differentiation in the two virulence models. Therefore, other virulence factors than InlA must be involved in the virulence of the *L. monocytogenes* in *C. elegans* worms.

5.3.2 *Drosophila melanogaster* as a model for *Listeria monocytogenes* infection

The fruit fly, *Drosophila melanogaster*, has been used to study the principle of genetics and heritable characters such as eye color and size of the wing. *D. melanogaster* has also been used as a virulence model for several bacterial human pathogens like *Pseudomonas aeruginosa* (D'Argenio *et al.,* 2001) and *Staphylococcus aureus* (Needham *et al.,* 2004) and *L. monocytogenes* (Mansfield *et al.,* 2003; Jensen *et al.,* 2007b; Jensen *et al.,* 2007d).

It is similar to *C. elegans* in being easy to handle and low laboratory expenses. But also this virulence model has some disadvantages. The most common route is injection in the thorax which could be a problem for e.g. oral infections (Mansfield *et al.,* 2003; Jensen *et al.,* 2007b; Jensen *et al.,* 2007d). Injections of bacteria and parasites in abdomen or hemocoel have also been seen (Schneider *et al.,* 2000; Brandt *et al.,* 2004). Oral infection have been tried with the parasite *Plasmodium gallinaceum*, a close relative of the human malaria parasite, but the flies were cleared (Schneider *et al.,* 2000). When injected with the parasite in the hemocoel, the flies became infected, which lead to the conclusion, that the parasite was not able to cross the gut-barrier.

Mansfield *et al.* (2003) developed the *D. melanogaster* model to study the host-pathogen interaction between *D. melanogaster* and *L. monocytogenes*. The route of infection with *L. monocytogenes* is injection of an overnight culture into the flies instead of the natural oral route which includes adhesion and invasion to the intestinal cells. Virulence genes important for infection in a human host were also necessary when infecting *D. melanogaster*, and the fruit fly has the potential to serve as a human comparable host for *L. monocytogenes* infection (Mansfield *et al.*, 2003). Recently, Jensen *et al.* (Jensen *et al.*, 2007d) found limitations in the use of *D. melanogaster* as a model host for gram-positive bacterial infection was observed. Several gram-positive human non-pathogen bacteria caused killing of the flies. Also the non-virulent *Listeria innocua* caused death with the same rate as the human clinical *L. monocytogenes* strain Scott A (Jensen *et al.*, 2007b; Jensen *et al.*, 2007d). *L. innocua* may not be an appropriate species to use as negative control, even though the genome of *L. innocua* does not contain the cluster of virulence genes that are regulated by PrfA (Glaser *et* *al.,* 2001). Therefore other genes than the PrfA-regulated genes have an influence on the virulence of *L. innocua*.

We were the first to compare the virulence potential for several wild type strains of *L. monocytogenes* in *D. melanogaster* (Jensen *et al.,* 2007b) (Figure 5.4).

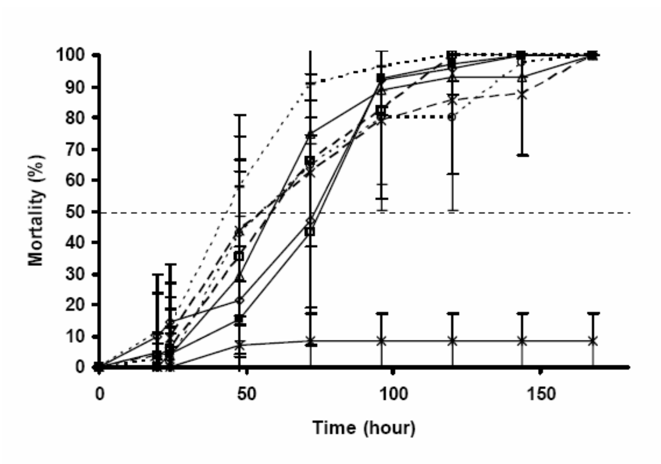

Figure 5.4: Mortality of *Drosophila melanogaster* injected with strains of *Listeria monocytogenes*, *Listeria innocua* and *Escherichia coli* OP50. *Listeria* strains were grown in BHI before injection and *E. coli* was grown in LB. Strains are as followed: Food strain (−−◊−−), RAPD type 9 strain (−−×−−), human clinical strain (—Δ—), human, clinical strain (---——---), reference strain (—□—) and RAPD type 9 strain (---○---) and *E. coli* OP50 (—*—), *L. innocua* (—◊—). Error bars are based on standard deviations from four independent experiments. Reference: Jensen *et al.* (2007b).

At different periods of time 50% mortality was reached. The two human clinical strains were the most efficient killers, and the reference strain showed to be less efficient. The group in between these two groups contained two RAPD type 9 strains and a food strain. *L. innocua* killed the flies with a similar pattern as the reference strain as shown by Jensen *et al.* (2007d) . Our data show that a part of the fruit fly model correlates with the other virulence models such as invasion into caco-2 cells, killing of *C. elegans*, but more work is needed on the *D. melanogaster* model to investigate the lethal action of presumable non-pathogenic bacteria.

5.4 Mammalian hosts

Earlier, mammalian hosts were widely used when studying *L. monocytogenes* infection. The non-mammalian hosts have been developed because of the large amount of experimental work required and a stricter regulation on use of experimental animals. Even though non-mammalian organisms are widely used, a model closer to the human host is still needed to minimize the number of factors that could be different between the human host and the model host. Mammalian hosts are also necessary when *L. monocytogenes* infection of fetuses in the host is described.

5.4.1 Infection methods of the mammalian hosts

Different mammalian hosts have been used to study virulence of *L. monocytogenes* (Gray *et al.,* 1966), included mice, guinea pigs and monkeys. The animals have been injected intraperitoneally (i.p.), intravenously (i.v.), or intragastric or exposed to an oral injection. The exposure methods have turned to orally feeding of the experimental animals instead of the injection methods, as it was discovered that *L. monocytogenes* was a food borne pathogen in the 1980s (Farber *et al.,* 1991; Lecuit *et al.,* 2001; Smith *et al.,* 2003; Kim *et al.,* 2004; Williams *et al.,* 2007; Andersen *et al.,* 2007; Jensen *et al.,* 2007b; Jensen *et al.,* 2007c).

The preferred model host was mice until 1999, where Lecuit *et al.* (1999) discovered that the E-cadherin receptor from mice differed from that of humans/guinea pigs. E-cadherin is crucial for *L. monocytogenes* infection when the host is orally exposed to the organism (Mengaud *et al.,* 1996). This discovery led to optimization of oral exposure of pregnant guinea pigs, and also a pregnant monkey model has been developed (Smith *et al.,* 2003; Williams *et al.,* 2007), even though the monkey model previously was introduced by Farber *et al.* (1991).

Whether mice are the suitable model for i.p., i.v. or intragastric injection still needs to be demonstrated. The role of InlA and InlB in the uptake in other cells types than epithelial intestinal cells is still studied. Since *L. monocytogenes* is a food borne pathogen, the true route of infection is oral, and the best suited models are either guinea pigs or transgenic mouse expressing human-E-cadherin on their intestinal cells (Lecuit *et al.,* 2001; Williams *et al.,* 2007) (Figure 5.1).

Table 5.1: Several ways are used for infection of mice and guinea pigs with *Listeria monocytogenes*. Intraperitoneal, intravenous and intragastric are often used for mice and guinea pigs, whereas oral feeding only is suitable for guinea pigs.

	Intraperitoneal/intravenous/intragastric	Oral
Mice	The inoculum do not passages through intestines where the low-affinity between E-cadherin and InlA is crucial. The bacteria might be able to use the surface protein InlB to bind with high-affinity to receptors on the surface of organs and invade the organs.	The inoculum passages in the intestines where the low-affinity between E-cadherin and InlA is notable and the bacteria will not cross the intestinal barrier in a high level.
Guinea pigs	The inoculum do not passages through intestines where the high-affinity between E-cadherin and InlA is notable. The bacteria might not be able to use the surface protein InlB to bind to receptors on the surface of organs because of the low-affinity to receptors on the surface of organs and invade the organs.	The *L. monocytogenes* cells will invade the intestinal cells and spread intracellular in the host because of the high-affinity between E-cadherin and InlA is present. The bacteria might not be able to use the surface protein InlB to bind to receptors on the surface of organs because of the low-affinity to receptors on the surface of organs and invade the organs.

5.4.2 Mice and transgenic mice as a model for *Listeria monocytogenes* infection

Mice have been used as experimental animals for several decades, and are preferred because they are easy to handle, low costs (compared to other mammalian experimental animals) and they reproduce easily. Earlier, mice were used as host for *L. monocytogenes* infection, because *L. monocytogenes* was discovered in mice (Murray *et al.*, 1926).

The mouse model has been used to describe the virulence of different *L. monocytogenes* strains (Lammerding *et al.,* 1992; Takeuchi *et al.,* 2006), to describe the importance of prfA in virulence (Chakraborty *et al.,* 1992) or to describe effect and action of different virulence genes (Borezee *et al.,* 2001; Cabanes *et al.,* 2004; Khelef *et al.,* 2006; Le Monnier *et al.,* 2007). Some of the studies are from after 1999, where mutations in E-cadherin from mice when compared to human/guinea pig E-cadherin were discovered but the majority of the studies have used intravenous or intraperitoneal injection. The discovery led to optimization and development of other virulence models, but research groups are still using the mouse as a virulence model for intravenously injections. In 2001, Lecuit *et al.* generated a transgenic mouse model. The mice did express human E-cadherin on their intestinal cells and it was therefore possible to use this model for oral infection of the mice. The disadvantage of this model is that only the intestinal cells are expressing human E-cadherin, and that other cell types in the mouse, where InlA—E-cadherin is of importance, do not express human E-cadherin. The use of the model is limited since few publications has been published (Lecuit *et al.,* 2001; Khelef *et al.,* 2006).

5.4.3 Pregnant guinea pig as a model for *Listeria monocytogenes* infection

Guinea pigs have been used as models for *L. monocytogenes* infection since the 1970's. Gray and Killinger (1966) suggested rabbits as the most ideal animal, whereas mice varied in susceptibility among the various genetic strains. The guinea pig was not suggested as a model since it appeared to be less susceptible than the rabbit and mouse. However, in 1977, the guinea pigs were used to describe how the infection spread in the host organism (Dustoor *et al.,* 1977). In earlier studies with guinea pigs, the animals were infected intravenously, intracardially or intraperitoneally, but neither reflect the natural oral infection route (Dustoor *et al.,* 1977; Bakardjiev *et al.,* 2004; Garner *et al.,* 2006). Recently, a method of orally feeding guinea pigs with *L. monocytogenes* has been developed by Williams *et al.* (2007), where the guinea pigs are fed with a mixture of sterilized whipping cream and *L. monocytogenes*. The disadvantage of the oral feeding is that the bacterial dose has to be high (two following days with 10^{10} CFU) to get countable *L. monocytogenes* in feces and tissues (Andersen *et al.*, 2007; Jensen *et al.*, 2007b). It is not possible to get any bacterial counts from the maternal and fetal tissue samples and the majority of feces samples if the level is lower (10^{8} CFU on one day) (Williams *et al.,* 2007; Jensen *et al.,* 2007c). Feeding of guinea pigs have been optimized (Williams *et al.*, 2007) and this animal model has now been used to assess virulence potentials between strains (Jensen *et al.,* 2007b; Jensen *et al.,* 2007c), influence of pre-grow conditions (Andersen *et al.*, 2007; Jensen *et al.*, 2007b) and the lethal dose of *L. monocytogenes* (Williams *et al.*, 2007). Several benefits can be listed for the use of guinea pigs as model hosts. They are natural sensitive to *L. monocytogenes* infection (Dustoor *et al.,* 1977; Bakardjiev *et al.,* 2004; Williams *et al.,* 2007; Andersen *et al.,* 2007; Jensen *et al.,* 2007b; Jensen *et al.,* 2007c), they express the intestinal surface protein E-cadherin similar as the human E-cadherin (Lecuit *et al.*, 1999) and they have a hemochorial placenta, which means that fetally derived cells called trophoblasts invade the uterus and are in direct contact with maternal blood (Bakardjiev *et al.,* 2004). Of all the rodent placentas, guinea pigs placenta resembles the human placenta most closely (Leiser *et al.,* 1994).

Even though the guinea pig is selected as one of the most appropriate models for the infection of humans with *L. monocytogenes*, there are still some disadvantages. InlB is not able to recognize or activate the guinea pig or rabbit cells, since the amino acid sequence of its receptor, Met, is different from the human/mouse Met receptor (Figure 5.5) (Khelef *et al.*, 2006).

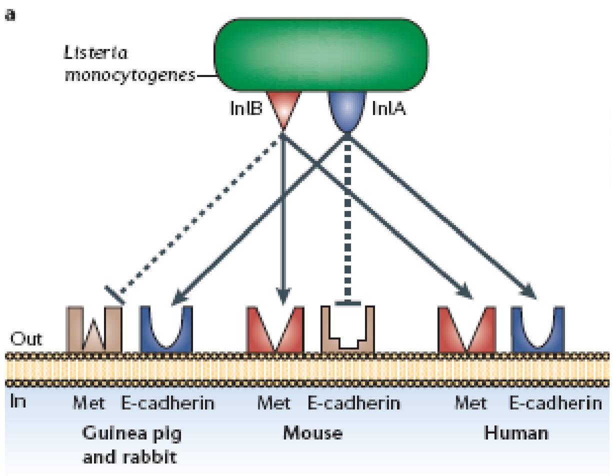

Figure 5.5: Host specificity of *Listeria monocytogenes* proteins internalin A (InlA) and InlB. InlA is able to bind to the surface protein, E-cadherin, on the human intestinal cells and on guinea pig and rabbit intestinal cells. The E-cadherin from mice is not compatible with InlA. The opposite is seen for InlB, where InlB is able to bind to the Met-receptor from mice and humans, but not from guinea pigs and rabbits. Reference: Hamon *et al.* (2006).

Fecal shedding of *L. monocytogenes* have been used as an indicator for infection in pregnant guinea pigs and monkeys (Smith *et al.,* 2003; Williams *et al.,* 2007), because a positive correlation was seen between the numbers of *L. monocytogenes* in feces and the birth outcome in the pregnant animals. However, when using a food processing persistent RAPD type 9 strain with hypothesized incorrect folded InlA, a lot of bacteria were shed in the beginning of the period and after a short time the fecal samples did not contain any *L. monocytogenes* (Jensen *et al.,* 2007b; Jensen *et al.,* 2007c) (Figure 5.6). We believe that the RAPD type 9 strains are not able to colonize the intestinal cells and are therefore almost washed out of the intestines. The human clinical strain was in both studies able to colonize the intestines and was isolated from fecal samples throughout the study (Jensen *et al.,* 2007b; Jensen *et al.,* 2007c) (Figure 5.6). Addition of 5% NaCl to the growth medium was shown to enhance adhesion and aggregation to a plastic surface. Fecal shedding of *L. monocytogenes* from non-pregnant guinea pigs was not influenced by addition of 5% NaCl to the bacterial growth medium before infection of the guinea pigs. Also, 5% NaCl in the bacterial growth medium did not change the virulence potential in adhesion to and invasion into Caco-2 cells and the mortality of *D. melanogaster*. However, oxygen restriction of *L. monocytogenes* has shown to increase the infective potential of *L. monocytogenes* against guinea pigs (Andersen *et al.,* 2007).

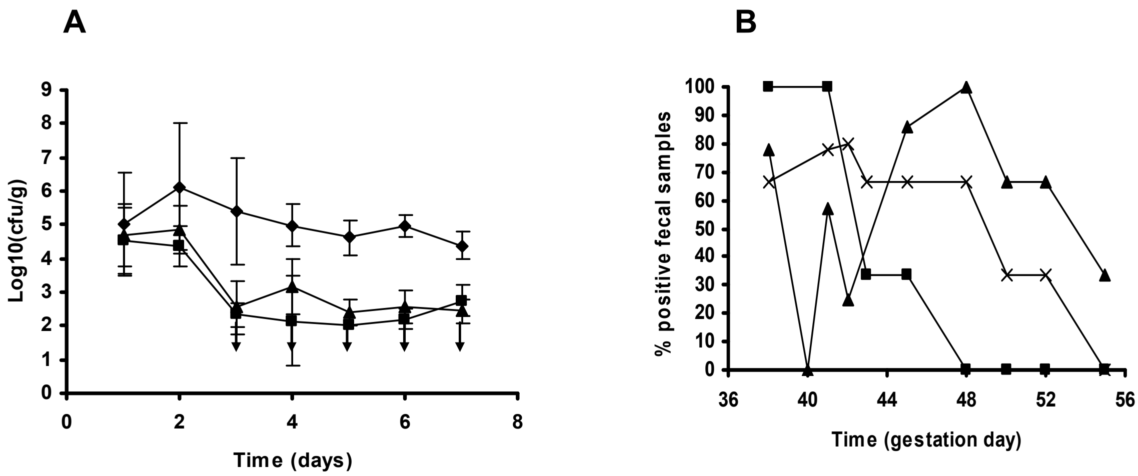

Figure 5.6: Guinea pigs shedding *Listeria monocytogenes*. (A): The human clinical strain Scott A (♦) and the food processing persistent RAPD type 9 strain N53-1 grown with 5% NaCl (▲) or without NaCl (■) shed from non-pregnant guinea pigs. Reference: Jensen *et al.* (2007b). (B): The human clinical strain Scott A (▲), the monkey clinical strain 12443 (×) and the food processing persistent RAPD type 9 strain La111 (■) shed from pregnant guinea pigs. Reference: Jensen *et al.* (2007c).

