Oxygen restriction increases the infective potential of Listeria monocytogenes in vitro in Caco-2 cells and in vivo in guinea pigs

Andersen, Jens Bo; Roldgaard, Bent; Christensen, Bjarke Bak; Licht, Tine Rask

Published in:
BMC Microbiology

Link to article, DOI:
10.1186/1471-2180-7-55

Publication date:
2007

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Andersen, J. B., Roldgaard, B., Christensen, B. B., & Licht, T. R. (2007). Oxygen restriction increases the infective potential of Listeria monocytogenes in vitro in Caco-2 cells and in vivo in guinea pigs. BMC Microbiology, 7, 55. DOI: 10.1186/1471-2180-7-55

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Oxygen restriction increases the infective potential of *Listeria monocytogenes* in vitro in Caco-2 cells and in vivo in guinea pigs

Jens Bo Andersen, Bent B Roldgaard, Bjarke Bak Christensen and Tine Rask Licht*

Address: National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

Email: Jens Bo Andersen - jeban@food.dtu.dk; Bent B Roldgaard - bebro@food.dtu.dk; Bjarke Bak Christensen - bbc@food.dtu.dk; Tine Rask Licht* - trl@food.dtu.dk

* Corresponding author

Abstract

**Background:** *Listeria monocytogenes* has been implicated in several food borne outbreaks as well as sporadic cases of disease. Increased understanding of the biology of this organism is important in the prevention of food borne listeriosis.

The infectivity of *Listeria monocytogenes* ScottA, cultivated with and without oxygen restriction, was compared in vitro and in vivo. Fluorescent protein labels were applied to allow certain identification of *Listeria* cells from untagged bacteria in in vivo samples, and to distinguish between cells grown under different conditions in mixed infection experiments.

**Results:** Infection of Caco-2 cells revealed that *Listeria* cultivated under oxygen-restricted conditions were approximately 100 fold more invasive than similar cultures grown without oxygen restriction. This was observed for exponentially growing bacteria, as well as for stationary-phase cultures.

Oral dosage of guinea pigs with *Listeria* resulted in a significantly higher prevalence (p < 0.05) of these bacteria in jejunum, liver and spleen four and seven days after challenge, when the bacterial cultures had been grown under oxygen-restricted conditions prior to dosage. Additionally, a 10–100 fold higher concentration of *Listeria* in fecal samples was observed after dosage with oxygen-restricted bacteria. These differences were seen after challenge with single *Listeria* cultures, as well as with a mixture of two cultures grown with and without oxygen restriction.

**Conclusion:** Our results show for the first time that the environmental conditions to which *L. monocytogenes* is exposed prior to ingestion are decisive for its in vivo infective potential in the gastrointestinal tract after passage of the gastric barrier. This is highly relevant for safety assessment of this organism in food.
Background
During the last two decades, *Listeria monocytogenes* has been implicated in several food borne outbreaks and sporadic cases of disease [1,2]. Foods implicated in food-borne listeriosis are generally highly processed foods with prolonged shelflives, supportive of growth of the organism [3]. Several attempts have been made to establish quantitative microbiological criteria for the presence of *L. monocytogenes* in foods [4,5], by use of dose-response predictions. However, there are indications that the number of bacteria ingested is not the only important determinant for the development of illness. For many enteric pathogens also including *Listeria*, it is well known that given environmental conditions induce the expression of identified virulence genes and/or contribute to their invasive potential in *in vitro* models [6-15]. However, to our knowledge, no reports describe a direct effect of such conditions on the virulence phenotype of these bacteria *in vivo*. It has thus not been shown whether an induced expression of virulence factors in intestinal pathogens at the time of ingestion affects their virulence *in vivo* after passage of the gastric barrier.

The objective of the present study was to investigate whether the physiological state of *L. monocytogenes* prior to ingestion (i.e. determined by the food environment), here exemplified by oxygen availability, could influence its ability to cause infection.

