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The effect of sublethal concentrations of benzalkonium chloride on invasiveness and intracellular proliferation of *Listeria monocytogenes*

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ABSTRACT

Listeria monocytogenes, which survives improper cleaning and disinfection processes are a major source of recontamination of food products, where they can survive or even multiply until consumption. In this study we investigated the effect of sublethal concentration of benzalkonium chloride (BAC) on the virulence of *L. monocytogenes* using human intestinal, hepatocytic and macrophage-like cells, three cell types involved in the human infection pathway of *L. monocytogenes*. Therefore we used four different strains, the type strain EGDe and three isolates from food or food processing environment, which revealed different susceptibility to BAC. Incubation of *L. monocytogenes* with sublethal concentrations of BAC reduced the invasiveness of all four strains in all three types of cells, respectively. However, a significant decrease could only be observed in the strains more sensitive to BAC. Furthermore sublethal concentration of BAC increased significantly the intracellular proliferation for all four strains in all three different human cell types. Interestingly, this effect of disinfectants on the virulence of *L. monocytogenes* was maintained even after 24 h incubation in a nutrient-rich broth, which mimics the food matrix. In conclusion we showed that exposure of *L. monocytogenes* to sublethal concentrations of disinfectants, although reducing invasion, supports the survival and growth of intracellular bacteria.

This study underlines the importance of proper disinfection in food processing environment to limit the number of *L. monocytogenes* foodborne illnesses.

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1. Introduction

The aim of cleaning and disinfection in the food industry is the elimination of microorganisms present on food-contact surfaces, thereby avoiding contamination of food products and reducing the risk of foodborne diseases. An efficient cleaning and disinfection procedure consists of a sequence of rinses and applications of detergents and disinfectants in various combinations of concentration and temperatures (Wirtanen & Salo, 2003). Disinfectants based on quaternary ammonium compounds (QAC), like benzalkonium chloride (BAC), are widely used in the food industry, also in dairies, and are known to be mainly effective against Gram-positive bacteria (Aase, Sundheim, Langsrud, & Rørvik, 2000; Best, Kennedy, & Coates, 1990). QAC are cationic biocides, which act on general membrane permeability leading to cell damage (McDonnell & Russell, 1999).

Listeria monocytogenes, a Gram-positive facultative intracellular food-borne pathogen, has a remarkable ability to survive and persist in food processing environments (Carpentier & Cerf, 2011; Latorre et al., 2011). *L. monocytogenes* can cause listeriosis, a rare but severe infection disease, in immune-compromised individuals, elderly, pregnant women and newborns, which is characterized by meningitis, encephalitis, septicemia, mother-to-fetus infection, and abortion (Freitag, Port, & Miner, 2009; Vazquez-Boland et al., 2001).

Using disinfectants at concentrations recommended for the food industry *L. monocytogenes* will be completely inactivated; however different factors like food debris or biofilm formation can significantly reduce the efficiency of disinfectants (Best et al., 1990; Pan, Breidt, & Kathariou, 2006; Saa Ibusquiza, Herrera, & Cabo, 2011). Furthermore inadequate cleaning and disinfection procedures like insufficient cleaning before disinfection, disinfection of wet surfaces and dosage failure in food processing plants can expose bacteria regularly to sublethal concentration of disinfectants. This might not only lead to resistance to the used disinfectants, but also to a resistance to a range of other antimicrobial compounds

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including antibiotics (Rajkovic et al., 2009; Rakic-Martinez, Drevets, Dutta, Katic, & Kathariou, 2011).

It has been reported that *sigB*, which encodes a major transcriptional regulator of stress response genes, plays a role in the resistance and adaption to disinfectants of both planktonic cells and biofilm (Ryan, Gahan, & Hill, 2008; van der Veen & Abee, 2010). There is evidence growing that *sigB* influences the virulence of *L. monocytogenes* by directly regulating the expression of specific virulence genes (Chaturongakul, Raengpradub, Wiedmann, & Boor, 2008; Garner, James, Callahan, Wiedmann, & Boor, 2006). Furthermore, *sigB* also contributes to the transcription of PrfA, the global virulence gene regulator in *L. monocytogenes* (Nadon, Bowen, Wiedmann, & Boor, 2002).

Data on the effect of disinfectants on the expression of virulence factors in *L. monocytogenes* are limited and contradictive (Fox, Leonard, & Jordan, 2011; Kastbjerg, Larsen, Gram, & Ingmer, 2010) and the influence of disinfectants on the *in vitro* virulence of *L. monocytogenes* has not been investigated yet. Therefore the main aim of our study was to investigate the effect of sublethal concentration of BAC on the invasiveness and intracellular proliferation of different *L. monocytogenes* strains using human intestinal, hepatocytic and macrophage-like cells, three cell types involved in the human infection pathway of *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Four *L. monocytogenes* strains (all serovar 1/2a) were used in this study: The type strain EGDe (ATCC BAA-679) and three strains isolated from food products; Lm 1 (strain 4423) from cheese smear water, Austria; Lm 2 (strain 6179) from cheese, Ireland (Fox et al., 2011); Lm 3 (strain R479a) from smoked salmon, Denmark (Fonnesbech Vogel, Huss, Ojeniyi, Ahrens, & Gram, 2001). All *L. monocytogenes* strains were activated on tryptic soy agar with yeast extract (TSAY, Merck, Germany). Single colonies were inoculated in 8 ml brain heart infusion (BHI, Merck) and incubated for 6–8 h at 37 °C with shaking at 120 rpm. The bacterial suspension was diluted in a ratio 1 to 10 in minimal media (MM), consisting of RPMI-1640 (PAA Laboratories GmbH, Austria) supplemented with 1% L-glutamine (PAA Laboratories GmbH) and 0.08 mg/ml ferric citrate (Merck) and incubated over night at 37 °C with shaking at 120 rpm.