Even though InlA from the food processing persistent RAPD type 9 strain is hypothesized to be incorrectly folded and therefore not able to attach effectively to the intestines of a pregnant guinea pig, the strain was still able to spread to the liver, spleen and gall bladder of the guinea pigs, although not to the same level as the monkey, clinical strain (Figure 5.7).

The food processing persistent strain did not spread to maternal organs as fast as the two clinical strains did, since none of the maternal organs contained *L. monocytogenes* at gestation day 42 (GD 42). *L. monocytogenes* have been detected in the stomach of orally infected mice just after the treatment, but from day 1 to day 3 post-treatment it was not possible to detect *L. monocytogenes* from any of the organs in the mice (Hardy *et al.,* 2004). *L. monocytogenes* was detected in the gall bladder of the mice on the following days, and it was suggested that *L. monocytogenes* may be carried in the human gall bladder. In our study, we could also detect *L. monocytogenes* in the gall bladder although our results indicate that the liver is the primary site of infection, since almost all livers were infected with *L. monocytogenes*.

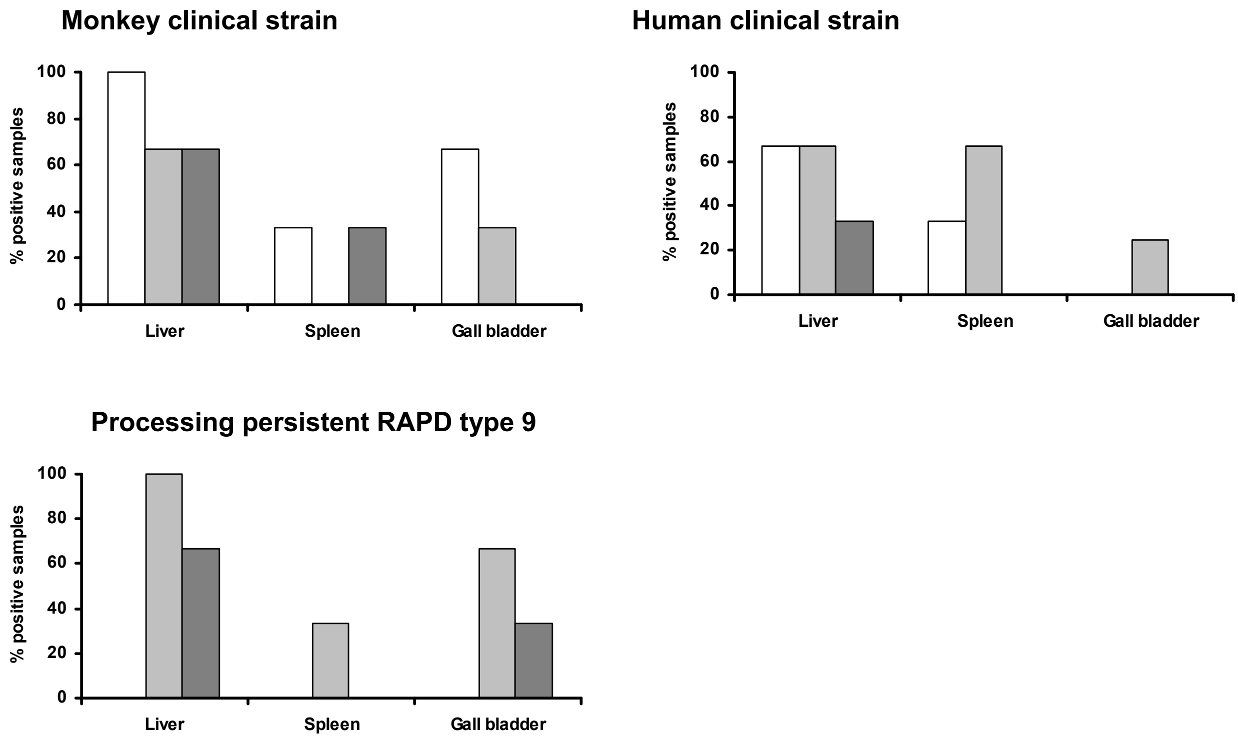

Figure 5.7: Percent of guinea pigs positive for *Listeria monocytogenes* strains in maternal liver, spleen and gall bladder at days of sacrifice. Gestation day 42 (□), gestation day 45 (■) and gestation day 56 (■), corresponding to 6, 9, and 21 days post-treatment. Each column is an average of 3 animals. Reference: Jensen *et al.* (2007c).

Surprisingly, we were able to detect *L. monocytogenes* from placentas originating from animals dosed with the RAPD type 9 strain at gestation day GD 45 (Table 5.2), which was not the case for animals dosed with the monkey clinical strain. The invasion of the placenta occurs early after the injection and allows growth of bacteria in the placenta, when *L. monocytogenes* is infected intravenously, (Le Monnier *et al.,* 2006). It can be hypothesized that the food processing persistent strain possesses some special characteristics that enhances its ability to cause placenta infection.

Also, a higher number of guinea pigs carried an infected fetus when dosed with the RAPD type 9 strain compared to the monkey clinical strain. Interestingly, the human clinical strain was not able to infect the placentas and thereby any fetuses, even though it was detected in maternal organs in just as high levels as the monkey clinical strain.

Table 5.2: Fetal infection after maternal oral exposure to *Listeria monocytogenes*. Monkey clinical strain (12443), food processing persistent strain RAPD type 9 strain (La111), human clinical strain (Scott A). Gestation day 42, gestation day 45 and gestation day 56, corresponding to 6, 9, and 21 days post-treatment. Reference: Jensen *et al.* (2007c).

Strain	No. guinea pigs with infected fetus [A] / total (%)	No. infected fetuses / total (%)	No. infected samples / total samples (%)		
			Placenta	Fetal liver	Fetal brain
12443	2/9 (22%)	4/31 (13%)	5/31 (16%)	4/31 (13%)	1/31 (3%)
GD 42	0/3 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
GD 45	0/3 (0%)	0/14 (0%)	0/14 (0%)	0/14 (0%)	0/14 (0%)
GD 56	2/3 (67%)	4/9 (44%)	5/9 (56%)	4/9 (44%)	1/9 (11%)
La111	5/9 (56%)	6/49 (12%)	10/49 (20%)	6/49 (12%)	0/49 (0%)
GD 42	0/3 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
GD 45	3/3 (100%)	4/20 (20%)	5/20 (25%)	4/20 (20%)	0/20 (0%)
GD 56	2/3 (67%)	2/14 (14%)	5/14 (36%)	2/14 (14%)	0/14 (0%)
Scott A [B]	0/9 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)

[A] An infected fetus is a fetus that had either an infected liver or brain.
[B] No placentas or fetuses from dams treated with Scott A were positive for *L. monocytogenes*.

The human clinical strain is able to infect the guinea pigs, both pregnant and non-pregnant, but was not able to cross the feto-maternal barrier. The monkey clinical strain is able to infect both non-pregnant and pregnant guinea pigs, and also the placentas and unborn fetuses. The food processing persistent strains are able to infect both non-pregnant and pregnant guinea pigs but to a smaller degree when compared to the other two strains. However, the placentas and unborn fetuses were infected to the same level as the monkey clinical strain did. Hence, the different strains can encode for different virulence potential against different hosts. The reason for this difference in virulence potential between the strains and between the hosts is unknown but needs to be investigated further.

5.5 Conclusion

The present chapter has outlined a range of models used to study the virulence of *L. monocytogenes* infection and virulence factors. Neither of the models studied is an ideal reflection of the human infection, since different results were reached from the different models.

A correlation was seen between invasion into Caco-2 cells, time to kill 50% *D. melanogaster* and *C. elegans* and the content of *L. monocytogenes* in fecal shedding of both non-pregnant and pregnant guinea pigs. Food processing persistent RAPD type 9 strains showed to be less virulent in these models when compared to human clinical strains. Surprisingly, a RAPD type 9 strain was infecting placenta and fetus to the same level as the monkey clinical strain. Because of these results one should be cautious when drawing conclusions on the degree of virulence, when using the simple models.

6 Concluding remarks

Many types of bacteria are capable of “colonizing” food processing plants and may reside for many years. However, in recent years focus has been directed to persistence of bacteria causing spoilage or diseases, such as *Listeria monocytogenes* in food processing industry.

L. monocytogenes is able to colonize equipment surfaces and indoor environment in food processing plants. A sub-type is defined as persistent when the same DNA-sub-type can be isolated repeatedly in the same plant, and even the same places inside the processing plant, over a longer period of time. Recently, one group of genetically similar strains (RAPD type 9) was identified as persistent in several Danish fish slaughter- and smokehouses. The reason for this persistence is not known, but several hypotheses have been suggested. From a risk analysis perspective, it is important to assess the virulence potential of strains that are likely contaminants of food products, such as strains persisting in the food processing environment.

In this thesis it was hypothesized that persistence of RAPD type 9 strains may be caused by higher or lower growth rate, or by increased adhesion to surfaces as compared to sporadic isolated strains of *L. monocytogenes*. The growth pattern of RAPD type 9 strains did not differ from that of other strains, and therefore a different growth pattern cannot explain persistence. All strains were able to adhere to a plastic surface, and a difference in the adhesion ability was seen between the strains. However, the food processing persistent RAPD type 9 strains did not adhere to a higher level than the sporadic isolated strains. Interestingly, addition of 5% NaCl to the growth medium enhanced the adhesion ability of several of the strains, and also caused formation of aggregates. The enhanced adhesion and aggregation was not only seen for RAPD type 9 strains, but also for sporadic isolated strains, human clinical strain and strains isolated from food. Addition of moderate levels of NaCl did enhance the adhesion, but is not the only reason for persistence, therefore other factors may influence the adhesion of *L. monocytogenes* to surfaces.

The exposure of *L. monocytogenes* to dehydration, cleaning or disinfection agents may also facilitate the persistence of specific DNA-sub-types of *L. monocytogenes* in a food processing plant. Further, the presence of co-cultures with *Pseudomonas* spp. or *Staphylococcus* spp. could enhance the ability to persist in the food processing environment. Further studies on these aspects are needed for the explanation for the presence of persistent *L. monocytogenes* DNA-sub-types in the food processing environment. A method for this could be full-genome sequencing of a food processing RAPD type 9 strain. By comparative genomics compare the sequences from the reference strain EGD, that showed a complete different adhesion pattern to a plastic surface when 5% NaCl was added to the growth medium.

In this thesis, also the virulence potential of food processing persistent RAPD type 9 strains was assessed. Further, we wanted to determine if the ethical controversial mammalian animal models could be substituted by more simple non-mammalian animal models or cell lines. The virulence potential of RAPD type 9 strains differed with respect to the used model. The invasion of *L. monocytogenes* into an intestinal epithelial cell is the first step in infection of a human host. RAPD type 9 strains invaded the intestinal cell line, Caco-2, in a significantly lower level than the human clinical strains. An explanation could be the presence of two single point mutations in the *L. monocytogenes* surface protein InlA, which is responsible for the invasion into intestinal cells. The folding of InlA might be incorrect, because of these mutations and thereby lead to lower affinity to the E-cadherin receptor molecule on the Caco-2 cells. Further studies are needed, since other research groups have identified nonsense mutations in InlA leading to lower invasion ability.

To study the virulence potential of RAPD type 9 strains, we have also used the two newly introduced virulence models, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. The strains separated into different groups regarding the time to kill 50% of the animals, where RAPD type 9 strains took a longer time to kill 50% of the animals than human clinical strains of *L. monocytogenes*. However, the *D. melanogaster* model may be inappropriate since human non-pathogenic bacteria are also able to kill the flies. More work is needed to investigate the killing ability of non-pathogenic bacteria, if *D. melanogaster* should be used as a virulence model.

A correlation was seen between invasion into Caco-2 cells, the time to kill 50% of *C. elegans* and the fecal shedding of *L. monocytogenes* in guinea pigs. RAPD type 9 strain were not able to colonize the intestines of both non-pregnant and pregnant guinea pigs, and also RAPD type 9 strains spread to organs with a lower rate when compared to a human, and a monkey clinical strain. Surprisingly, a RAPD type 9 strain was able to spread to placenta and cross the feto-maternal barrier in the same level as a monkey clinical strain. The reason for this high affinity for placental and fetal tissues is not known an further studies is important. The ability of *L. monocytogenes* to cross the feto-maternal barrier is still a newly investigated area and not all factors implicated in the infection are identified.

Because of this inconsistency in the results regarding the virulence potential of RAPD type 9 strain, the simple models to measure the virulence potential cannot be used, and one should be cautious when drawing conclusions on the degree of virulence when using only simple models.

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Paper 1

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Sodium Chloride Enhances Adherence and Aggregation and Strain Variation Influences Invasiveness of *Listeria monocytogenes* Strains

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ABSTRACT

Some subtypes of *Listeria monocytogenes* can persist in the food-processing industry, but the reasons for such persistence are not known. In the present study, 10 strains of *L. monocytogenes* representing known persistent randomly amplified polymorphic DNA (RAPD) types from fish processing plants were compared to eight strains of different RAPD type and origin (clinical, food, and animal). All 18 strains of *L. monocytogenes* had similar growth patterns at different temperatures (5 or 37°C) or different salinities (0.5 or 5% NaCl), and all strains formed a thin layer of adhered cells on a plastic surface when cultured in tryptone soya broth (TSB) with a total of 1% glucose. Many ready-to-eat foods, such as cold-smoked fish, contain NaCl at concentrations of 2 to 5%, and NaCl is present in the processing environment. Adding NaCl to TSB changed the adhesion patterns of all strains, and all adhered better when NaCl was added. Also, the addition of NaCl caused a marked aggregation of 13 of the strains; however, 5 of the 18 strains did not aggregate in the presence of up to 5% NaCl. The aggregates stuck to the plastic surface, and this occurred in all but one of the persistent RAPD types. Four strains represented one particular RAPD type that has been isolated as a persistent RAPD type in several fish processing plants for up to 10 years. Because this RAPD type often can contaminate fish products, it is important to address its potential virulence. The 18 strains differed markedly in their ability to invade Caco-2 cells, and the four strains representing the universal persistent RAPD type were the least invasive (10^2 to 10^3 CFU/ml), whereas other strains invaded Caco-2 cells at levels of 10^4 to 10^5 CFU/ml. Five of the 18 strains belonged to the genetic lineage 1 and were the most invasive. Although the most commonly isolated persistent RAPD type was low invasive, it is important to understand why moderate salinity facilitates aggregation and biofilm formation, for this understanding can be beneficial in developing procedures to reduce processing plant contamination.

Listeria monocytogenes can cause human listeriosis (meningitis, septicemia), which is a rare foodborne disease with a high fatality rate of approximately 25 to 30% *(10)*. Often, ready-to-eat food products with extended shelf lives have been vehicles of the infection *(37, 40),* and the contamination of these foods typically occurs in the processing environment *(2, 13, 28, 38)*. Some *L. monocytogenes* strains can persist in food processing plants over many years *(13, 31, 43)*; for instance, several fish processing plants appear to have their own ‘‘in-house’’ *L. monocytogenes* population *(2, 13, 31, 39, 43)*. We recently characterized 231 *L. monocytogenes* strains isolated from four fish slaughterhouses and four fish smokehouses *(14, 43)*. Eighty-six of the strains belonged to one particular randomly amplified polymorphic DNA (RAPD) type (RAPD type 9) that was isolated in five of the plants and was the dominant, persistent RAPD type in three plants. This RAPD type has been isolated from several other fish smokehouses as far back as 1996 *(14, 43)*. These data indicate that certain subtypes of *L. monocytogenes* may be specifically adapted to persistence, and understanding the genetic and physiological factors determining persistence would be crucial in reducing contamination, e.g., by allowing development of targeted cleaning and disinfection procedures. Also, it would be important from a risk-assessment perspective to determine if such persistent isolates that often contaminated food products are more or less virulent than the average of *L. monocytogenes* isolates.

One could hypothesize that such dominant persistent subtypes simply were the most prevalent in the outdoor environment; however, *L. monocytogenes* strains isolated from water, fish, or soil do not cluster in the same RAPD groups as the factory isolates *(16)*.