Recent reports point out that mice and rats are not suitable as animal models for human listerial infectivity, since the bacterium does not interact with the epithelial receptor of these animals [16,17]. A guinea pig model [BB Roldgaard, JB Andersen, TR Licht and BB Christensen, submitted] was therefore used for *in vivo* studies of infectivity in the present study, while Caco-2 cells were applied to assess the infective potential *in vitro*. In any animal model, variation between individuals is unavoidable. In order to circumvent this variation in the comparative study, challenge of the animals with a mixture of *L. monocytogenes* ScottA cells cultivated under conditions of different oxygen availability, was included in the investigation. For this purpose, a recently developed fluorescence labeling system [18] was applied, making it possible to distinguish between otherwise isogenic *Listeria* cells originating from cultures grown with and without oxygen-restriction.

The obtained results reveal that oxygen-restriction clearly increases the infective potential of *L. monocytogenes in vitro* and *in vivo*.

Results

**Invasiveness of oxygen-restricted and un-restricted *L. monocytogenes* in Caco-2 cells**
Caco-2 cell assays revealed that cultures of *L. monocytogenes*-CFP, grown to selected densities under oxygen-restricted conditions were approximately 100 fold more invasive than corresponding cultures grown without oxygen restriction (Figure 1). Similar results were obtained with *L. monocytogenes*-YFP, as well as with stationary-phase overnight cultures grown with and without oxygen restriction (data not shown).

**Infection of guinea pigs with monocultures of oxygen-restricted and un-restricted *L. monocytogenes***
Exponentially growing cultures (OD<sub>600</sub> = 1.0) were used for oral dosage of guinea pigs. *L. monocytogenes*-CFP was recovered from the liver and jejunum of half of the 12 animals dosed twice with the un-restricted bacterial cultures, and was found in the spleen of a single animal. The occurrence of *Listeria* in organs of animals dosed with oxygen-restricted bacteria was significantly higher (p < 0.05); the pathogen was recovered from jejunum of all of the 12 animals, and was found in the liver and spleen of ten and seven of the guinea pigs, respectively. The average concentrations of *Listeria* found in positively infected organs were not different in the two groups (Table 1).

Throughout the experiment, fecal counts of *Listeria* remained significantly higher in the animals dosed with oxygen-restricted *L. monocytogenes*-CFP than in those dosed with the un-restricted, identical strain. The concentration of *Listeria* in feces reached a level of 10<sup>8</sup>–10<sup>7</sup> per million bacteria per milled in the bacterial cultures prior to infection. Each bar represents an average of three different experiments. Error bars designate standard deviations. OD600 refers to optical density at 600 nm.

![Figure 1](http://www.biomedcentral.com/1471-2180/7/55)
gram on the days immediately after dosage, which was carried out on Day 0 and Day 1 of the experiment. Hereafter, the concentration in animals dosed with \textit{L. monocytogenes} grown under unrestricted conditions dropped and remained at a level of approximately $10^3$, while the fecal \textit{Listeria} concentration in animals dosed with oxygen-restricted cells stayed between 1 and 3 logs higher (Figure 2A).

**Competitive infection of guinea pigs with oxygen-restricted and un-restricted \textit{L. monocytogenes}**

Oral dosage of 24 guinea-pigs with a mixture of oxygen-restricted and un-restricted, exponentially growing \textit{L. monocytogenes} revealed that also in a mixed challenge experiment, the occurrence of the oxygen-restricted strain in internal organs was significantly higher ($p < 0.05$) than observed for the un-restricted strain. \textit{Listeria}, which had been grown under un-restricted conditions, was detected in the liver and jejunum of 2 animals, and in the spleen of 4 animals, while \textit{Listeria} grown under oxygen-restricted conditions prior to dosage was recovered from liver, spleen and jejunum of 18, 12, and 14 animals, respectively. It had no influence on the infectivity ($p = 0.165$) whether the \textit{L. monocytogenes} cells were labeled with CFP or YFP. The average concentrations of \textit{Listeria} found in positively infected organs were similar for oxygen-restricted and un-restricted bacteria (Table 2).