2.2. Growth of L. monocytogenes in the presence of BAC

Overnight cultures of the four different *L. monocytogenes* strains were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and were grown at 37 °C in MM containing different concentrations of BAC (0.625, 1.25, 2.5 and 5 mg/l; Sigma–Aldrich, Germany) to determine the minimal inhibitory concentration (MIC), the concentration of BAC, where no growth could be observed. The optical density at 600 nm was measured at different time points. *L. monocytogenes* grown in MM without BAC was used as a control.

2.3. Viability of L. monocytogenes cells

In order to determine the viability of *L. monocytogenes* in the presence of sublethal concentration of BAC, overnight cultures of the four strains were diluted to an OD_{600} of 0.2, grown at 37 °C for 2 h to reach mid logarithmic phase and incubated in MM in the absence and presence of 1.25 mg/l BAC for 30 min at 37 °C. Surviving bacteria were determined by serial plating on TSAY plates and colony forming units (CFU) were counted after 24 h incubation at 37 °C.

2.4. In vitro virulence assay

For the *in vitro* virulence assay three human cell lines were used: Caco-2 (human intestinal epithelial cells), HepG2 (human hepatocytic cells) and THP-1 (human macrophage-like cells). The cell lines were cultivated in Eagle's minimum essential medium (MEM for Caco-2 and HepG2) or RPMI-1640 (for THP-1) containing 2 mM L-Glutamine, 10% fetal bovine serum (FBS), 100 units/ml Penicillin, 100 μg/ml Streptomycin sulfate, 0.25 μg/ml Amphotericin B and 1% non essential amino acids (NEAA; all PAA) at 37 °C in a humidified atmosphere (95% relative humidity) containing 5% CO_2. The cells were seeded two days prior the experiment in a 24 well-plate at a mean cell density of 10^5 cells per well. THP-1 cells were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) for 48 h. Cells were starved 24 h prior experiments in MEM or RPMI-1640 containing 0.1% bovine serum albumin (BSA; PAA).

Overnight cultures of the four *L. monocytogenes* strains were diluted to OD_{600} of 0.2, grown at 37 °C for 2 h to reach mid logarithmic phase and incubated with and without BAC (1.25 mg/l) in MM for 30 min at 37 °C. Cell monolayers were infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 25 bacteria per cell at 37 °C. After 1 h of incubation, the monolayers were washed twice with Dulbecco's Phosphate Buffered Saline (PBS; PAA) to remove non-adhering bacteria. For the determination of invasion and intracellular growth cells were incubated with MEM containing gentamicin (100 μg/ml, PAA) to kill extracellular bacteria for 45 min (invasion) and 4 h (proliferation), respectively. Cells were washed twice with PBS and lysed with 1 ml of cold 0.1% Triton X-100 (Merck). Intracellular bacteria were determined by serial plating on TSAY plates and colony forming units (CFU) were counted 24 h after incubation at 37 °C. Values for invasiveness and intracellular proliferation were calculated as mean CFU/ml per 100,000 cells with MOI of 25 for one experiment. Each experiment was performed at least 4 independent times.

The intracellular proliferation was in addition calculated as the number of recovered bacteria after 4 h of gentamicin treatment per well divided by the number of recovered bacteria after invasion, respectively.

2.5. The maintenance of the effect of benzalkonium chloride on the virulence of L. monocytogenes in vitro

Overnight cultures of the different *L. monocytogenes* strains were diluted to an OD_{600} of 0.2, grown at 37 °C for 2 h to reach mid logarithmic phase and incubated with and without BAC (1.25 mg/l) in MM for 30 min at 37 °C. Subsequently bacteria were incubated for 24 h at 4 °C in PBS or BHI and *in vitro* virulence assay using Caco-2 cells was performed as described above. Each experiment was performed at least 3 independent times.

2.6. Determination of cytotoxic effects of BAC on human cells

Possible cytotoxic effects of BAC were ruled out using a commercially available assay for photometric determination of lactate dehydrogenase (LDH) activity (Sigma-Aldrich), which measured LDH leakage. Therefore we incubated the cells without and with BAC at different concentrations [0.001–10 mg/l] for 1 h at 37 °C. No differences in LDH leakage were observed between controls (no BAC) and cells treated with BAC.

2.7. Statistical analysis

Data were calculated using Microsoft Excel 2007 software. Values were compared statistically using *t*-test (independent variables); *P*-values <0.05 were considered to be significant.

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