It has been suggested that differences in adherence ability could explain the ability of some strains of *L. monoogenes* to persist *(33)*. *L. monocytogenes* does adhere to stainless steel, plastic, or rubber surfaces *(4, 5, 7, 9, 24, 33)*. However, some studies have concluded that persistent strains of *L. monocytogenes* adhere better to stainless steel surfaces than nonpersistent strains *(5, 34),* whereas others have found no relationship between environmental persistence of strains and biofilm formation *(9)*.

Persistent strains could also be more tolerant to cleaning and disinfection procedures, and to our knowledge, only one study *(1)* has investigated the relationship between persistence and tolerance to cleaning or disinfection agents. Aase et al. *(1)* found that 50% of *L. monocytogenes* strains

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TABLE 1. *Origin, serotype, and lineage of strains of* Listeria monocytogenes *used in the present study*[a]

Strain	Origin	Serotype	Lineage	RAPD type	Frequently occurring, persistent RAPD type in processing plants	% NaCl causing: Increased adherence	% NaCl causing: Aggregation	Reference
R479a	Cold-smoked salmon	1/2a	2	2	+	2–5	NP	*13*
6895	Ham	1/2a	2	6	+	2–4	NP	*25*
N53-1	Smokehouse equipment	1/2a	2	9	+	2–5	2–5	*43*
H13-1	Smokehouse equipment	1/2a	2	9	+	2–5	2–5	*43*
La111	Cold-smoked salmon	1/2a	2	9	+	2–5	2–5	*14*
M103-1	Slaughterhouse equipment	1/2a	2	9	+	2–5	3–5	*43*
La22	Cold-smoked salmon	1/2a	2	12	+	2–5	2–5	*13*
7418	Spreadable sausage	1/2b	1	14	−	2–4	3–4	*25*
V518a	Smokehouse equipment	4b	1	15	+	2–5	2–5	*13*
7291	Pasta with chicken	4b	1	15	+	2–5	2–5	*25*
4459	Human, clinical	1/2a	2	22	−	2–5	NP	*25*
4239	Human, clinical	1/2a	2	27	−	2–4	NP	*25*
No40-1	Smokehouse equipment	1/2a	2	57	+	2–5	2–5	43
O57	Gravad salmon	1/2a	2	67	−	2–5	2–5	*3*
EGD	Rabbit, 1926	1/2a	2	68	−	2–4	NP	W. Goebel[b]
LO28	Human, fecal	1/2c	2	69	−	2–5	3–4	*40*
4666	Human, clinical	1/2b	1	70	−	2–4	3	*25*
4446	Human, clinical	4b	1	71	−	2–5	3–5	*25*

[a] +, the RAPD type was frequently occurring and persistent; −, the RAPD type was not frequently occurring and persistent; NP, aggregation was not present.

[b] The strain was kindly provided by Werner Goebel, University of Würzburg.

isolated from the production environment, raw products, and finished products were resistant to benzalkonium chloride. Interestingly, strains that were benzalkonium chloride resistant and from the Norwegian fish processing plant were all persistent, indicating that such resistance could be a factor correlated to persistence.

Persistent strains are, logically, common contaminants of food products, and from a food safety perspective, it is important to know if such strains are more or less pathogenic than nonpersistent strains. Assessing virulence of *L. monocytogenes* requires expensive animal models, but some steps in the infectious process, such as invasiveness or cell-to-cell spread, can be measured in model systems *(8, 15, 18, 25, 32).* Persistent strains from a smoked-fish industry or strains isolated from foods appear to have a lower ability to form plaques in a cell-to-cell spread plaque assay as compared with epidemic strains and clinical strains *(15, 32).*

The purpose of the present study was to determine if strains of *L. monocytogenes* that are repeatedly isolated from fish processing environments in Denmark differ from other strains in phenotypic characteristics that could explain their ability to persist. To this end, we investigated their growth under food-relevant stress conditions (such as low temperature and NaCl) and their adhesion to surfaces. We used laboratory substrates but added NaCl to mimic the water activity values typically found in many delicatessen-type ready-to-eat foods. Also, to address the risk perspective, we determined their invasive capability in a mammalian cell line.

MATERIALS AND METHODS

Bacterial strains and media. Experiments were carried out with 18 *L. monocytogenes* strains (Table 1), representing different serotypes, RAPD types, and origins (food-processing environment, human, animal, and food). Ten of the strains represent RAPD types 2, 6, 9, 12, 15, and 57 *(43)* that have been or are still persistent in the fish-processing industry. Four strains belong to RAPD type 9, and strains of this RAPD type frequently dominate and persist in several types of fish processing plants *(13, 14, 43).* Clustering of these strains has been determined by RAPD with four primers and verified with AFLP, PFGE, and ribotyping *(12–14, 43).* The strains were genetically similar as revealed by all subtyping methods but belonged to different clones. Five strains, including LO28, were human isolates that belonged to five different RAPD types, one strain was the EGD strain (RAPD type 68 in our system), and two strains were isolated from foods and belonged to separate RAPD types. Strain O57 was isolated from a fish product and belonged to an RAPD type that we have only rarely encountered. This strain becomes sensitive to selective enrichment in Palcam agar when plated after exposure to stressful conditions *(12).* Strain 7418 is a lineage 1 strain isolated from food products, and it represents an RAPD type often isolated from foods *(12).* We deliberately do not use the term ''nonpersistent'' because strains that belong to RAPD types that have not been isolated repeatedly, in principle, could be persistent in other settings. The strains were obtained from The Danish Institute for Fisheries Research, The Royal Veterinary and Agricultural University, The Danish Institute for Food and Veterinary Research, Statens Serum Institute and University of Wurzburg, Germany. Stock cultures were stored in −80°C in 4% (wt/vol) glycerol. The bacteria were grown in brain heart infusion (BHI) broth (CM0225, Oxoid, Basingstoke, UK), Luria-Bertani (LB) broth (244620, Difco, Becton Dickinson, Sparks, Md.), and tryptone soya broth

(TSB; CM129, Oxoid, Basingstoke, UK), and in some trials broths were supplemented with glucose to a final concentration of 1% (wt/vol) and/or NaCl to 2, 3, 4, or 5% (wt/wt).

Characterization of the strains. The strains were serotyped with commercial antisera (Mast Diagnostic, Merseyside, UK) following the manufacturer's directions with minor modifications. Strains were grown for 24 h in BHI broth at 37°C, and 1.5 ml of culture was boiled in a water bath for 1 h, centrifuged at approximately 9,500 × *g* for 2 min, and the pellets resuspended in a small amount of the supernatant. One drop of resuspended cell material was mixed with one drop of O-antigen antiserum on a glass slide, and coagulation indicated a positive result. For determination of the H antigens, strains were passed through semisolid (0.2%) BHI agar three times each over 24 h at 30°C. Cells were inoculated in 5 ml of BHI broth and incubated for 24 h at 25°C, and 5 ml of 1% formal saline was added. Suspensions were mixed with each of the tested antisera in Eppendorf tubes and placed at 50 to 52°C for 1 h. Coagulation indicated a positive result.

RAPD analysis was performed as described previously *(13)*, and the *L. monocytogenes* lineage was determined according to Fonnesbech Vogel et al. *(12)*.

Growth under different environmental conditions. *L. monocytogenes* strains were grown in LB or TSB with or without 1% glucose and with or without 5% NaCl. To standardize the inoculum level, an optical density at 450 nm (OD_{450}) CFU curve was prepared for each of the strains grown for 24 h in LB at 37°C. *L. monocytogenes* strains were diluted in LB to a concentration of 10^4 to 10^5 CFU/ml and inoculated in LB (±1% glucose and ±5% NaCl) to an initial cell density of approximately 10^2 CFU/ml. Two hundred microliters of inoculated media was pipetted into honeycomb microtiter plates (Honeycomb 2, 950 2550, Labsystems, Helsinki, Finland), and growth was followed by OD_{420} measurements in a BioScreen C (Labsystems, Helsinki, Finland). The plates were incubated at either 5 or 37°C. Plates were shaken for 10 s before OD_{420} measurements, which were taken every 10 min (at 37°C) and every 4 h (at 5°C). All strains were tested in triplicate at all conditions.

Five strains (N53-1, La111, EGD, 7418, and 4446, representing RAPD types 9, 9, 68, 14, and 71, respectively) were selected for more detailed growth experiments. The strains were cultured in LB at 37°C for 24 h and inoculated in LB (5% NaCl) or LB (1% glucose plus 5% NaCl) at approximately 10^3 CFU/ml. The samples were incubated at 5°C, and cell density followed by colony count determined by surface plating onto BHI agar plates that were incubated at 30°C. Experiments were done in duplicate.

Microtiter plate biofilm assay. Adhesion was studied by using the assay described by Djordjevic et al. *(9)* with a few modifications. Each *L. monocytogenes* strain was grown overnight at 37°C in 4 ml of the TSB medium to be used for adhesion. TSB was supplemented to a final concentration of 1% glucose and up to 5% NaCl. An overnight culture was diluted 1:10 in fresh medium, and 100 μl of diluted culture was pipetted into each of eight microtiter wells of a microtiter plate (Nuncleon 163320, Nunc, Roskilde, Denmark). Sterile media was used as a control. Plates were incubated at 37, 15, or 5°C, and growth was measured as optical density (wavelength 600 nm) on a Versamax Tunable microplate reader (Molecular Devices, Sunnyvale, Calif.) or by surface plating onto BHI agar. The culture medium and nonadherent bacteria were removed after incubation by washing each plate with 200 ml of demineralized water. Plates were air dried for 5 min, and 125 μl of crystal violet solution (1%, wt/vol; 1.01408.0100, Merck, Glostrup, Denmark) was added to each well and left for 20 min. Plates were washed with 3 × 200 ml of distilled water after being stained and photographed. Subsequently, 200 μl of 95% ethanol was added to each well to dissolve the crystal violet, and 100 μl from each well was transferred to a new microtiter plate after 30 min. The intensity of crystal violet was measured at OD_{590}. Eight vials of each strain at each condition were assayed. The assays at 37°C were carried out in two independent trials, whereas the assays at 15 and 5°C were carried out once. In each trial, all strains were included in eight replicates, except for testing adhesion at different salt concentrations where four replicates were included.

Invasion assay. Measurement of invasion was performed as described by Larsen et al. *(25)*. Caco-2 cells (ATCC HTB 37) were propagated in Eagle's minimum essential medium (41090-028, Invitrogen, Taastrup, Denmark) with GlutaMAX and HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid) and supplemented with 20% fetal bovine serum, 0.1 mM nonessential amino acids, and 50 μg/ml gentamicin (15750-037, Gibco, Grand Island, N.Y.). For the invasion assay, the concentration of cells was adjusted to approximately 2 × 10^5 CFU/ml and grown in 96-well tissue culture plates to a monolayer (36 h at 37°C with 5% CO_2).

L. monocytogenes strains were grown in BHI at 37°C for 24 h before infection. The bacterial cultures were adjusted to approximately 1.5 × 10^7 CFU/ml, and 200 μl was added to each well. The cells were washed once with 200 μl of saline water (0.9% NaCl, pH 7.2) after 1 h of incubation at 37°C. To kill extracellular bacteria, 200 μl of Eagle's minimum essential medium with 50 μg/ml gentamicin was added to the wells, and the mixture was incubated at 37°C for 1 h. The cells were washed with 200 μl of saline water followed by the addition of 200 μl of 0.1% Triton X-100. The bacteria were then diluted before being plated onto BHI agar to determine the number of intracellular bacteria. These experiments were carried out in three independent trials, one in duplicate and two in triplicate.

Statistical analysis. Student's *t* test was used with a significance level of $P < 0.05$.

RESULTS

Similar growth of *L. monocytogenes* strains under different stress conditions. We hypothesized that strains belonging to RAPD type 9 and other persistent RAPD types could be more tolerant to food preservation conditions; therefore, we compared growth of these strains with a number of reference strains. We used 37°C as a reference condition but also performed the experiments at 5°C and, at both temperatures, supplemented with 5% NaCl to mimic several of the ready-to-eat products, where *L. monocytogenes* can be a problem. All strains grew equally well in all media examined, both at 5 and 37°C, as well as in media supplemented with up to 1% glucose and/or 5% NaCl when an optical density–based assay was used (Fig. 1). The maximum cell density increased when LB or TSB was supplemented with up to 1% glucose, as compared with growth in nonsupplemented LB and TSB. The strains were diluted and adjusted to 10^8 CFU/ml before inoculation based on absorbance at 450 nm (OD_{450}). Minor differences in lag time were noted (Fig 1); however, because the inoculum was adjusted to the same CFU per milliliter, these differences are likely explained by the initial differences in the adjusted optical density that varied from 0.08 to 0.289 at

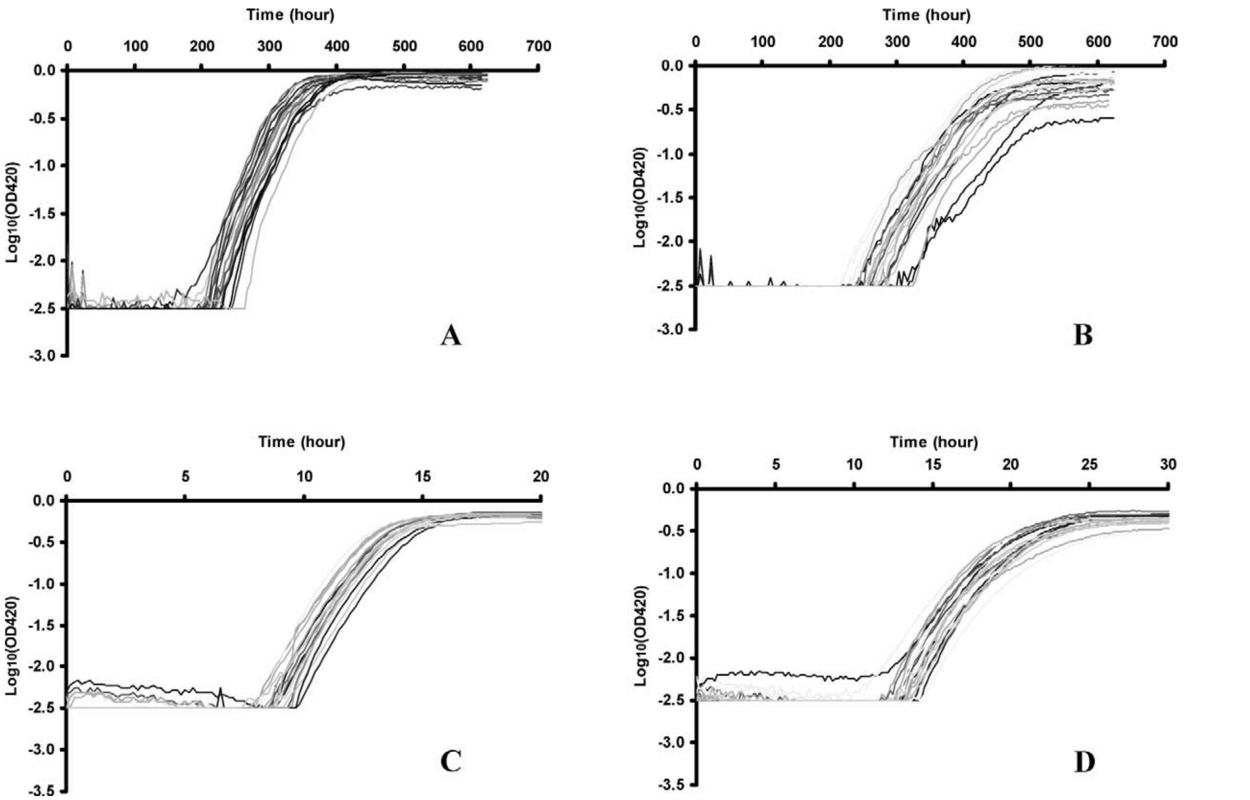

FIGURE 1. *Growth of* Listeria monocytogenes *strains La22, V518a, N53-1, No40-1, R479a, O57, H13-1, La111, M103-1, EGD, LO28, 4666, 4459, 7418, 4446, 6895, 7291, and 4239 in LB (1% glucose) at 5°C (A), LB (1% glucose plus 5% NaCl) at 5°C (B), LB (1% glucose) at 37°C (C), and LB (1% glucose plus 5% NaCl) at 37°C (D). Growth was followed by absorbance at 420 nm. Curves are average of three wells. All measurements below OD_{420} of 0.005 are considered below the detection level of the instrument and are therefore not included in the figure.*

450 nm. Growth rates were highest in LB with 1% glucose and lowest in LB with 1% glucose supplemented with 5% NaCl. Growth was similar in LB and TSB. Strains belonging to persistent RAPD types did not differ in any way from the remaining strains.

The bioscreen measurements represent the latter part of the growth curve, and we therefore chose five strains (N53-1, La111, EGD, 7418, and 4446) to confirm the homogeneous growth patterns by using colony count determinations. The strains were of different origin and RAPD types (except N53-1 and La111) (Table 1), had different adhesion patterns (see below), and had different invasive potential (see later). Generation times at 5°C in LB with NaCl varied from 23 to 30 h and were not statistically significantly different. The addition of glucose shortened the generation time to 15 to 16 h, and again no statistical difference was seen between the strains (data not shown).