In all animals, fecal concentrations of \textit{Listeria} grown under oxygen-restricted conditions prior to dosage were higher than concentrations of cells grown without oxygen restriction (Figure 2B). This was observed independently of which fluorescent label was used to identify the bacteria (Figure 2C and 2D). The kinetics of the pathogen occurrence in feces observed in the mixed infection experiment were quite similar to what was observed after dosage with monocultures (Figure 2).

**Discussion**

We have shown that oxygen-restriction prior to ingestion significantly ($p < 0.05$) enhances the \textit{in vivo} infective potential of \textit{L. monocytogenes} ScottA (Table 1 and 2, Figure 2). This has to our knowledge not previously been shown, and signifies that gene expression occurring in \textit{Listeria} before intake influences its infective potential even after passage of the oral/gastric barrier. The trend in the results is that the prevalence of \textit{Listeria} in internal organs

![Figure 2](http://www.biomedcentral.com/1471-2180/7/55)

**Figure 2**

**Fecal densities.** Concentration of \textit{L. monocytogenes} in fecal samples of animals dosed with un-restricted (open symbols) and/or oxygen-restricted (closed symbols), fluorescence-labeled bacteria. (A): Animals dosed with monocultures of CFP-labeled bacteria. (B): Integrated presentation of data from C and D. (C): Animals dosed with a mixture of CFP-labeled, un-restricted \textit{Listeria} and YFP-labeled, oxygen-restricted \textit{Listeria}. (D): Animals were dosed with a mixture of YFP-labeled, un-restricted \textit{Listeria} and CFP-labeled, oxygen-restricted \textit{Listeria}. All animals were dosed at Day 0 and again at Day 1. Each data point in panel A and B represents the average of samples from 12 animals, while each data point in panel C and D represents the average of samples from 6 animals. Error bars designate standard errors of the means.

| Days post first dosage | Un-restricted | Oxygen-restricted |
|------------------------|--------------|------------------|
|                        | 4            | 7                |
| **Liver**              | 3/6          | 3/6              |
|                        | 6/12 (50%)   | 6/6              |
|                        | 2.2 ± 0.34   | 4/6              |
|                        |              | 10/12 (83%)      |
|                        |              | 3.0 ± 0.88       |
| **Spleen**             | 0/6          | 1/6              |
|                        | 1/12 (8%)    | 4/6              |
|                        | 2.8          | 3/6              |
|                        |              | 7/12 (58%)       |
|                        |              | 2.6 ± 0.67       |
| **Jejunum**            | 2/6          | 4/6              |
|                        | 6/12 (50%)   | 6/6              |
|                        | 2.1 ± 0.47   | 6/6              |
|                        |              | 12/12 (100%)     |
|                        |              | 3.7 ± 0.70       |

Numbers of guinea pigs where \textit{L. monocytogenes}-CFP was recovered from liver and spleen after oral dosage with un-restricted or oxygen-restricted \textit{L. monocytogenes}-CFP, respectively. Mean log (CFU/g) designate the mean levels found in animals positive for \textit{Listeria} in the given organs, followed by standard deviations.
animals carrying

While oxygen restriction clearly affected the number of
gut.
are all exposed to similar environmental conditions in the
expected to approach each other over time, since the cells
present in the cultures at the time of ingestion must be
which is not surprising because the expression patterns
reports indicate that anaerobic physiology contributes sig-
ificantly to an enhanced production of InlA in the human gut [26], and may therefore be
important for bacterial attachment to the epithelial wall. We observed that oxygen-restriction significantly
increased the prevalence of L. monocytogenes in the jeju-
num of guinea-pigs (Table 1 and 2), suggesting that an
increased InlA-expression occurring prior to ingestion
causd increased attachment of L. monocytogenes to the
jejunal mucosa. We speculate that the observed increased
translocation to spleen and liver (Tables 1 and 2), as well
as the increased invasion of Caco-2 cells (Figure 1) may be
attributed to an increased initial InlA-mediated attach-
ment to the epithelial receptors. However, also other
genes are reported to be induced under oxygen restricted
conditions and to affect attachment of Listeria [27].

