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Short communication

The effect of milk components and storage conditions on the virulence of *Listeria monocytogenes* as determined by a Caco-2 cell assay

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ABSTRACT

Nearly all cases of human listeriosis have been associated with consumption of contaminated food, therefore the investigation of the virulence of *Listeria* (*L.*) *monocytogenes* after exposure to environmental conditions in food matrices is critical in order to understand and control its impact on public health. As milk and dairy products have been implicated in more than half of the listeriosis outbreaks, we investigated the *in vitro* virulence of *L. monocytogenes* incubated in different milk types at various storage conditions.

Incubation in pasteurized milk at refrigeration conditions (4 °C) revealed a higher invasion and intracellular proliferation of four different *L. monocytogenes* strains compared to raw milk using human intestinal epithelial Caco-2 cells. Furthermore the period of storage, which increased *L. monocytogenes* cell numbers, decreased *in vitro* virulence. However, *L. monocytogenes* stored for 3 weeks at 4 °C in milk are still able to invade and proliferate into the host cell. Interestingly abused storage temperatures (25 °C and 30 °C) for a short time period (2 h) revealed an attenuated impact on the *in vitro* virulence of *L. monocytogenes* compared to the storage temperature of 4 °C. Regarding the major milk compounds, the level of milk fat significantly affected the *in vitro* virulence of *L. monocytogenes*. Pre-incubation in milk with high fat content (3.6%) resulted in a lower invasion capability compared to milk with low fat content. In contrast casein and lactose did not influence the invasiveness of *L. monocytogenes* into the host cell.

In conclusion our study shows that the milk environment and different storage conditions influence the *in vitro* virulence of *L. monocytogenes*, both of which have to be considered in the risk assessment of contaminated food.

1. Introduction

Listeria (*L.*) *monocytogenes* is a Gram-positive facultative intracellular food-borne pathogen responsible for listeriosis, a rare but severe infection in humans and animals with a mortality rate of 25–30%. There are two forms of listeriosis: non-invasive self-limited, gastrointestinal listeriosis in healthy individuals and invasive, systemic listeriosis in immunocompromised individuals, pregnant women and newborns, leading to meningitis, encephalitis, septicemia, mother-to-fetus infection, and abortion (Ooi and Lorber, 2005; Stavru et al., 2011; Vazquez-Boland et al., 2001).

According to the last EFSA report the overall notification rate was 0.35 listeriosis cases per a population of 100,000 (EFSA, 2012). In several European countries like Austria and Spain an increasing trend in listeriosis cases was reported, whereas a significant decreasing trend was noted in Belgium, Czech Republic and Slovakia (Allerberger and Wagner, 2010; EFSA, 2012).

Milk and dairy products have been implicated in more than half of the reported listeriosis outbreaks and in several sporadic cases (Lunden et al., 2004; Rebagliati et al., 2009). Most of these outbreaks have been linked to consumption of raw milk or products made from raw milk. However some listeriosis cases have been associated with pasteurized milk, although heat treatment eliminates *L. monocytogenes* during the pasteurization process (Centers for Disease Control and Prevention, 2007; Dalton et al., 1997; Koch et al., 2010; Lyytikainen et al., 2000). This indicates that these dairy products have been contaminated with *L. monocytogenes* in subsequent stages of production from sources in the plant environment.

The infection pathway of *L. monocytogenes* is very complex and has been extensively studied in the last years (Stavru et al., 2011). There is growing evidence that virulence is not a stable property, but can be influenced by environmental conditions. For example, it has been shown that acid and salt stress increase the expression of virulence genes and *in vitro* virulence (Conte et al., 2000; Garner et al., 2006; Olesen et al., 2009). Moreover temperature, the presence or absence of oxygen, osmotic stress and pH influence the virulence

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potential (Bo Andersen et al., 2007; Conte et al., 1994; Gameiro et al., 2007; Walecka et al., 2011).