Adhesion of *L. monocytogenes* to a plastic surface. The adhesion of five strains (N53-1, La111, EGD, 7418, and 4446) grown in different media was tested to select a basic reference medium in which good adhesion occurred. Only a thin layer of adhered bacteria was present when cells were grown in either LB or TSB, and staining with crystal violet resulted in a crystal violet absorption at 590 nm of 0.05 to 0.15 (data not shown). The adhesion increased when the medium contained 1% glucose, resulting in crystal violet OD_{590} values of 0.11 to 0.42 (data not shown). This could be due to the increase in biomass, which was measured before crystal violet staining as OD_{600}, and this value increased from 0.3 to 0.8 when glucose was added. TSB with a total of 1% glucose was used as reference medium in subsequent adhesion experiments.

All 18 strains formed a thin homogeneous layer of adhered cells on the plastic surface when grown in TSB with 1% glucose, and crystal violet absorbance values ranged from OD_{590} of 0.15 to 0.40 (Fig. 2). Strains EGD and LO28 formed the thinnest layer, but no systematic difference in adhesion depending on RAPD type, genetic lineage, or origin was seen.

Sodium chloride stimulates adhesion of *L. monocytogenes*. To mimic the NaCl level during the processing of ready-to-eat foods, we measured the adhesion of *L. monocytogenes* to a plastic surface of cells grown in TSB containing 1% glucose and NaCl. The addition of NaCl to the TSB medium enhanced the adhesion of all strains at 37°C and caused pronounced aggregation of several strains (Table 1 and Fig. 3). We have defined adhesion as a homogeneous crystal violet stainable layer of cells, whereas aggregates are pellets of stained cells. Both types stick to the surface even after repeated washings. The aggregates especially could not be redissolved in ethanol after crystal violet staining. Therefore, measurement of crystal violet optical density (OD_{590}) could not be used to quantify biofilm formation, and the aggregation and adhesion had to be

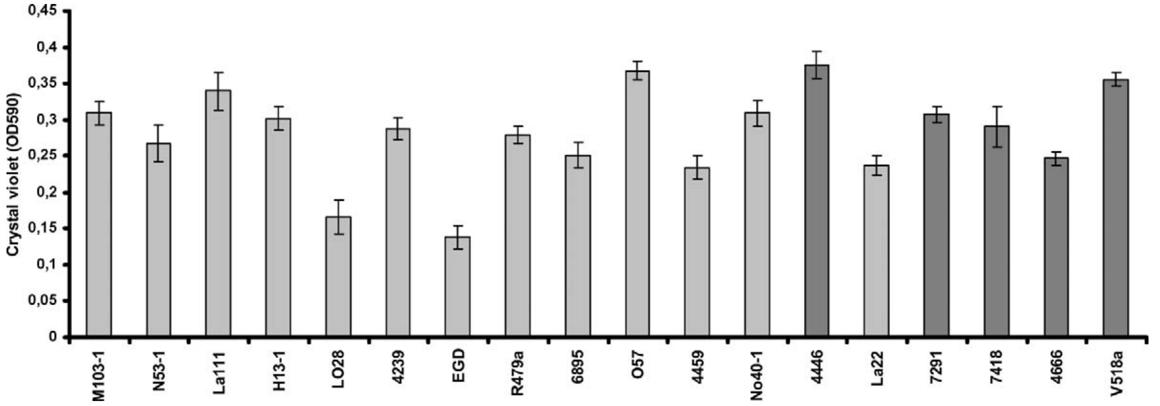

FIGURE 2. *Adhesion to microtiter wells of* Listeria monocytogenes *grown in TSB with 1% glucose for 48 h at 37°C. Adhesion was measured by crystal violet adhesion assay. Lineage 1 strains are in dark gray, and lineage 2 strains are in light gray. Columns are average of eight replicate determinations, and error bars indicate standard deviations.*

scored visually. In some strains (EGD, 6895, and 4239), the addition of 2 to 4% NaCl caused formation of a slightly thicker homogeneous layer of adhered bacteria, whereas the addition of 5% NaCl completely abolished adhesion (Table 1 and Fig. 3). Two strains (R479a and 4459) also formed a homogeneous layer of adhered cells with no aggregation, but the thin layer of bacteria was also seen at 5% NaCl. In the remaining strains, the addition of NaCl caused both increased adherence as well as formation of cell aggregates. This was seen in the range of 2 to 5% NaCl, and the optimal NaCl concentration for aggregation varied slightly between the strains (Table 1).

The marked NaCl effect on adhesion that was seen in TSB was also present when the strains were cultured in LB with a total of 1% glucose and 5% NaCl at 37°C, although the patterns were not as distinct as in TSB (1% glucose and 5% NaCl) (data not shown).

Lack of adhesion of *L. monocytogenes* strains at low temperatures. Most food processing takes place below room temperature, and we therefore determined if the addition of NaCl also enhanced *L. monocytogenes* adhesion at 15°C. Five strains (N53-1, La111, 2063, 7418, and 4446) were chosen, but only a thin biofilm layer was formed at 15°C (crystal violet OD_{590} 0.02 to 0.1) and the addition of NaCl did not result in the distinct aggregation seen at 37°C.

Differences in invasion capacity of *L. monocytogenes* strains in Caco-2 cells. To investigate if the RAPD type 9 strains and other persistent strains were more or less invasive than strains of *L. monocytogenes* belonging to other RAPD types, the invasion of the human cell line Caco-2 was examined. Although all strains invaded Caco-2 cells, their invasive capacity differed significantly. Strains N53-1, H13-1, La111, and M103-1, all of RAPD type 9, were low invasive, and cell densities in Caco-2 cells were approximately 10^2 to 10^3 CFU/ml, whereas the remaining *L. monocytogenes* strains invaded Caco-2 cells more efficiently, i.e., between 10^3 and 10^5 CFU/ml (Fig. 4). The difference between the invasion of the group of four RAPD type 9 strains (N53-1, H13-1, La111, and M103-1) and the group of the five most invasive strains (4446, 7418, 7291, 4666, and V518a) was statistically significant ($P < 0.05$). The latter group of strains consisted of two strains isolated from humans, two strains isolated from foods, and one strain isolated from smokehouse equipment. The invasion assays were carried out in three independent trials, and levels and the ranking of the strains was very similar in all three independent experiments (data not shown).

DISCUSSION

Several studies have demonstrated that fish (and other food) processing plants often harbor an in-house *L. mono-*

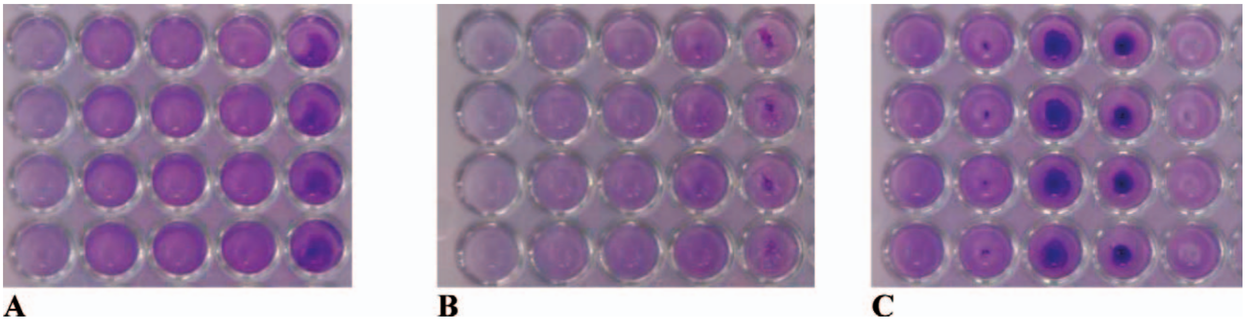

FIGURE 3. *Adhesion of* Listeria monocytogenes *strain N53-1 (A), EGD (B), and 4446 (C) to plastic surfaces (microtiter plates). Photos were taken after strains were grown at 37°C in following media: TSB (1% glucose) (column 1), TSB (1% glucose plus 2% NaCl) (column 2), 1% glucose plus 3% NaCl (column 3), 1% glucose plus 4% NaCl (column 4), and 1% glucose plus 5% NaCl (column 5). Each strain in each medium was grown in four replicates in the microtiter plate.*

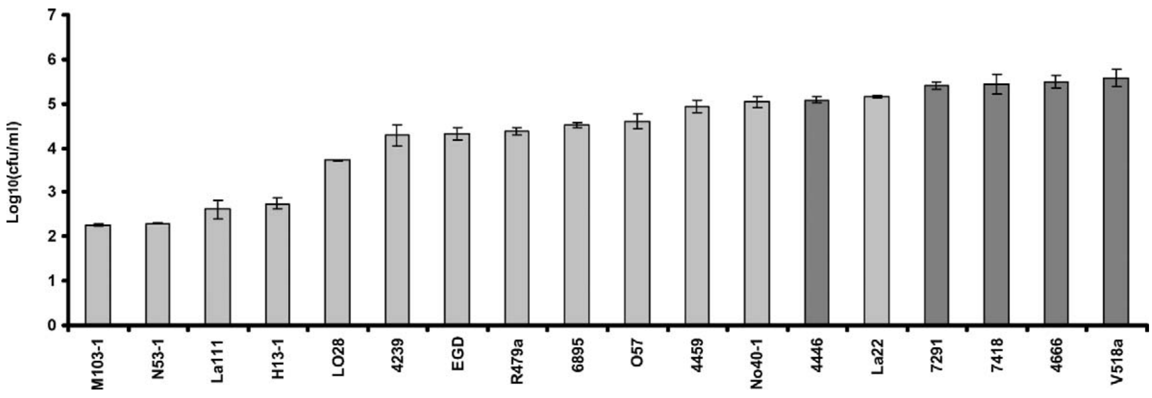

FIGURE 4. *Invasion of strains of* Listeria monocytogenes *in Caco-2 cells. Strains have been sorted according to invasiveness. Lineage 1 strains are in dark gray, and lineage 2 strains are in light gray. Columns are the average from one trial carried out in triplicate. Error bars are based on the standard deviation from the triplicate measurements. The result is representative of two independent experiments.*

cytogenes population, and the ability of the organism to persist in food processing environments for years is well known *(2, 13, 39).* We recently found in a cross-plant comparison that one particular RAPD type (RAPD type 9) was dominant in several processing plants *(43),* and we hypothesized that these strains may have one or more phenotypic characteristics that enable this persistence. In the present study, we demonstrate that these RAPD type 9 strains and other strains representing other persistent types do not have a growth advantage as compared with other *L. monocytogenes* strains. Vialette et al. *(41)* found that clinical strains were more resistant to 8% NaCl as compared with environmental strains, but we did not note such a difference, which could be a consequence of the lower level (5% NaCl) used in our study or due to strain differences.

Different adhesion patterns could also explain persistence of particular strains, and we therefore compared adhesion of *L. monocytogenes* strains with the crystal violet microtiter plate assay *(9)* by using TSB or LB with different supplements. We found no systematic difference in adhesion in these two basic substrates, and, similarly, Moltz and Martin *(30)* did not find any systematic difference when comparing *L. monocytogenes* adhesion in TSB and modified Welshimer's broth. The addition of glucose (to 1%) to LB and TSB resulted in thicker layers of adhered cells, but this is probably due to a simple increase in biomass rather than a specific biofilm enhancing effect, for Kim and Frank *(24)* did not find that glucose (0.1 to 2%) had a consistent effect on the area of a stainless steel slide covered by a biofilm of *L. monocytogenes.* The addition of glucose to the growth media also increased biofilm formation in *Staphylococcus aureus (26),* but biomass data were not reported.

The 18 strains of *L. monocytogenes* varied in their ability to adhere to plastic surfaces when grown in TSB with a total of 1% glucose, and this is consistent with results of previous studies *(8, 9, 29, 34).* Norwood and Gilmour *(33)* found that adhesion values of persistent strains were higher than that of sporadic strains when grown in diluted TSB (6.67%) at 25°C; however, we found only marginal adherence in diluted TSB (data not shown).

Many ready-to-eat products associated with listeriosis contain moderate levels of NaCl, and we therefore investigated the effect of NaCl on *L. monocytogenes* adhesion. The addition of 2 to 5% NaCl caused a dramatic change in surface attachment of all the strains, for all adhered to a greater degree and several formed tightly bound aggregates of cells. The four RAPD type 9 strains as well as several others formed cell aggregates that adhered to the plastic surface. Also, biofilm formation of *S. aureus* is increased by the addition of 3% NaCl to the growth medium *(23, 35).* An enhanced adhesion capacity of *L. monocytogenes* at higher levels of NaCl has been noted *(6),* although it was examined only for one strain (Scott A) and did not include strains isolated repeatedly in food processing environments. Zaika and Fanelli *(44)* and Jørgensen et al. *(21)* observed that Scott A changed cell morphology when grown in 6 and 9% NaCl, respectively, at 37 and 30°C, where the cells became long and filamentous; such change may have influenced the adhesion properties. The cell aggregation and increased adherence of *L. monocytogenes* to the plastic surface when NaCl was added has, to our knowledge, not been reported before. In particular, the group of strains which included all the four RAPD type 9 strains (N53-1, H13-1, La111, and M103-1) changed adhesion patterns dramatically upon the addition of 5% NaCl. The aggregation of cells seen when NaCl was added could be a factor enhancing the ability of *L. monocytogenes* to persist in food processing, for the aggregates adhered well to the surface and may be comparable to a biofilm state, which is more resistant to cleaning and disinfection agents than planktonic cells *(42).*

Some strains of *L. monocytogenes* may become more hydrophilic when grown in the presence of 5% NaCl *(4),* and because the microtiter plates used in this study have a hydrophilic surface this could explain the increased adhesion. Preliminary experiments measuring surface hydrophibicity by using a microbial adhesion to solvent assay have demonstrated that some of our strains become more hydrophilic in the presence of 5% NaCl *(21).* The cell numbers were similar in TSB with and without NaCl and, hence, the difference in the crystal violet patterns cannot be attributed to differences in cell biomass.

The marked change in adhesion seen at 37°C with 5% NaCl was not seen at 5 or 15°C. No noteworthy adhesion was seen in this temperature interval; other studies have also found that adhesion of *L. monocytogenes* decreases with decreasing temperature *(6, 7, 36)*. Ongoing experiments in our laboratory have shown that the addition of NaCl to TSB does increase adhesion of *L. monocytogenes* to stainless steel at 20°C *(25)*. Hence, the effect of NaCl on aggregation and adhesion may be very relevant in a food-processing environment.

Invasion of Caco-2 cells by the four RAPD type 9 strains (N53-1, H13-1, La111, and M103-1) was much lower than invasion by the remaining strains, but other strains representing persistent RAPD types were ranked as high invaders. Norton et al. *(32)* found that a collection of isolates that persist in fish processing factories had lower invasion ability as compared with strains from humans, clinical cases, and foods. Strain 4446 was classified as a highly invasive strain similar to results from another study *(25)*, and the two strains representing RAPD type 15 had identical high invasive ability. Strain LO28 was almost as poor at invading Caco-2 cells as the RAPD type 9 strains, and this is expected because LO28 has a nonsense mutation in *inlA* gene encoding InlA *(20)*, which is an 800 amino acid protein required for entry into epithelial cells, such as the Caco-2 cell line, expressing the InlA receptor, E-cadherin *(27)*. The reason for the low invasion of the four RAPD type 9 strains could be caused by a mutation (insertion or deletion) of some of the virulence genes. Truncated *inlA* is found in 35% of tested strains isolated from different foods *(17)*, and this is a likely explanation for its low invasive capability.

Strains belonging to the genetic lineage 1 were more invasive as compared with strains belonging to lineage 2; however, the average invasiveness of the two lineages was not statistically different because lineage 2 strains spanned a wider invasive spectrum than lineage 1. This result is consistent with those of Norton et al. *(32)*, who found that strains in lineage 1 create a greater plaque size in mouse L cells compared with strains from lineage group 2.

The virulence of a strain is a combination of its invasion ability and its ability to grow and spread intracellularly. We do not know if the persistent RAPD type 9 strains survive and grow intracellularly, and, hence, statements about their virulence as such must be made with care.

In conclusion, the present study revealed that there are phenotypic differences between different strains of *L. monoogenes*, and some characteristics may be consistent with the clustering into RAPD types. The four RAPD type 9 strains are representative of a group of highly persistent strains, which do not appear to persist in the production environment because of enhanced growth capacity under stressful conditions. Instead, the persistence could be due to cell-surface characteristics (proteins, carbohydrates) because these strains alter their adhesion patterns when exposed to substrates containing 5% NaCl. However, adhesion and aggregation of strains clustering in other RAPD groups also were influenced by the addition of NaCl. Fortunately, the four RAPD type 9 strains seem to have a lower invasion capacity for Caco-2 cells as compared with other strains of *L. monocytogenes*.