We suggest that the observed increased infectivity of L. monocytogenes grown under oxygen-restricted conditions can be attributed to an increased expression of the Intern-
alinA (InlA) protein, which is known to be a key factor for
virulence of L. monocytogenes [23], on the surface of the
bacterial cells. In concordance with this hypothesis, recent
reports indicate that anaerobic physiology contributes sig-
ificantly to an enhanced production of InlA in the human gut [26], and may therefore be
important for bacterial attachment to the epithelial wall. We observed that oxygen-restriction significantly
increased the prevalence of L. monocytogenes in the jeju-
num of guinea-pigs (Table 1 and 2), suggesting that an
increased InlA-expression occurring prior to ingestion
causd increased attachment of L. monocytogenes to the
jejunal mucosa. We speculate that the observed increased
translocation to spleen and liver (Tables 1 and 2), as well
as the increased invasion of Caco-2 cells (Figure 1) may be
attributed to an increased initial InlA-mediated attach-
ment to the epithelial receptors. However, also other
genes are reported to be induced under oxygen restricted
conditions and to affect attachment of Listeria [27].

The percentage of animals in which Listeria was recovered
from internal organs was significantly lower (p < 0.05) in
animals infected with mixed cultures, than in animals
infected with monocultures (Tables 1 and 2). The sensitiv-
ity of the mixed culture infection approach was thus
slightly lower than observed for infection with monocul-
tures, probably because the dosed numbers of cells grown
under a given condition in the mixed infections were only
half of the corresponding numbers dosed as monocul-
tures. Total numbers of cells in each dosage were similar
in the two approaches. In spite of this, the observed differ-
ence between oxygen-restricted and un-restricted Listeria
was more significant in the mixed culture experiment
(\( P_{\text{liver}} < 0.0001; P_{\text{spleen}} = 0.0143; P_{\text{jejunum}} = 0.0002 \)) than in
the monoculture infections (\( P_{\text{liver}} = 0.0833; P_{\text{spleen}} =
0.0094; P_{\text{jejunum}} = 0.0047 \)) due to the higher amount of

increases from Day 4 to Day 7 post challenge with the un-
restricted bacteria, while a decrease in prevalence is seen
in the same period after dosage with the oxygen-restricted
bacteria (Table 1). The effect of oxygen-restriction prior to
dosage is thus larger on Day 4 than on Day 7 post dosage,
which is not surprising because the expression patterns
present in the cultures at the time of ingestion must be
expected to approach each other over time, since the cells
are all exposed to similar environmental conditions in the
gut.

While oxygen restriction clearly affected the number of
animals carrying L. monocytogenes in their internal organs,
the concentration of bacteria present in positively infected
organs was not affected (Table 1 and 2). This suggest that
oxygen restriction increases the initial translocation of Lis-
teria from the gut lumen to internal organs, but does not
influence the ability of the bacteria to proliferate inside
the investigated organs.

Obviously, an increased ability to survive the gastric bar-
ger will increase the probability of causing an infection
[19,20]. It is well known, that genes involved in many
kinds of stress-responses are co-regulated, and that expos-
ure to one type of stress therefore improves the ability to
survive another type of stressful condition [21,22]. It
could thus be speculated, that exposure to oxygen restric-
tion would increase the viability of L. monocytogenes under
low pH and/or its resistance to gastric enzymes. This is
however not the full explanation for our observations,
since also the ability of this strain to infect Caco-2 cells in
vitro is significantly increased by oxygen restriction (Figure 1).
In the in vitro studies, the effect of oxygen restriction
was seen for exponentially growing cultures as well as for
cultures in stationary phase, and the induction of the vir-
ulent phenotype was thus expected to be independent of
the growth stage of the bacterial cells (Figure 1). Still, we
chose to use exponentially growing cultures for the ani-
mal studies in order to eliminate putative contributions to
in vivo infectivity from the many genes known to be
induced in stationary phase.