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Paper 2

Anne Jensen, Line E. Thomsen, Rikke L. Jørgensen, Marianne H. Larsen, Bent B. Roldgaard, Bjarke B. Christensen, Birte F. Vogel, Lone Gram, Hanne Ingmer (2007).

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Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models

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Abstract

Listeria monocytogenes is an important food borne bacterial pathogen that can colonize food processing equipment. One group of genetically similar *L. monocytogenes* strains (RAPD type 9) was recently shown to reside in several independent fish processing plants. Persistent strains are likely to contaminate food products, and it is important to determine their virulence potential to evaluate risk to consumers. We compared the behaviour of food processing persistent and clinical *L. monocytogenes* strains in four virulence models: Adhesion, invasion and intracellular growth was studied in an epithelial cell line, Caco-2; time to death in a nematode model, *Caenorhabditis elegans* and in a fruit fly model, *Drosophila melanogaster* and fecal shedding in a guinea pig model. All strains adhered to and grew in Caco-2 cells in similar levels. When exposed to 10^6 CFU/ml, two strains representing the persistent RAPD type 9 invaded Caco-2 cells in lower numbers (10^2-10^3 CFU/ml) compared to the four other strains (10^4-10^6 CFU/ml) including food and human clinical strains. In the *D. melanogaster* model, the two RAPD type 9 strains were among the slowest to kill. Similarly, the time to reach 50% killed *C. elegans* worms was longer (110 h) for the RAPD type 9 strains than for the other four strains (80 h). The Scott A strain and one RAPD type 9 strain were suspended in whipping cream before being fed to guinea pigs and the persistent RAPD type 9 strain was isolated from feces in a lower level (approx. 10^2 CFU/g) than the Scott A strain (approx. 10^5 CFU/g) ($P < 0.05$). Addition of NaCl have shown to cause autoaggregation and increases adhesion of *L. monocytogenes* to plastic, however, growth in the presence of NaCl did not alter the behaviour of the tested *L. monocytogenes* strains in the virulence models.

Overall, the two strains representing a very common fish processing plant persistent group (RAPD type 9) had a lower virulence potential in all four virulence models than Scott A and a strain isolated from a clinical case of listeriosis.

1. Introduction

Listeria monocytogenes is a gram-positive pathogenic bacterium, which can cause listeriosis (meningitis, septicaemia) in humans. The fatality rate is very high at approximately 25-30% (Farber et al., 1991), but this food borne disease is rare and affects primarily immunosuppressed people, or during pregnancy, the developing fetus. The vehicles of infection are typically ready-to-eat food products (Rocourt, 1996; Vazquez-Boland et al., 2001), in which the organism can grow to high numbers.

L. monocytogenes has a remarkable ability to reside in the food processing environment (Autio et al., 1999; Norton et al., 2001b; Rørvik et al., 1995; Vogel et al., 2001a), and specific molecular subtypes can repeatedly be isolated from the processing environment (Wulff et al., 2006). We recently demonstrated that a particular Random Amplified Polymorphic DNA (RAPD) type (RAPD type 9) was found as a persistent type in several fish processing facilities (Wulff et al., 2006), although, this RAPD type is not common in the outside environment (Hansen et al., 2006). The reason for the persistence of this particular subtype is not known.

From a risk analysis perspective, it is important to assess the virulence potential of strains that are very likely contaminants of food products, such as strains persisting in the food processing environment. Several *in vitro* models and animal models have been used to investigate pathogenicity and virulence of *L. monocytogenes. In vitro* models using tissue culture cell lines such as the epithelial cell line Caco-2 have simplified the study of particular virulence functions and are widely used to compare adhesion, invasion and intracellular growth of different strains. A more complete analysis of virulence is obtained using animal models, and for the study of *L. monocytogenes*, the mouse and the guinea pig models have been used (Andersen et al., 2007; Bakardjiev et al., 2004; Dustoor et al., 1977; Garner et al., 2006a; Lecuit et al., 2001; Takeuchi et al., 2006; Williams et al., 2007). In contrast to the guinea pig, the mouse model has some limitations for oral infections as the E-cadherin receptor is different from the human and guinea pig E-cadherin (Lecuit et al., 1999) and *L. monocytogenes* Internalin A does not bind properly to the mouse E-cadherin.

To avoid the ethically controversial animal models, simpler eukaryotic models have recently been developed to study host-pathogen interactions. The fruit fly model, *Drosophila melanogaster*, was introduced by Mansfield et al. (2003) for *L. monocytogenes* and the nematode *Caenorhabditis elegans* appears to be an appropriate host for *L. monocytogenes* infection (Thomsen et al., 2006) since known non-virulent mutants kill the worms more slowly than the wild-types.

The purpose of the present study was to determine if strains representing the common persistent RAPD type isolated from fish processing environments have a higher or lower virulence potential than clinical strains. This was done using an array of virulence model assays and, hence, the study also serves as an inter-comparison of these different models. Further, the role of NaCl in the bacterial growth medium for the adhesion, invasion and virulence potential of *L. monocytogenes* was investigated, as we previously have observed that addition of 2-5% NaCl to the growth medium dramatically changed cell aggregation and adhesion to plastic surfaces of *L. monocytogenes* (Jensen et al., 2007a).

2. Materials and methods

2.1 Bacterial stains and growth conditions. The behaviour of six strains of *L. monocytogenes* belonging to different serotypes, lineages and RAPD types in four virulence models was compared in this study (Table 1). N53-1 and La111 belongs to a group of genetically similar strains, which frequently dominates and persists in fish processing environments (Wulff et al., 2006). This group of strains appear phenotypically similar (Jensen et al., 2007a) and N53-1 and La111 were chosen as representatives for RAPD type 9. *L. monocytogens* EGD was obtained from Werner Goebel (1999) and Scott A was obtained from Campden Food and Drink Association (1989) and are both used as reference strains. Strain 7418 was isolated from spreadable sausages, and strain 4446 was isolated from a human case of listeriosis (Larsen et al., 2002). The strains also represent genetic lineage 1 (Scott A, 7418, 4446) and lineage 2 (N53-1, La111, EGD) and the three serotypes (1/2a, 1/2b, 4b) typically involved in disease.

Escherichia coli strain OP50 was used as food for the *C. elegans* nematodes, and as a negative control in both the *D. melanogaster* and the *C. elegans* models. *Listeria innocua* strain Div-A8 (culture collection, Department of Veterinary Pathobiology, University of Copenhagen) was used as a non-virulent *Listeria* control in the *D. melanogaster* model. For the sequencing of *inlA*, *L. monocytogenes* strains H13-1 and M103-1 belonging to RAPD type 9 (Jensen et al., 2007a) and strain LO28 were included.

Stock cultures were stored in -80°C in a medium containing 4% (wt/vol) glycerol, 2% (wt/vol) skim milk powder and 3% (wt/vol) Tryptone Soya Broth (TSB) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and grown in either Lauria-Bertani (LB) broth (Difco, Becton, Dickinson, Sparks, Md, USA), Brain Heart Infusion (BHI) broth (Oxoid

Ltd., Basingstoke, Hampshire, United Kingdom), or TSB supplemented with glucose to a final concentration of 1% (wt/vol) with or without 5% NaCl (wt/wt) (Jensen et al., 2007a). The cell adhesion and invasion as well as the virulence potential of strains were studied with and without addition of NaCl to the growth medium. Numbers of *L. monocytogenes* were determined by spread plating on Palcam agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) or BHI agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) followed by two days of incubation at 37°C. LB agar (Difco, Becton, Dickinson, Sparks, Md, USA) was used as grow medium for all bacterial strains in *C. elegans* trials.

2.2 Adhesion, invasion and intracellular growth in Caco-2 cells. Strains N53-1, EGD and Scott A were grown either in TSB (1% glucose) or TSB (1% glucose and 5% NaCl) for 24 h at 37ºC before Caco-2 cell adhesion studies. Strains were sub-cultured twice, prior to the addition to Caco-2 cells, that were grown and prepared as previously described (Jensen et al., 2007a). The Caco-2 cells (ATCC HTB 37) were grown in a 24 well tissue culture plate (TPP, Trasadingen, Switzerland) for 36 h at 37°C with 5% CO_2 to reach a monolayer. The bacterial cultures were adjusted to approx. 5×10^6 CFU/ml by dilutions in Modified Eagle Medium with Glutamax and HEPES (MEM, Invitrogen, Carlsbad, CA), supplemented with 20% Fetal Bovine Serum (FBS), 0.1 mM non essential amino acids (NEA) and 1 ml was added to each well of the 24 well plate. After 1 h of incubation at 37ºC, the Caco-2 cells were washed twice with 1 ml saline water (0.9% NaCl, pH 7.2). One ml of 0.1% TritonX-100 was added to each well to loosen and lyse the adhered Caco-2 cells. The mixture of lysed Caco-2 cells and bacteria were diluted and plated on BHI-agar to determine the number of adhered bacteria.

Invasion was assessed with strains N53-1, EGD and Scott A grown either in TSB (1% glucose) or TSB (1% glucose and 5% NaCl). The number of bacteria invading the cells were determined as previously described (Jensen et al., 2007a) with a few modifications. The Caco-2 cells were infected as described above and after 1 hour of infection, the monolayer was washed twice in saline water and extracellular bacteria were killed by incubation with MEM with 100 µg/ml gentamicin for 1 hour at 37°C. The wells were washed with saline water and lysed with 1 ml 0.1% TritonX-100. The number of bacteria released was expressed in CFU/ml by plating appropriate dilutions on BHI agar plates.

The ability of *L. monocytogenes* to grow intracellularly in Caco-2 cells was studied using all six strains N53-1, La111, EGD, 7418, 4446 and Scott A, all grown in TSB (1% glucose) prior to addition to the Caco-2 cells. The Caco-2 cells were infected as described above, the monolayer was washed twice in saline water and the extracellular bacteria were killed by incubation with MEM with 100 µg/ml gentamicin for 1 hour at 37°C. The media was removed and MEM was added to each well. The plates were incubated for 0 h, 2 h, 3.5 h or 5 h at 37°C. At each time MEM was removed and cells were lysed with 1 ml 0.1% TritonX-100, and the number of intracellular bacteria was determined by plating serial dilutions on BHI agar plates. All experiments were carried out in duplicate in two independent trials.

2.3 *Listeria monocytogenes* infection of the fruit fly *Drosophila melanogaster*. Experiments with fruit flies were done according to Jensen et al. (2007b). Six *L. monocytogenes* strains (N53-1, La111, EGD, 7418, 4446, Scott A), *L. innocua* and *E. coli* were tested in the *D. melanogaster* model. The bacterial strains were grown at 37°C in BHI, TSB (1% glucose) or TSB (1% glucose and 5% NaCl) before injection into the dorsal thorax of the flies. Each assay was carried out four independent times.

2.4 *Listeria monocytogenes* infection of the nematode *Caenorhabditis elegans*. Experiments with *C. elegans* were done with the temperature-sensitive sterile *C. elegans* pha-1 (e2123ts) (Schnabel et al., 1990) as described by Thomsen et al. (2006). Six strains of *L. monocytogenes* (N53-1, La111, EGD, 7418, 4446, Scott A) and *E. coli* OP50 were grown on LB plates. The worms were fed on a lawn of bacteria. The effect of each strain on survival of *C. elegans* was studied in three independent trials for each strain.

2.5 *Listeria monocytogenes* colonization and infection of guinea pigs. The guinea pig model was developed by Andersen et al. (2007). Twenty four Dunkin Hartley guinea pigs (12 males and 12 females), 3-4 weeks old, were obtained from Charles River Laboratories (Germany). The animal experiments were approved and conducted according to Danish legislation.

L. monocytogenes strains N53-1 and Scott A were tested in the guinea pig model. Cultures were grown during two successive overnight transfers at 37°C with shaking at 200 rpm, where N53-1 was grown with or without 5% NaCl and Scott A was grown without NaCl. At day 0 and day 1, 0.5 ml of a whipping cream/*L. monocytogenes* cocktail (approx. 10^{11} CFU/ml) was given orally, with a syringe to each guinea pig. Bacterial cell counts were confirmed on Palcam Agar for the inoculation culture.

Fecal samples were taken from the animals every day until day 7. On day 4 half of the guinea pigs from each group were sacrificed by decapitation and cell count was made on liver, spleen, jejunum and fresh feces. Samples were diluted in 0.9% NaCl water and *L. monocytogenes* was enumerated on Palcam agar. The remaining guinea pigs were sacrificed on day 7.

2.6 Sequencing of *inlA*. DNA from *L. monocytogenes* was extracted with Dynabeads DNA direct universal (Dynal Biotech ASA, Oslo, Norway). PCR amplification of *inlA* was done according to Nightingale et al. (2005) with primer inlA seq F and inlA R under the described conditions. PCR products were separated by gel electrophoresis and purified with GFX PCR DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK). Sequencing was done by MWG-Biotech AG (Ebersberg, Germany) and the DNA-sequences were aligned with ClustalW (http://www.ch.embnet.org/software/ClustalW.html), and translated to protein sequences with ExPASy (http://www.expasy.ch/tools/dna.html).

2.7 Statistical analysis. Students t-test was used with a significance level of $P < 0.05$.

3. Results

We focused on comparing the virulence potential of food processing persistent strains, reference strains and strains isolated from food or a human clinical case of listeriosis in four different virulence model systems. We also determined if addition of NaCl could have an influence of the virulence potential of the strains since NaCl affects *Listeria* adhesion to inert surfaces (Jensen et al., 2007a).

3.1 Adhesion, invasion and intracellular growth in Caco-2 cells. We examined the ability of *L. monocytogenes* strains to adhere to, invade and survive in Caco-2 cells. The first step in the infection process is adhesion of the bacteria to the enterocytes. Strains N53-1 and EGD adhered to a similar level of 10^4-10^5 CFU/ml to Caco-2 cells, while Scott A adhered slightly better (10^6 CFU/ml) (Fig. 1). The next step in the infection process is the invasion of the eukaryotic cell. The two RAPD type 9 strains showed reduced invasion into Caco-2 cells as also previously described (Jensen et al., 2007a) compared to the other strains. Addition of 5% NaCl to the bacterial growth medium prior the infection assay did not change adhesion ($P < 0.05$) or invasion ($P < 0.05$) of N53-1, EGD or Scott A ($P < 0.05$) (Fig.1 and results not shown). Following invasion, *L. monocytogenes* must survive and proliferate in the intestinal cells, and the six strains grew with identical intracellular growth rates in the Caco-2 cells ($P < 0.05$) (Fig. 2).

3.2 *Listeria monocytogenes* infection of *Drosophila melanogaster*. To determine the virulence potential of *L. monocytogenes* strains, *D. melanogaster* were infected with *L. monocytogenes* by injection in dorsal thorax. All six *L. monocytogenes* strains caused killing of the fruit flies at a faster rate than the negative control strain *E. coli* OP50 (Fig 3A). The time to reach 50% mortality for the fruit flies was used as a virulence measurement and divided the strains into three groups: Scott A and 4446 were the most efficient killers. A middle group consisted of N53-1, La111 and 7418, followed by EGD which was the least efficient at causing death.

The time to reach 100% mortality also varied between the strains (Fig. 3A). Three groups were seen, where N53-1 and La111 were the less efficient strains causing 100% mortality whereas EGD and 4446 belonging to the middle group and Scott A and 7418 were the most efficient strains. The non-virulent *L. innocua* was also tested in the fruit fly model and was able to kill the files similar as the EGD strain.

To test if addition of NaCl to the bacterial growth medium (TSB with 1% glucose) had an enhanced effect on the killing kinetics, 5% NaCl was added to the growth medium. No notable changes in the killing kinetics of the *L. monocytogenes* strains were seen (results not shown).

3.3 *Listeria monocytogenes* infection of *Caenorhabditis elegans*. The six *L. monocytogenes* strains killed *C. elegans* after feeding the worms on a lawn of bacteria. *C. elegans* were killed more rapidly than the negative control strain *E. coli* OP50. Previous studies at our laboratory have shown that *L. innocua* kills *C. elegans* similarly to the negative feeding control strain *E. coli* OP50 (Thomsen et al., 2006). The six *L. monocytogenes* strains were separated into two groups with respect to time taken to reach 50% mortality of the worms (Fig. 3B). Scott A, 7418 and 4446 killed 50% of the worms in 80 h whereas N53-1, La111 and EGD took 110 h to reach 50% mortality. It was not possible to test the influence of NaCl on the killing as addition of 5% NaCl killed the *C. elegans* worms.