| Fluorescence | CFP | YFP | Total | Mean log(CFU/g) | CFP | YFP | Total | Mean log(CFU/g) |
|--------------|-----|-----|-------|----------------|-----|-----|-------|----------------|
| Liver        | 1/12| 1/12| 2/24 (8%) | 1.5 ± 0.21 | 10/12| 8/12| 18/24 (75%) | 1.9 ± 0.32 |
| Spleen       | 3/12| 1/12| 4/24 (17%) | 1.9 ± 0.76 | 7/12| 5/12| 12/24 (50%) | 2.1 ± 0.58 |
| Jejunum      | 1/12| 1/12| 2/24 (8%) | 2.2 ± 0.21 | 8/12| 6/12| 14/24 (58%) | 3.1 ± 0.65 |

Numbers of guinea pigs where L. monocytogenes was recovered from liver and spleen after oral dosage with a 1:1 mixture of un-restricted and oxygen-restricted L. monocytogenes, carrying either of the two different fluorescent labels CFP and YFP. Samples were taken either four or seven days post first dosage. Mean log (CFU/g) designate the mean levels found in animals positive for Listeria in the given organs, followed by standard deviations.

Table 2: L. monocytogenes mixed culture infections in guinea pigs

Increases from Day 4 to Day 7 post challenge with the un-
restricted bacteria, while a decrease in prevalence is seen
in the same period after dosage with the oxygen-restricted
bacteria (Table 1). The effect of oxygen-restriction prior to
dosage is thus larger on Day 4 than on Day 7 post dosage,
which is not surprising because the expression patterns
present in the cultures at the time of ingestion must be
expected to approach each other over time, since the cells
are all exposed to similar environmental conditions in the
gut.

While oxygen restriction clearly affected the number of
animals carrying L. monocytogenes in their internal organs,
the concentration of bacteria present in positively infected
organs was not affected (Table 1 and 2). This suggest that
oxygen restriction increases the initial translocation of Lis-
teria from the gut lumen to internal organs, but does not
influence the ability of the bacteria to proliferate inside
the investigated organs.

Obviously, an increased ability to survive the gastric bar-
ger will increase the probability of causing an infection
[19,20]. It is well known, that genes involved in many
kinds of stress-responses are co-regulated, and that expos-
ure to one type of stress therefore improves the ability to
survive another type of stressful condition [21,22]. It
could thus be speculated, that exposure to oxygen restric-
tion would increase the viability of L. monocytogenes under
low pH and/or its resistance to gastric enzymes. This is
however not the full explanation for our observations,
since also the ability of this strain to infect Caco-2 cells in
vitro is significantly increased by oxygen restriction (Figure 1).
In the in vitro studies, the effect of oxygen restriction
was seen for exponentially growing cultures as well as for
cultures in stationary phase, and the induction of the vir-
ulent phenotype was thus expected to be independent of
the growth stage of the bacterial cells (Figure 1). Still, we
chose to use exponentially growing cultures for the ani-
mal studies in order to eliminate putative contributions to
in vivo infectivity from the many genes known to be
induced in stationary phase.
data resulting from the mixed-infection approach. This shows that the co-infection model, which has the advantage of elimination of variations attributed to individual animals, additionally had a better discriminatory power than a monoculture approach involving the same number of animals.

**Conclusion**

Our results are of particular importance for the risk assessment of *Listeria* in food. For the first time, we have shown that the environmental conditions to which a bacterium is exposed before ingestion can be decisive for its infective potential when it reaches the gut. This means that not only the number of *Listeria* present in a given food item, but also the physiological condition of these bacteria is important for food safety. The *in vitro* and *in vivo* data suggest that an oxygen-restricted *L. monocytogenes* cell represents a significantly higher risk than a cell grown without oxygen restriction. This should be taken into account in future quantitative risk profiling and dose-response models.

**Methods**

**Strains and media**

The clinical isolate, *Listeria monocytogenes* ScottA, carrying erythromycin resistance, and labeled with either Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP) as previously described [18] was used in all experiments. Bacteria were cultivated on BHI-agar (Oxoid) or in liquid BHI (Oxoid) buffered with 100 mM 3-(N-Morpholino)-propanesulfonic acid (MOPS), pH = 6. When appropriate, Erythromycin (Sigma) was used at a final concentration of 10 µg/mL and Nalidixic acid at a final concentration of 100 µg/mL.