3.4 *Listeria monocytogenes* infection of guinea pigs. To investigate if a food processing persistent strain (N53-1) had a reduced virulence potential compared to Scott A in a more complex model, we examined virulence of these two strains in an orally fed, *in vivo* guinea pig model. The influence of NaCl on the virulence potential was studied by growing *L. monocytogenes* N53-1 in media with and without 5 % NaCl prior to the infection in guinea pigs. Fecal samples were collected every day from the guinea pigs, and the numbers of *L. monocytogenes* were enumerated. The persistent RAPD type 9 strain N53-1 was shed in a significantly lower level from the guinea pigs than Scott A. The shedding of N53-1 was approximately 3 log unit lower than the shedding of Scott A ($P < 0.05$) (Fig. 4). The level of N53-1 in the feces of the infected animals was 10^4 CFU/g during the first 2 days and thereafter the levels was reduced to 10^2 CFU/g. Scott A was shed at approximately 10^5 CFU/g during the 7 days of infection.

The content of *L. monocytogenes* in spleen, liver and jejunum was determined at day 4 and 7 post-treatment. At days of sacrifice, the number of animals positive for *L. monocytogenes* was higher for animals fed with Scott A than when fed with N53-1 (Table 2). The lower number of *L. monocytogenes* cells in the feces of guinea pigs fed with N53-1 was reflected in the presence of *L. monocytogenes* in the liver, spleen and jejunum (Table 2). N53-1 was detected in the jejunum of approximately 20% of the infected animal whereas Scott A was detected in 100% of the guinea pigs at day 4. Also in the liver and spleen, fewer animals were positive for *L. monocytogenes* when infected with N53-1 than Scott A (Table 2). The cell count for all the samples were between 0 and 10^3 CFU/ml (results not shown). The number of positive organs from animals dosed with Scott A did not change from day 4 to day 7, but surprisingly, there was an increase from day 4 to day 7 in the number of positive spleen and liver organ samples in animals dosed with N53-1 suggesting that N53-1 is a slower invader of the guinea pigs. The shedding of N53-1 was not influenced by the addition of NaCl to the bacterial growth medium prior the infection (Fig. 4), but the invasion of N53-1 into spleen and liver over time increased slightly with the addition of NaCl. Due to ethical considerations, these animal experiments were not repeated and the influence of NaCl on organ invasion needs to be investigated further (Table 2).

3.5 Sequencing of *inlA*. The *L. monocytogenes* strains were tested for the presence of premature stop codons (PMSC) in *inlA* to explain the low invasion into Caco-2 cells of the strains belonging to RAPD type 9. The 3' region of *inlA* from strains of *L. monocytogenes* used in this study (N53-1, La111, EGD, 7418 and 4446) were sequenced. Further two strains representing the RAPD type 9 were included (M103-1 and H13-1). These strains invaded Caco-2 cells as poorly as strains N53-1 and La111 (Jensen et al., 2007a). Also, strain LO28 which has a PMSC (Jonquieres et al., 1998) was included as control. The eight sequences were aligned against the reference *inlA* (accession no. NC_003210) from EGD-e (complete genome) with ClustalW, and we found the single point mutation in LO28 resulting in a frame shift mutation and creation of a nonsense codon (Jonquieres et al., 1998). None of the other tested strains showed a premature stop codon in their 3' region of *inlA*, but single point mutations were identified in the RAPD type 9 strains when aligned against the reference *inlA*. The translated protein sequences were aligned against the reference InlA with ClustalW. The four RAPD type 9 strains had the same two single amino acid changes when aligned to reference InlA, EGD InlA, 7418 InlA and 4446 InlA (Fig. 5). The first amino acid change was at position 539 where glutamine (Q) was changed to lysine (K), and the second amino acid change was at position 572 where phenylalanine (F) was changed to leucine (L).

4. Discussion

L. monocytogenes strains that persist in food processing environments are more likely food product contaminants than non-persistent strains and it is therefore important to evaluate the degree of risk they represent. Virulence is not a constant property and it has been speculated that it may be modulated e.g. by components or conditions in processing of food (Dallmer et al., 1990; Garner et al., 2006b; Myers et al., 1993). Addition of low levels of NaCl enhances the adhesion and aggregation forming properties of *L. monocytogenes* to a plastic surface, especially for some of the strains persisting in fish processing plants (Jensen et al., 2007a). In the present study, we addressed the issue of both the general virulence potential and the possible influence of NaCl on the virulence potential of *L. monocytogenes* strains.

As a surrogate for oral exposure in humans based on similarities in clinical symptoms, we chose the guinea pig model in which fecal shedding has been used as an indicator for *L. monocytogenes* infection, and has previously been shown to be an acceptable marker (Williams et al., 2007). Thus, there is a positive correlation between the numbers of *L. monocytogenes* in feces and birth outcome in pregnant guinea pigs and monkeys (Smith et al., 2003; Williams et al., 2007). We found a correlation between the number of shed bacteria in feces and the number of animals with detectable levels of *L. monocytogenes* in the tissues. Strain N53-1 representing the persistent RAPD type 9, was shed in significantly lower numbers than the Scott A strain. The *L. monocytogenes* infection is also indicated by detection of the bacteria in spleen, liver and jejunum (Lecuit et al., 2001; Takeuchi et al., 2006; Williams et al., 2007) and N53-1 was detected less frequently than the Scott A strain.

There is a need to develop new models to test differences in virulence potential, because of the ethical controversial and expensive animal models. The nematode *C. elegans* has been used as a model to describe virulence of *L. monocytogenes* (Thomsen et al., 2006), *Pseudomonas aeruginosa* (Tan et al., 1999) and *Staphylococcus aureus* (Sifri et al., 2003). The *L. monocytogenes* strains differed in time taken to cause killing and the division into two groups was very similar to the division of strains based on invasion of Caco-2 cells. Thomsen et al. (2006) suggested that *L. monocytogenes* remains extracellular in *C. elegans*, but we saw the same strain differentiation as for invasion into caco-2 cells. Therefore, the *C. elegans* model may assess other virulence factors than invasiveness.

Although non-virulent *L. innocua* were able to kill fruit flies with the same rate as a virulent *L. monocytogenes* strain (Jensen et al., 2007b), we were able to use this model to differentiate the *L. monocytogenes* strains into two groups with the two persistent RAPD type 9 strains into one group and Scott A and strain 4446 into another. These were the same groups as was seen with *C. elegans* and invasion into Caco-2 cells. A major difference in these models is the route of infection in fruit flies which is injection and may result in other factors of importance compared to oral infection. To investigate the lethal action of presumable non-virulent bacteria more work is needed on this model.

The simple Caco-2 models allow testing of several steps in the infection cycle namely adhesion, invasion and intracellular growth. The strains did not show a noteworthy difference in their ability to adhere or grow intracellular, which is similar to findings by Chui et al. (2006). However, the strains differed markedly in their invasive ability, as reported earlier (Jensen et al., 2007a), as the two persistent RAPD type 9 strains invaded to a lower level than the other strains.

The reduced invasive capability could be caused by a truncation of the *L. monocytogenes* surface protein, internalin A (Nightingale et al., 2005). This 800-amino acid protein is promoting the entry into epithelial intestinal cells, such as the Caco-2 cell line, which express the InlA receptor, E-cadherin (Mengaud et al., 1996). *inlA* from RAPD type 9 strains, did not contain the previously hypothesised single point mutations causing stop codons (Jensen et al., 2007a), but instead we saw single point mutations resulting in two changes in amino acids in the InlA from the RAPD type 9 strains. These two changes can result in abnormal protein folding of InlA leading to a lower affinity to E-cadherin. The mutations were seen in all four persistent RAPD type 9 strains N53-1, La111, H13-1 and M103-1.

All together, the results suggest that the two processing plant persistent process strains (RAPD type 9) (N53-1 and La111) are less invasive and have a lower virulence potential in the tested models than Scott A and the human clinical strain. However, none of the models reflects the complete infection process seen in humans and results from simpler models may not completely show the infection process in immunocompromised and pregnant humans.

L. monocytogenes is separated into three different lineages (Rasmussen et al., 1995; Wiedmann et al., 1997) because of gene polymorphism, and each lineage contains several serotypes. Strains of lineage 1 are, in general, more invasive than lineage 2 (Jensen et al., 2007a; Norton et al., 2001a), which is consistent with our results where Scott A and strain 4446 (lineage 1) are more invasive and have a higher infection potential than strain N53-1 and La111 (lineage 2). Whether this theory is correct in more complicated models have not been tested systematically.

Addition of NaCl to ready-to-eat food is commonly used to add flavour and to decrease growth of unwanted bacteria. Also, addition of NaCl enhances the ability of *L. monocytogenes* to adhere and aggregate to a plastic surface (Jensen et al., 2007a). This enhanced ability to adhere could be a result of a changed expression of surface proteins. Further, elevated levels of NaCl and the availability of iron, regulate the stress response and modify the cell surface hydrophobicity and the expression of virulence factors (Conte et al., 1996; Kazmierczak et al., 2003). Expression of *inlA* is up-regulated when NaCl is added (Sue et al., 2004), and we therefore expected the invasion ability to be higher for NaCl-stressed cells. However, we did not find any significant difference in virulence potential between the tested strains grown either in media with or without 5% NaCl using the different virulence models. Although, there might be a minor increase in the level of spleens and livers positive from animals fed with N53-1, which was grown in the presence of 5% NaCl. Different levels of NaCl in the growth medium did not show any differences in the virulence of a *L. monocytogenes* strain in mice (Myers et al., 1993). On the other hand, addition of 2.2 % NaCl has enhanced the ability of *L. monocytogenes* to invade Caco-2 cells (Garner et al., 2006b). Discrepancy between the results could be due to the use of different concentrations of NaCl and different virulence models.

In conclusion, NaCl does not seem to affect the virulence potential of either the persistent RAPD type 9 strains or the human clinical strains. The persistent RAPD type 9 strains had a lower virulence potential as compared to the clinical strains, in invasion of Caco-2 cells, killing fruit flies and nematode worms and fecal shedding and infection of tissues of guinea pigs.

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Table 1. Origin and sub-type of *Listeria monocytogenes* strains used in the present study

Strain	Origin	Serotype	Lineage	RAPD-type	Reference
N53-1	Smoke house equipment	1/2a	2	9	(Wulff et al., 2006)
La111	Cold smoked salmon	1/2a	2	9	(Vogel et al., 2001b)
EGD	EGD	1/2a	2	68	[A]
Scott A	Human, clinical	4b	1	72	[B]
7418	Spreadable sausage	1/2b	1	14	(Larsen et al., 2002)
4446	Human, clinical	4b	1	71	(Larsen et al., 2002)

[A] The strain was kindly provided by Werner Goebel, University of Würzburg, Germany.

[B] The strain was kindly provided by Campden Food and Drink Association, UK

Table 2: Guinea pigs (% of total animals treated with each strains) positive for *Listeria monocytogenes* (100 CFU/ml is detection limit) in spleen, liver and jejunum after oral exposure to 5×10^{10} CFU/ml per day on two consecutive days. Guinea pigs were fed with Scott A or N53-1 grown without NaCl (-NaCl) or with N53-1 grown with 5% NaCl (+NaCl). Animals were sacrificed at day 4 or day 7 post-treatment.

Strain	Sacrificed at day	No. of animals	-/+ NaCl	% animals with higher than 100 CFU/ g		
				Spleen	Liver	Jejunum
Scott A	4	3	-	100	67	100
	7	3	+	100	67	67
N53-1	4	4	-	0	0	25
	7	5	+	20	40	20
N53-1	4	5	-	20	20	20
	7	4	+	100	50	0

Figure legends

Figure 1: Adhesion of *Listeria monocytogenes* strains (N53-1, EGD and Scott A) to Caco-2 cells. Strains were grown in TSB (1% glucose) (▨) or TSB (1% glucose + 5% NaCl) (▩) before the assays. Error bars are based on standard deviations from two independent experiments in duplicate.

Figure 2: Invasion and survival of *Listeria monocytogenes* strains in Caco-2 cells. Strains were grown in TSB (1% glucose) before beginning of the assay. Strains are as followed: 7418 (––◊––), N53-1 (––×––), 4446 (—Δ—), Scott A (---—---), EGD (—□—) and La111 (---○---). Hours is the number of hours after addition of gentamicin. Error bars are based on standard deviations from duplicate measurements. The figure is representative of two independent experiments.

Figure 3: Mortality of *Drosophila melanogaster* (A) injected with and *Caenorhabditis elegans* (B) fed on strains of *Listeria monocytogenes*, *Listeria innocua* and *Escherichia coli* OP50. *Listeria* strains were grown in BHI before injection and *E. coli* was grown in LB. Strains are as followed: 7418 (––◊––), N53-1 (—×––), 4446 (**—Δ—**), Scott A (---**—**---), EGD (—□—), La111 (---○---), *L. innocua* (**——**) and *E. coli* OP50 (**—*—**). *L. innocua* was only given to *D. melanogaster*. Error bars are based on standard deviations from four independent experiments (*D. melanogaster*) and three independent measurements (*C. elegans*).

Figure 4: Content of *Listeria monocytogenes* in fecal samples collected from infected guinea pigs over a period of 4-7 days after dosing. From day 1 to 4, numbers are average of 6

guinea pigs dosed with Scott A, and 9 guinea pigs dosed with N53-1 grown in either TSB (1% glucose) or TSB (1% glucose and 5% NaCl). From day 5-7, numbers are average of 3 guinea pigs dosed with Scott A, and 4-5 guinea pigs dosed with N53-1 grown in either TSB (1% glucose) or TSB (1% glucose and 5% NaCl). Scott A (---——---), N53-1 (TSB+1% glucose) (——×——) and N53-1 (TSB+1% glucose + 5% NaCl) (—Δ—).

Figure 5: InlA from different *Listeria monocytogenes* strains were aligned with the ClustalW program. InlA from following strains were aligned: InlA (reference, accession no. NC_003210), EGD, N53-1, La111, H13-1, M103-1, 7418 and 4446. The two mutations are boxed in black, and their position in the InlA protein is indicated. Glutamine (Q) is changed to lysine (K), and phenylalanine (F) is changed to leucine (L) Stars indicates identity, semi-colon indicates similarity.

Figure 1

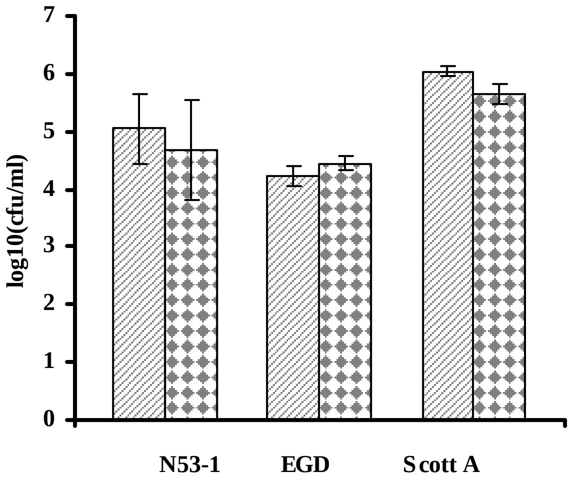


Figure 2

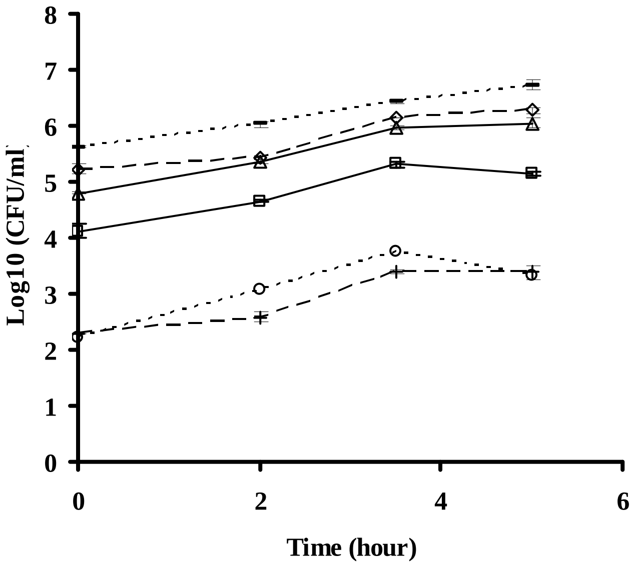
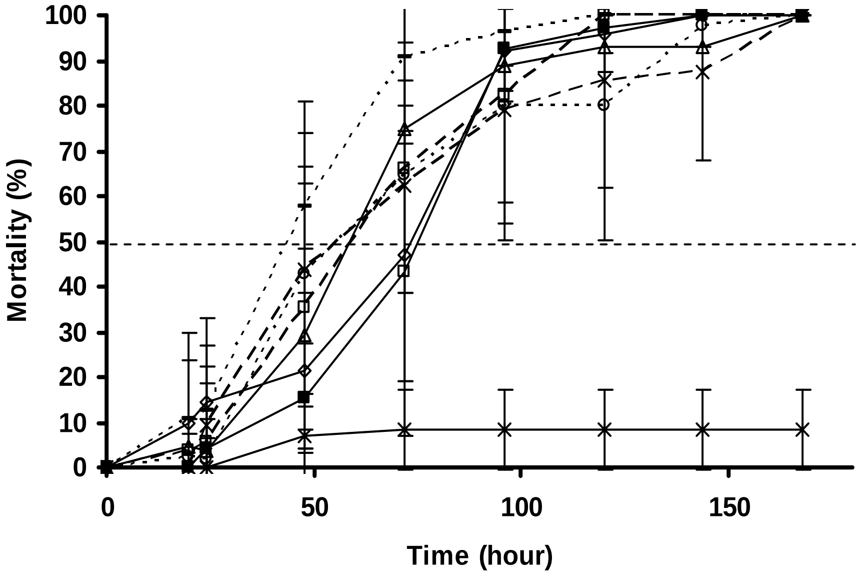