**Preparation of *L. monocytogenes* for infection studies**

A fluorescent single colony of *L. monocytogenes* was inoculated into 10 mL MOPS buffered BHI media supplemented with Erythromycin and incubated at 37°C for 8 hours. The resulting cultures, for which the optical densities (OD600) ranged between 1.5 and 2, were subsequently diluted between 10^4 and 10^5 fold into 2 L Bluecap flasks containing 450 mL MOPS buffered BHI medium supplemented with Erythromycin.

To obtain oxygen-restricted cultures of *Listeria monocytogenes* ScottA, atmospheric air above the diluted cultures was exchanged with sterile Nitrogen and the lid of the Bluecap flasks was tightened and sealed prior to incubation. To obtain non-restricted cultures, the Bluecap flasks were incubated with the lid loosely tightened allowing free exchange of atmospheric air. All cultures were incubated at 37°C in a rotary shaker set at 200 rpm and samples for *in vitro* invasion studies and *in vivo* infection studies were taken after approximately 20 hours of incubation, when they reached the optical densities reported below.

**Conclusion**

Samples for *in vitro* invasion studies were taken at OD_{600} = 0.5 (exponential phase), OD_{600} = 1.0 (exponential phase), and from cultures that had been in stationary phase for approximately 10 hours (referred to as over night cultures). The samples were diluted directly into 37°C MEM medium (Gibco) to a concentration of 10^7 bacteria/mL and immediately used for challenge of Caco-2 cells as described below.

**Caco-2 cell infection experiments**

Enterocyte-like Caco-2 cells were cultivated and prepared as previously described [28]. Bacteria were cultivated as described above to the desired optical density and diluted in 37°C MEM medium (Gibco) to a concentration of 10^7 bacteria/mL immediately before the invasion assays. One ml of bacterial culture was applied to each well, resulting in a multiplicity of infection of approximately 25 bacteria per Caco-2 cell. Following 1 hour of invasion and 2 hours of gentamycin treatment to kill extracellularly located bacteria, Caco-2 cells were lysed and the numbers of *Listeria* present in each well was estimated as described in the section 'Enumeration of *L. monocytogenes* in samples'. Sampling was done in triplicate, and the experiments were performed twice.

**Animal experiments**

Male and female Hartley guinea pigs (Charles River Laboratories; Germany) with a weight of 275 g (± 10 g) were used. After seven days of acclimatization in pens (custom made, 90 × 130 × 61 cm), animals were randomized and housed individually in Polycarbonate cages, Eurostandard Type III H (425 × 266 × 185 mm) with Tapvei bedding (peeled Aspen hardwood, Tapvei Kaavi, Finland) in negatively pressurized isolators. Fecal samples from the guinea pigs were tested by plating on Palcam agar (Oxoid) to verify the absence of *Listeria* prior to dosage.
A total of 48 guinea pigs were dosed with *L. monocytogenes* ScottA. Two groups of 12 animals were dosed with monocultures of CFP-labelled bacteria, cultivated either with or without oxygen-restriction. Two other groups of 12 animals were dosed with a 1:1 mixture of oxygen-restricted and un-restricted bacteria carrying the two different fluorescent labels CFP and YFP. In one of these groups, it was the oxygen-restricted *Listeria*, which carried the CFP label, while in the other group the labels were reversed so that the unrestricted bacteria were labelled with CFP.

All animals were dosed with 0.5 ml double cream containing 38 % milk fat and approximately $5 \times 10^{10}$ *L. monocytogenes* at Day 0 and again at Day 1. The number of cells in the inoculum was approximately the same in the mono- and mixed cultures.

Dosage was done directly in the oral cavity, between the incisors and the molars. Following dosage the animals had access to food and water *ad libitum* throughout the duration of the study. Fresh fecal samples were collected every day, avoiding bedding and traces of urin from the cages. Six animals from each of the groups were euthanized on Days 4 and 7, respectively, for investigation of intestinal segments, liver and spleen. Samples of jejunal content from the middle part of the small intestine were squeezed out with tweezers, and samples from spleen and liver of approximately 0.6 g were homogenized prior to investigation as described below.

**Ethical aspects**

Animal experiments were carried out under the supervision of the Danish National Agency for Protection of Experimental Animals

The recently developed guinea-pig model [BB Roldgaard, JB Andersen, TR Licht and BB Christensen, submitted] is much less stressful to the animals than previously published models [17], since it involves no intubation, injection or anesthetizing. Additionally, the approach of treating each animal with a mixture of cultures reduces the number animals needed.

**Estimation of L. monocytogenes in samples**

Samples of lysed Caco-2 cells and homogenized organs were cultivated on BHI-agar supplemented with Erythromycin. Samples from faeces and jejunum were cultivated on BHI-agar supplemented with Erythromycin. Samples from faeces and jejunum were cultivated on BHI-agar supplemented with Erythromycin. Samples of lysed Caco-2 cells and homogenized organs were cultivated on BHI-agar supplemented with Erythromycin. Samples of lysed Caco-2 cells and homogenized organs were cultivated on BHI-agar supplemented with Erythromycin.

**Statistics**

Statistical analysis was performed using the software package JMP (SAS institute, Copenhagen, Denmark). Pearson’s Chi² test was used to compare the numbers of infected animals after dosage with either oxygen-restricted or unrestricted *L. monocytogenes*. The same test was used to compare the results obtained with either mono infection or mixed infection, and with either YFP or CFP labelling. A significance level of 0.05 was used in all cases.

**Authors’ contributions**

JBA carried out the sample preparation and the in vitro invasion assays, while BBR was responsible for the animal experiments. BBC and TRL conceived the study and participated in its design and coordination. TRL drafted the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

We thank Grethe Fisher and Mansour Badaki for excellent technical assistance, and Tina Beck Hansen for help with statistical analysis. Anne Ørnsen and her staff are acknowledged for assistance with animal handling. The Danish Councils for Independent Research and The Danish Council for Strategic Research supported this work.

**References**

1. Low JC, Donachie W: A review of *Listeria monocytogenes* and listeriosis. Vet J 1997, 153:5-29.
2. Authority EFSA: Trends and Sources of zoonoses, zoonotic agents and antimicrobial resistance in the European Union in 2004. The EFSA Journal 2005 - 310 2006.
3. Foundation ILIR, Institute RS: Achieving continuous improvement in reductions in foodborne listeriosis—a risk-based approach. J Food Prot 2005, 68:932-994.
4. Rocourt J, BenEmbarek P, Toyofuku H, Schlundt J: Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat foods: the FAO/WHO approach. FEMS Immunol Med Microbiol 2003, 35:263-267.
5. Norrung B: Microbiological criteria for *Listeria monocytogenes* in foods under special consideration of risk assessment approaches. Int J Food Microbiol 2000, 62:217-221.
6. Kim H, Marquis H, Boor KJ: SigmaB contributes to *Listeria monocytogenes* invasion by controlling expression of inlA and inlB. Microbiology 2005, 151:3215-3222.
7. Kim H, Boor KJ, Marquis H: *Listeria monocytogenes* (sigma)B Contributes to Invasion of Human Intestinal Epithelial Cells. Infect Immun 2004, 72:7374-7378.
8. Sue D, Fink D, Wiedmann M, Boor KJ: (sigmaB)-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. Microbiology 2004, 150:3843-3853.
9. Lee CA, Falkow S: The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc Natl Acad Sci U SA 1990, 87:4304-4308.
10. Ernst RK, Dombrowski DM, Merrick JM: Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect Immun 1990, 58:2016-2018.
11. Mekalanos JJ: Environmental signals controlling expression of virulence determinants in bacteria. J Bacteriol 1992, 174:1-7.
12. Chaturangkul S, Boor KJ: SigmaB activation under environmental and energy stress conditions in *Listeria monocytogenes*. Appl Environ Microbiol 2006, 72:5197-5203.
13. Atkins JN, Mottaz HM, Norbeck AD, Gustin JK, Rue J, Claus TR, Purvins SO, Rodland KD, Heffron F, Smith RD: Analysis of the *Salmonella typhimurium* proteome through environmental
14. Rychlik I, Barrow PA: Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection. FEMS Microbiol Rev 2005, 29:1021-1040.