Figure 3

A



Mortality (%)
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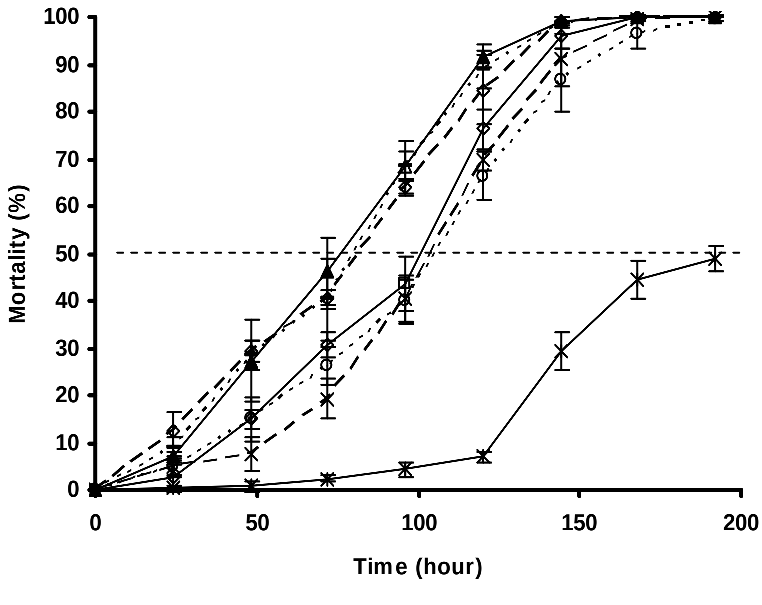
10
20
30
40
50
60
70
80
90
100
0
50
100
150
Time (hour)

B



Mortality (%)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
Time (hour)

Figure 4

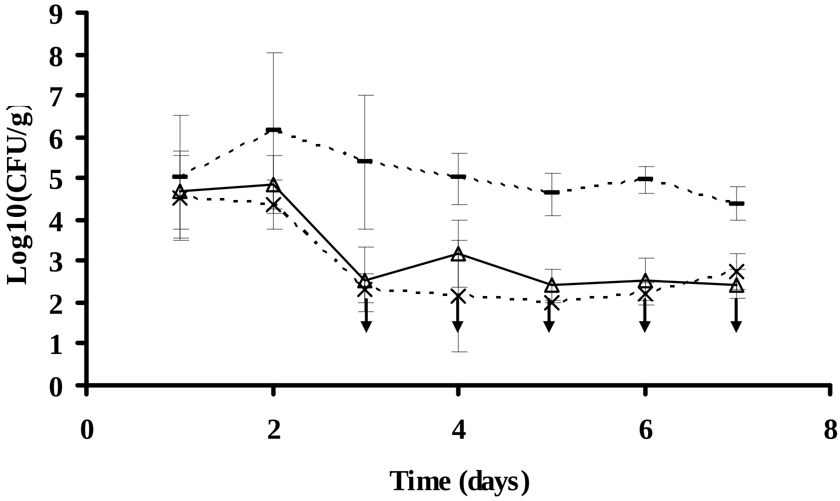


Figure 5

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Aminoacid                              539                               572
                                        |                                 |
InlA (ref)       PAKPVKEGHTFVGWFDAQTGGTKWNFSTDKMPTNDINLYAQFSINSYTATFDNDGVTT
EGD              PAKPVKEGHTFVGWFDAQTGGTKWNFSTDKMPTNDINLYAQFSINSYTATFDNDGVTT
N53-1            PAKPVKEGYTFIGWFDAKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
La111            ----------------AKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
H13-1            -------------WFDAKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
M103-1           PAKPVKEGYTFIGWFDAKTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATLDNDGVTT
7418             PAKPVKEGYTFVGWFDAQTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATFDNDGVTT
4446             ---------------DAQTGGTKWNFSTDKMPTNDIDLYAQFSINSYTATFDNDGVTT
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Paper 3

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A processing plant persistent strain of *Listeria monocytogenes* crosses the feto-placental barrier in a pregnant guinea pig model.

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A processing plant persistent strain of *Listeria monocytogenes* crosses the feto-placental barrier in a pregnant guinea pig model

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Abstract

The food-borne pathogen, *Listeria monocytogenes*, can cause infection in immunocompromised humans and in pregnant women, where the fetus is the primary target. Food is often contaminated from processing equipment, and previously, we have demonstrated that one group of genetically similar *L. monocytogenes* strains (RAPD type 9) dominate and persist in several independent fish processing plants. Strains belonging to this RAPD type showed a smaller virulence potential when investigated in Caco-2 cells, *Caenorhabditis elegans* and non-pregnant guinea pigs compared to human clinical strains.

The purpose of the present study was to determine virulence of one RAPD type 9 strain (La111), one human clinical strain (Scott A) and one monkey clinical strain (12443) in a pregnant guinea pig model. Animals were fed 10^8 CFU *L. monocytogenes* in whipping cream on gestation day (GD) 36 and sacrificed on GD 42, GD 45 and GD 56. Strains 12443 and Scott A were shed from the treated animals for 20 days whereas La111 was shed only in the first 10 days. Strains 12443 and Scott A were recovered from maternal liver, spleen and gall bladder at all three days of sacrifice, whereas La111 only was recovered at GD 45 and GD 56. Scott A was not isolated from any placentas or fetuses. When treated with 12443, 22% of the guinea pigs carried fetuses that were positive for *L. monocytogenes* and surprisingly, animals treated with La111 resulted in 56% guinea pigs with infected fetuses. *L. monocytogenes* was isolated from 16% and 20% of placentas for 12443 and La111, respectively.

In conclusion, the study demonstrates that a food processing plant persistent strain of *L. monocytogenes* is able to cross the feto-placenta barrier in pregnant guinea pigs. Furthermore, we demonstrate that although information can be gained from model virulence assays, assessment of the infective potential of a strain may require more complex hosts.

Introduction

Listeria monocytogenes is a gram-positive food borne pathogenic bacterium, and upon invasion, can result in listeriosis in humans. Outbreaks are typically associated with ready-to-eat food (RTE) products including fish products *(23, 29)*, in which the organism can grow to high numbers. The disease is relatively rare, 3.4 cases per million inhabitants in Europe *(28)*, but with a high fatality rate (25-30%) *(8)*. The disease primarily affects immunosuppressed people with underlying conditions, pregnant women, neonates and the elderly *(8)*. Between 1991-1992, the percentage of perinatal cases of listeriosis worldwide was 31-38% *(24)*.

L. monocytogenes is commonly isolated from fish processing plants, including fish slaughterhouses and smokehouses *(2, 22, 25)*. We recently demonstrated that the same Random Amplified Polymorphic DNA (RAPD) type was found as a persistent type in several fish industries *(32)*, even though this particular RAPD type is not common in the outside environment *(10)*. It is not known why strains of particular DNA sub-types persist in the food industry, but it is important to know the infection potential of these persistent strains, as they are likely contaminants of food products. The virulence potential of a strain can be assessed in several ways. This includes polymerase chain reaction (PCR) detection of virulence genes, production of virulence factors, and behavior in the epithelial cell line Caco-2 *(5, 12, 13, 15)*. Recently, the nematode *Caenorhabditis elegans (27)* and the fruit fly *Drosophila melanogaster (14, 21)* have been used to test the virulence potential of *L. monocytogenes*. *C. elegans* seems to be a good model for virulence of *L. monocytogenes*, while the *D. melanogaster* have some limitations because the non-pathogenic *Listeria innocua* are able to kill the flies *(13, 14)*.

Strains belonging to the fish processing persistent RAPD type (RAPD type 9) appear as low virulent strains in the different models described above. They do not invade Caco-2 cells as well as clinical strains of *L. monocytogenes,* and in *C. elegans* nematodes are killed at a slower rate after infection with the time to kill 50% of the worms was longer for the RAPD type 9 strains *(13)*. Also, when fed to guinea pigs, they are rapidly shed from the animals *(13)*.

Guinea pigs are an excellent model to study the virulence potential of *L. monocytogenes* strains because they are naturally sensitive to *L. monocytogenes* infection *(1, 3, 6, 31)*. Also in guinea pigs, the intestinal cell E-cadherin, receptor molecule of the *L. monocytogenes* invasion protein internalin A, has an active site that is identical to human E-cadherin *(18)*. In contrast, E-cadherin in mice has one amino acid substitution (from proline to glutamic acid) as compared to the human E-cadherin, and mice are therefore not a good animal model for oral exposure to food-borne *L.* monocytogenes.

In early studies with guinea pigs, the animals were infected intravenously, intracardially or intraperitoneally *(3, 6, 9, 20)*, but none of these reflect the oral exposure most common in humans. Recently whipping cream (38% fat) has been used as a vehicle for delivering *L.* monocytogenes in feeding studies using monkeys *(26)* and guinea pigs *(1, 13, 31)*. Guinea pigs, exposed to a whipping cream/*L. monocytogenes* cocktail, have been used to compare the infection by different *L. monocytogenes* strains *(13)* and to determine the LD50 for fetal death, which is approximately 10^7 CFU *(31)*.

The purpose of the present study was to determine the infectivity of strains representing the common persistent RAPD type 9 isolated from fish processing environments and to compare the infectivity to strains isolated from clinical cases of listeriosis in humans and monkeys. Infectivity was compared by orally exposing pregnant guinea pigs to a single strain of *L. monocytogenes* and examining invasion of maternal and fetal tissues, fecal shedding and pregnancy outcome.

Materials and methods

Bacterial strains and growth conditions: Three strains of *L. monocytogenes* were compared in this study and the origin and subtype are listed in Table 1. One of the strains (La111) belongs to a RAPD type, RAPD type 9, which is a group of bacteria frequently isolated from fish processing environments in which they often persist *(32)*. La111 was isolated from a fish product in 1996 and is, by the more discriminating sub-typing method, AFLP, identical to strains that during the last 10 years have been isolated as fish processing plant persistent strains *(32)*. Scott A has been chosen as the reference strain but is also a human clinical strain. Strain 12443 was isolated from a *Listeria*-induced stillbirth from a rhesus monkey, and has subsequently been used to induce still-births in a primate study *(26)* and to determine the dose response in a pregnant guinea pig model *(31)*. All strains were stored on CryoBank beads (CryoBank, Copan Diagnostics, Corona, CA, USA) at -80°C. Preparation of the inoculum was done as previously described by Williams et al *(31)*, with a few modifications. Briefly, each strain was grown in 10 ml Tryptic Soy Broth (TSB) (BD, Sparks, MD, USA) at 37°C for 24h with gentle shaking. Following three successive trans-fers, cultures were harvested by centrifugation (9,000 × *g* at 4°C for 10 min), washed twice and resuspended in phosphate buffer saline (PBS) (BD, Sparks, MD, USA). The washed culture was added to sterilized whipping cream (38% milk fat) to give a final concentration of 2.5×10^7 CFU/ml, and the inoculum was sweetened with 8.25% (wt/vol) of the artificial sweetener Splenda®. The control animals were treated with sweetened whipping cream plus PBS.

Bacterial cell counts of each inoculum were determined on Trypic Soy Agar (TSA) (Difco, Sparks, MD, USA). Plates were incubated for 24 h at 37°C before colony enumeration.

Bacterial cell counts isolated from fecal and tissue samples were determined on Listeria Selective Agar (LSA) (EMD, Darmstadt, Germany) to which Oxford Listeria Selective Supplement (EMD, Damstadt, Germany) was added.

Animals and treatments: Timed-pregnant Hartley guinea pigs were purchased from Elm Hill Breeding Laboratories (MA, USA) on gestation day (GD) 28. Guinea pigs were housed in cages fitted with air-filters, maintained on a 12 hour light/dark circle, and temperature and humidity were 21°C ± 2°C and 55% ± 15%, respectively. The animals were provided sterilized water and food *ad libitum*.

During a one week period of acclimation at the animal facility, weights of the guinea pigs were recorded. On GD 34 and 35 the guinea pigs were trained to drink sweetened whipping cream from a plastic transfer pipette. At GD 36, animals were treated with the *L. monocytogenes* inoculum containing approx 10^8 CFU per 4 ml of sweetened whipping cream.

After treatment, control animals and *L. monocytogenes* treated animals were kept in two separate rooms to prevent cross contamination, and they were observed daily for changes in behavior, fecal output, weight loss, still birth or any other signs of listeriosis. Fecal samples were collected every second day during the week. Guinea pigs were sacrificed by asphyxiation with CO_2 on GD 42, 45 or 56 and tissue samples were collected for further analysis.

***L. monocytogenes* confirmation in fecal tissue samples:** Fecal and tissue samples were analyzed both quantitatively and qualitatively for the presence of *L. monocytogenes*. Samples were diluted 1:10 in UVM broth (Sparks, MD, USA) and mixed in a stomacher bag. Quantitative analysis was made from the UVM enriched sample, diluted in PBS and directly plated onto LSA.

Qualitative analysis was completed after incubation of the sample in UVM broth for 24 h at 30°C. One-hundred µl of the UVM broth was transferred to a tube containing 9.9 ml Fraser broth (Oxoid, Basingstoke, England), and the sample was incubated at 37°C for 24 h. Streaks were made from the UVM enriched sample and from the Fraser enriched sample onto LSA. To obtain isolated colonies, positive samples were streaked onto TSA and incubated for 24 h at 37°C. Single colonies were streaked onto Rapid'L mono plates (Bio-Rad, Hercules, CA, USA) (24 h at 37°C) for confirmation as *L. monocytogenes*.

Statistical analysis: A student's t-test was used to determine weights and lengths differences between control and treated groups of animals with a significance level of $P < 0.05$.

Ethical considerations: All animals used in this study were handled in accordance the National Institutes of Health guidelines, and their use was approved by the University of Georgia Institutional Animal Use and Care Committee. The study was designed to avoid the use of a high number of animals. Each strain was given to a group of nine animals and each group resulted from at least two different sets of experiments.

Results

Detection of *L. monocytogenes* in fecal samples. All strains of *L. monocytogenes* were excreted in the feces of orally exposed pregnant guinea pigs. *L. monocytogenes* were detected and if possible, enumerated in fecal samples. Most of the fecal samples contained *L. monocytogenes* below our detection limit (10 CFU/g), thus fecal samples were enriched in a selective medium followed by identification of *L. monocytogenes* using the selective Rapid'L mono agar plates. Two days after the treatment (GD 38) 66% of animals treated with strain 12443, 77% of animals treated with Scott A and 100% of animals treated with La111 shed *L. monocytogenes* in their feces (Figure 1). Although Scott A shed fewer *L. monocytogenes* than the other two strains during GD 40-42, approximately 80-100% of the animals treated with Scott A were shedding this strain on GD 45-48, decreasing to 60-70% on GD 50-52 and to 33% on GD55. The number of animals shedding *L. monocytogenes* 12443 was stable at 66% for ten days after dosing (GD 48), followed by a decrease to 33% and to 0% at GD 55. Interesting, La111 was shed by 100% of the animals in the beginning of the period, followed by a decrease to 33% (GD 43-45), and then a further decrease to 0% (GD 48-55). When the results from all fecal samples were combined by strain, there was very little difference in the total number of days where *L. monocytogenes* was isolated from the feces (Table 2). Only a few fecal samples could be enumerated from direct plate counts, of these, strain 12443 and Scott A were present in approximately 10^5 CFU/g feces whereas La111 was present in approximately 10^2 CFU/g feces (Table 2).

Invasion of maternal liver, spleen and gall bladder. All strains of *L. monocytogenes* were able to cross the intestinal barrier and invade the livers, spleens and gall bladders of orally exposed pregnant guinea pigs (Figure 2). Monkey clinical strain 12443 invaded the tissues more consistently than the other strains, and was isolated from seven of the nine livers (Figure 2). The human clinical strain Scott A was isolated from six of the nine livers, while La111, the food processing persistent strain was isolated in only five of the nine livers (Figure 2). Interestingly, the food processing persistent strain La111 was not detected in any of the maternal or fetal tissues at GD 42 (Figure 2 and Table 3).

Visible hepatic lesions were observed on some of the maternal livers, but animals having these lesions did not shed more *L. monocytogenes* than animals with normal livers (results not shown).

Invasion of placental and fetal tissue. The three strains differed in their ability to invade the placenta and fetus. Invasion of the placenta and fetus occurred mainly by GD 45 for strain La111, by GD 56 for strain 12443, whereas Scott A was not detected in any placentas or fetuses at the time periods examined (Table 3). Twenty-two percent (2 of 9) of the guinea pigs treated with strain 12443 did carry at least one infected fetus even though strain 12443 not was isolated from placentas and fetal tissues at GD 42 or at GD 45. But at GD 56, 12443 was detected in placenta, fetal liver and fetal brain.

When treated with the food processing persistent La111, 56% (5 of 9) of the guinea pigs carried an infected fetus (Table 3). At the first day of sacrifice (GD 42) *L. monocytogenes* La111 could not be isolated from any placentas or fetuses, but at GD 45 and GD 56, *L. monocytogenes* were isolated from both placentas and fetal livers.

Size and weight of the outcome

Weights and lengths were measured for all the fetuses (Table 4). When pregnant guinea pigs were treated with strain 12443, the weights of the infected fetuses sacrificed at GD 56 were not different from the control fetuses ($P < 0.05$). Similar was seen for infected fetuses from guinea pigs treated with La111 at GD 56 ($P < 0.05$), but the weight of La111 positive fetuses were smaller at GD 45 as compared to control ($P < 0.05$). The lengths of all the fetuses (infected or not infected) were the same as the lengths of the respective controls.

Discussion

Determining the virulence of *L. monocytogenes* is difficult because of the high mortality rate (20-30%) precluding the use of human volunteers. Thus, it is important to choose an animal model where the exposure and disease process is similar to humans. Challenging pregnant guinea pigs with *L. monocytogenes* suspended in a food matrix closely mimics human exposure in foods. Similarly, pregnant guinea pigs have been shown to have stillbirths in response to *L. monocytogenes* infection *(31)* in a similar manner to humans. Virulence depends on several different factors such as strain differentiation, modes of infection, and status of the host (including pregnancy). We report here, for the first time, the out-come of testing a representative of a food processing plant persistent *L. monocytogenes* strain in an orally exposed pregnant guinea pig model. Because the persistent strains are likely to remain in the food processing environment over long periods of time and serve as a constant source of contamination, it is very important to understand the risk they represent. Previously, we have demonstrated that food processing persistent RAPD type 9 strains invade Caco-2 cells to a lesser degree, are lower in virulence in the nematode *C. elegans* and the fruit fly *D. melanogaster*, and shed fewer numbers in fecal from infected guinea pigs as compared to human clinical strains of *L. monocytogenes (13)*. In this study, we demonstrate that in the pregnant guinea pig model, these strains may represent a high risk group due to their remarkable ability to cross the placental barrier and invade fetal tissues. The three different strains of *L. monocytogenes* used in this study behave differently in the pregnant guinea pig model and their infectivity may not be reflected by simpler virulence models.

The pregnant guinea pig model was used as a surrogate model for oral exposure in humans based on similarities in the clinical symptoms. Fecal shedding has previously shown to be an acceptable marker for infection of *L. monocytogenes*, because a positive correlation was seen between the number of *L. monocytogenes* in feces and the birth outcome in pregnant guinea pigs and monkeys *(26, 31)*. However, in this study, fecal shedding was not correlated with isolation of *L. monocytogenes* from fetal tissue samples, although no stillbirths occurred in any of the groups. This may have been due to the small number of pregnancies (n = 3) allowed to proceed until near term. Earlier pregnancies may simply have not had enough time for fetal death to occur.

The presence of white foci on the surface of livers was seen at GD 45 and GD 56 for animals treated with Scott A and strain 12443 and this is in agreement with Dustoor et al. *(6)*. It has been suggested that serum alanine aminotransferase levels can serve as an indicator of liver damage, but no correlation was seen between visual liver damage and the measured ALT level *(31)*. Another indicator of tissue damage is detection of apoptosis. Measurement of apoptosis in placentas from animals treated with strain 12443 has been done by Irvin et al *(11)*. The amount of placentas positive for apoptosis increased from 73% at GD 42 and GD 45 to 100% at GD 56 and was at all times 55% for the control. Therefore, even though no visible tissue damage was seen, a high number of placentas were starting to degenerate.

One could expect a smaller weight of fetuses from infected mothers, but no systematic differences between weight and length of the fetuses depending on *L. monocytogenes* strain were seen. Williams et al *(31)* did see a significant increase of fetal weight from animals treated with a low level (10^4 CFU) of strain 12443. For higher levels of treatment (10^5 – 10^8 CFU), there were no differences between weights and lengths of treated animals.

Invasion assay into the epithelial intestinal cell line Caco-2 cells was done according to Jensen et al *(13)* and La111 did not invade Caco-2 cells as well as strains 12443 and Scott A (results not shown). We have demonstrated that *inlA* in La111 and other strains of the same RAPD subtype contain two single point mutations at the 3' region of *inlA* as compared to other strains with a higher ability to invade Caco-2 cells *(13)*. These mutations could result in an altered protein structure leading to lower affinity of InlA to E-cadherin on the surface of Caco-2 cells. Despite a decreased ability of La111 to invade the type of cells first encountered when *L. monocytogenes* is infecting a host, La111 and strain 12443 were detected in placenta and fetus at the same level. Also, La111 was detected in placentas and fetuses at both GD 45 and GD 56, where strain 12443 was detected in placentas and fetuses only at GD 56. The physical barrier separating fetal and maternal blood in the placenta is mainly formed by fetally derived trophoblastic cells. There is evidence that the trophoblast cells play a central role in vertical transmission of *L. monocytogenes* from the mother to the fetus *(17)*. Several research groups have studied the virulence factors required to cross the fetoplacental barrier *(3, 16, 17, 19)*. *Listeria* internalization protein InlA is required for internalization in the human trophoblastic cell line (BeWo) *(3)*, but in pregnant guinea pigs and pregnant mice, InlA is not required for invasion into the placenta *(3, 16)*. La111 encodes for two single mutations in *inlA* and is still able to invade placenta and fetal liver suggesting that *inlA* is not required for placental and fetal invasion. Both LLO and ActA are important factors in fetoplacental invasion *(16)*. LLO is necessary when bacteria are escaping the phagosomes of trophoblastic cells, and therefore a LLO deletion mutant is unable to spread from placenta to fetus *(16)*. Crossing the murine fetoplacental barrier requires ActA-dependent cell-to-cell spreading that allows bacteria to cross the trophoblastic cell layers separating fetal and maternal blood vessels *(16)*. An enhanced ActA production in La111 might result in an increase in the spread of La111 to placenta and fetus. However, La111 was not able to spread to the maternal tissues as rapidly as strain 12443, since La111 was not detected in maternal liver, spleen, gallbladder at GD 45, where strain 12443 was detected in liver and gallbladder at GD 45. Therefore the reason for enhanced ability of La111 to spread to the placenta and fetus needs to be investigated further. It was unexpected that Scott A did not spread to the placentas and fetuses, because it is normally characterized as a highly virulent strain.

In conclusion, a food processing persistent strain belonging to RAPD type 9 was virulent in pregnant guinea pigs based on its ability to cross the feto-placental barrier and infect fetuses. Its invasive capability was similar to that of a monkey clinical strain, which in other studies have shown to be highly virulent. Using simpler virulence models, we have found that strains belonging to the RAPD type 9 were less invasive in Caco-2 cells, and less virulent against the nematode *C. elegans* and the fruit fly *D. melanogaster*. Therefore one should be cautious when drawing conclusions on degree of virulence when using the simple models.

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Figure legends

Figure 1: Percent of pregnant guinea pigs shedding *Listeria monocytogenes* in feces after oral exposure. One sample before treatment was collected to assure that animals were not shedding *L. monocytogenes* before treatment. Strains are as follows: strain 12443 (×), Scott A (▲) and La111 (■). Each number is an average of 3-9 animals.

Figure 2: Percent of guinea pigs positive for *Listeria monocytogenes* in maternal liver, spleen and gall bladder at days of sacrifice. Gestation day 42 (□), gestation day 45 (▒) and gestation day 56 (■), corresponding to 6, 9, and 21 days post-treatment. Each column is an average of 3 animals.

Table 1: Origin and sub-type of *Listeria monocytogenes* strains used in the present study.

Strain	Origin	Serotype	Lineage	RAPD type	Reference
La111	Cold smoked salmon	1/2a	2	9	*(30)*
Scott A	Human, clinical	4b	1	72	*(4)*
12443	Monkey, clinical	1/2a	2	73	*(26)*

Table 2: Isolation of *Listeria monocytogenes* from fecal samples and the maximum cell count in feces from pregnant guinea pigs orally exposed to *L. monocytogenes* strains

Strain	No. of samples positive/total samples collected[a] (%)	Maximum cell count in fecal sample (CFU/g)
12443	29/47 (62%)	1.6×10^5
Scott A	25/40 (63%)	3.3×10^5
La111	22/42 (52%)	4.0×10^2

[a] The number of positive fecal samples were added for all guinea pigs treated with a specific strain/the total number of fecal samples collected from all guinea pigs in that group.

Table 3: Fetal infection after maternal oral exposure to *Listeria monocytogenes*.

Strain	No. guinea pigs with infected fetus [a] / total (%)	No. infected fetuses / total (%)	No. infected samples / total samples (%)		
			Placenta	Fetal liver	Fetal brain
12443	2/9 (22%)	4/31 (13%)	5/31 (16%)	4/31 (13%)	1/31 (3%)
GD 42	0/3 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
GD 45	0/3 (0%)	0/14 (0%)	0/14 (0%)	0/14 (0%)	0/14 (0%)
GD 56	2/3 (67%)	4/9 (44%)	5/9 (56%)	4/9 (44%)	1/9 (11%)
La111	5/9 (56%)	6/49 (12%)	10/49 (20%)	6/49 (12%)	0/49 (0%)
GD 42	0/3 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
GD 45	3/3 (100%)	4/20 (20%)	5/20 (25%)	4/20 (20%)	0/20 (0%)
GD 56	2/3 (67%)	2/14 (14%)	5/14 (36%)	2/14 (14%)	0/14 (0%)
Scott A [b]	0/9 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)	0/30 (0%)

[a] An infected fetus is a fetus that had either an infected liver or brain.

[b] No placentas or fetuses from dams treated with Scott A were positive for *L. monocytogenes*.

Table 4: Average weights and lengths of fetuses infected with different strains of *Listeria monocytogenes* and fetuses from the control. NI: Not-infected fetus, I: Infected fetus. Different letters in superscript denotes weights and length that are statistically independent ($P < 0.05$)

		Weight (g)			Length (mm)		
Strains		GD 42	GD 45	GD 56	GD 42	GD 45	GD 56
Scott A	I	-	-	-	-	-	-
	NI	17.0 ± 2.7^{A}	21.5 ± 2.7^{B}	66.3 ± 5.2^{D}	42.3 ± 4.9^{H}	44.25 ± 6.1^{I}	64.6 ± 4.1^{J}
12443	I	-	-	52.2 ± 4.4^{C}	-	-	98.0 ± 9.6^{J}
	NI	18.5 ± 2.1^{A}	22.7 ± 2.8^{D}	50.6 ± 5.4^{C}	43.9 ± 3.2^{H}	45.1 ± 3.7^{I}	75.8 ± 16.4^{J}
La111	I	-	21.7 ± 3.9^{E}	58.5 ± 7.8^{C}	-	41.3 ± 6.9^{I}	96.8 ± 1.4^{J}
	NI	17.4 ± 5.0^{A}	23.0 ± 4.7^{F}	69.8 ± 16.2^{G}	40.1 ± 6.6^{H}	42.8 ± 7.0^{I}	88.4 ± 8.7^{J}
Control		17.0 ± 1.9^{A}	31.6 ± 4.4^{B}	48.2 ± 11.5^{C}	45.2 ± 2.7^{H}	50.1 ± 6.1^{I}	90.3 ± 5.3^{J}

Figure 1

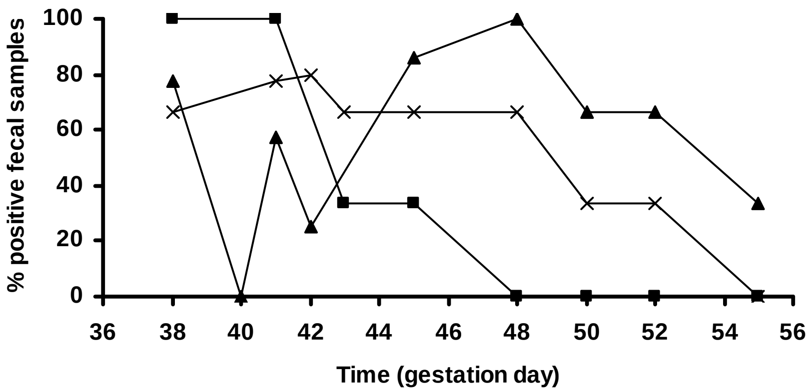
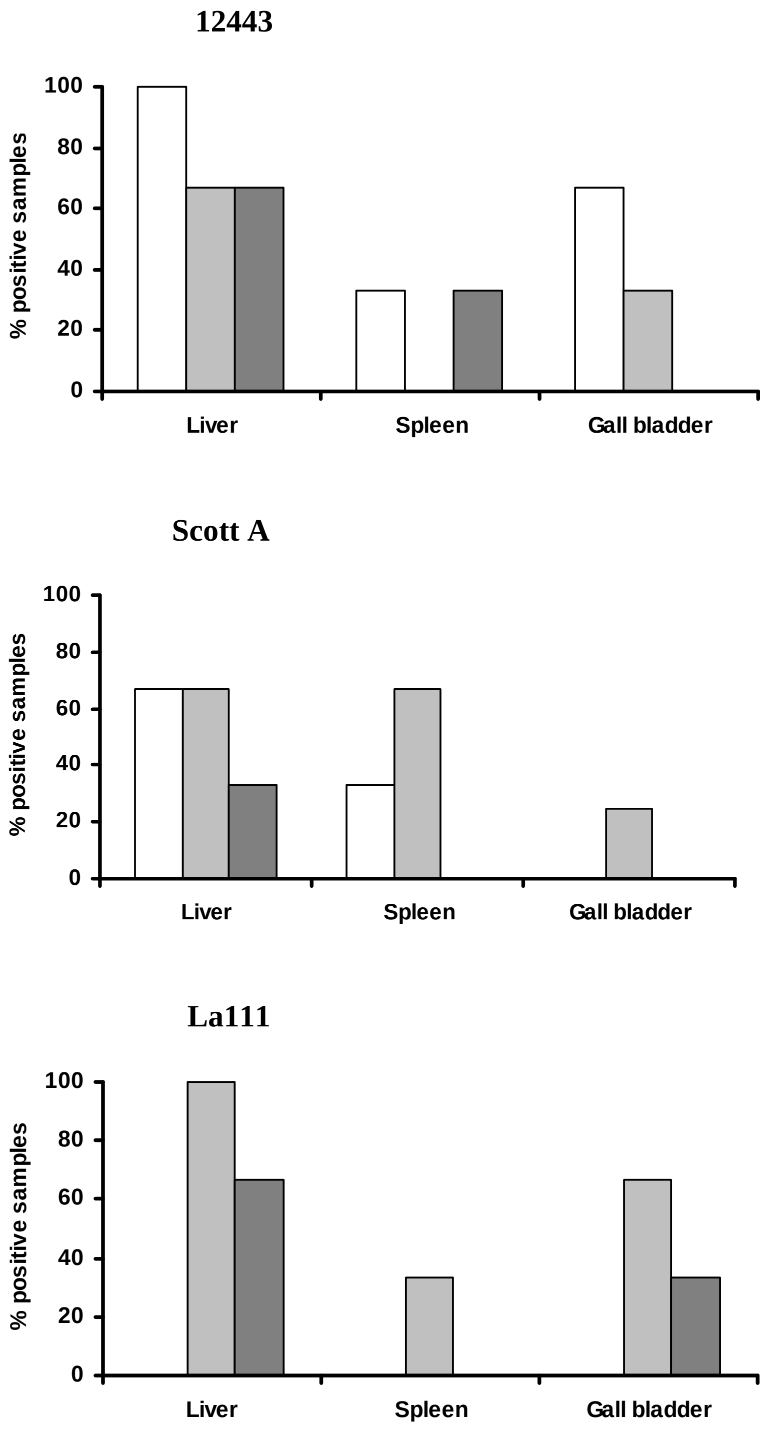

% positive fecal samples
100
80
60
40
20
0
36
38
40
42
44
46
48
50
52
54
56
Time (gestation day)

Figure 2


12443
% positive samples
100
80
60
40
20
0
Liver
Spleen
Gall bladder
Scott A
% positive samples
100
80
60
40
20
0
Liver
Spleen
Gall bladder
La111
% positive samples
100
80
60
40
20
0
Liver
Spleen
Gall bladder