15. Chaturongakul S, Boor KJ: RsbT and RsbV Contribute to (sigma)B-Dependent Survival under Environmental, Energy, and Intracellular Stress Conditions in Listeria monocytogenes. Appl Environ Microbiol 2004, 70:5349-5356.

16. Lecuit M, Dransi S, Gottardi C, Fedor-Chaiken M, Gumbiner B, Cossart P: A single amino acid in E-cadherin responsible for host specificity towards the human pathogen Listeria monocytogenes. EMBO J 1999, 18:3956-3963.

17. Lecuit M, Vandormael-Pournin S, Lefort J, Huere M, Gounon P, Dupuy C, Babinet C, Cossart P: A Transgenic Model for Listeriosis: Role of Internalin in Crossing the Intestinal Barrier. Science 2001, 292:1722-1725 [http://www.sciencemag.org/cgi/content/abstract/292/5522/1722]

18. Andersen JB, Roldgaard BB, Lindner AB, Christensen BB, Licht TR: Construction of a multiple fluorescence labelling system for use in co-invasion studies of Listeria monocytogenes. BMC Microbiology 2006, 6:86.

19. Audia JP, Webb CC, Foster JW: Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. Int J Med Microbiol 2001, 291:97-106.

20. Richard HT, Foster JW: Acid resistance in Escherichia coli. Adv Appl Microbiol 2003, 52:167-186.

21. Peronneau G, D’Ambrosio C, Arena S, Rullo R, Ledda L, Ferrara L, Scalon A: Differential proteomic analysis in the study of prokaryotes stress resistance. Ann Ist Super Sanita 2005, 41:459-468.

22. O'Driscoll B, Gahan CG, Hill C: Adaptive acid tolerance response in Listeria monocytogenes: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl Environ Microbiol 1996, 62:1693-1698.

23. Garner MR, Njaa BL, Wiedmann M, Boor Kj: Sigma B contributes to Listeria monocytogenes gastrointestinal infection but not to systemic spread in the guinea pig infection model. Infect Immun 2006, 74:876-886.

24. Stritzker J, Janda J, Schoen C, Taupp M, Pilgrim S, Gentschev I, Schreier P, Geginat G, Goebel W: Growth, Virulence, and Immunogenicity of Listeria monocytogenes aro Mutants. Infect Immun 2004, 72:5622-5629 [http://iai.asm.org/cgi/content/abstract/72/10/5622].

25. Stritzker J, Schoen C, Goebel W: Enhanced Synthesis of Internalin A in aro Mutants of Listeria monocytogenes Indicates Posttranscriptional Control of the inlAB mRNA. J Bacteriol 2005, 187:2836-2845.

26. Lecuit M, Nelson DM, Smith SD, Khan H, Huere M, Vacher-Lavenu MC, Gordon JJ, Cossart P: Targeting and crossing of the human maternofetal barrier by Listeria monocytogenes: Role of internalin interaction with trophoblast E-cadherin. PNAS 2004, 101:6152-6157.

27. Flanary PL, Allen RD, Downs L, Kariyous S: Insertional inactivation of the Listeria monocytogenes cheYA operon abolishes response to oxygen gradients and reduces the number of flagella. Can J Microbiol 1999, 45:646-652.

28. Larsen CN, Norrung B, Sommer HM, Jakobsen M: In Vitro and In Vivo Invasiveness of Different Pulsed-Field Gel Electrophoresis Types of Listeria monocytogenes. Appl Environ Microbiol 2002, 68:5698-5703.