Since nearly all cases of human listeriosis have been associated with consumption of contaminated food, the investigation of the virulence of *L. monocytogenes* after exposure to environmental conditions in food matrices is critical in order to understand and control the impact on public health. However, studies on the influence of food on the virulence are limited. Recently, it has been shown that meat and meat products modulate the virulence of *L. monocytogenes* both *in vitro* and *in vivo* (Larsen et al., 2010; Lin et al., 2010; Mahoney and Henriksson, 2003; Rantsiou et al., 2012). Furthermore Duodu et al. showed that the storage temperature has a strain dependent impact on the *in vitro* virulence of *L. monocytogenes* incubated on a salmon matrix (Duodu et al., 2010). Another study reported a modification of the virulence potential due to different food matrices like rillettes, raw salmon and ultra-high temperature treated (UHT) milk using a plaque forming assay (Midelet-Bourdin et al., 2006). In contrast Pang et al. showed that the exposure of *L. monocytogenes* to chocolate milk has no impact on the virulence potential as determined in a Caco-2 cell assay (Pang et al., 2007). Besides these two studies the effect of milk on the virulence of *L. monocytogenes* has not been studied, so far.

The aim of this study was to investigate the effect of the milk environment on the virulence of *L. monocytogenes*. Therefore we stored different *L. monocytogenes* strains for 24 h at 4 °C in pasteurized and raw milk and determined adhesion, invasion and intracellular proliferation using human intestinal epithelial Caco-2 cells. Furthermore we investigated the effect of storage time and temperature and the impact of fat, casein and lactose on the virulence potential of *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Four *L. monocytogenes* strains, all of serotype 1/2a, were used in this study: the type strain EGDe (ATCC BAA-679), LM 5318 isolated from a cheese production environment (2007, Czech Republic), LM 5380 (2010, Austria) and LM 5683 (2011, Austria) both isolated from cheese. For activation, *L. monocytogenes* strains were grown on tryptic soy agar complemented with yeast extract (TSA-Y, Merck, Darmstadt, Germany). Single colonies were inoculated in 8 ml brain heart infusion (BHI, Merck), incubated for 8 h at 37 °C, diluted 1:20 in a minimal growth media RPMI-1640 supplemented with 1% L-glutamine (both from PAA, Pasching, Austria) and 0.08 mg/ml ferric citrate (Merck) and incubated overnight at 37 °C with shaking at 120 rpm.

2.2. Types of milk and storage conditions

Cultures of *L. monocytogenes* were diluted to an OD_{600} of 0.2, which corresponded to a cell count of (2.0–3.5) × 10^8 CFU/ml, and incubated for 24 h at 4 °C in Dulbecco's phosphate buffered saline (DPBS; PAA), and pasteurized (3.6% fat) and raw milk. All types of pasteurized milk (Ja! Natürlich Naturprodukte Gmbh, Wr. Neudorf, Austria) were purchased from a local store and raw milk was kindly provided by the education and research farms of the University of Veterinary Medicine Vienna. All milk samples used in this study were tested negative for native *L. monocytogenes* using ALOA chromogenic (Microgen Bioproducts LTD, Camberly, United Kingdom) and PALCAM agar (Oxoid, Basingstoke, United Kingdom). We determined the total number of bacteria in the raw milk on TSA-Y plates and revealed (1.28 ± 0.43) × 10^4 CFU/ml. The composition of raw milk was determined using a Milk-Lab Pro® milk composition examiner with ultrasound spectroscopic technology (Milk-Lab, Oldham, UK). Table 1 shows the detailed milk composition of all types of milk used in this study. The milk was aliquoted, stored at −20 °C and tested for bacterial contamination prior to the experiments.

In order to investigate the effect of storage time and temperature on the virulence of *L. monocytogenes* the type strain EGDe was either incubated for 2 h, 24 h and 3 weeks at 4 °C (inoculation level: (1.0–2.5) × 10^5 CFU/ml) or after preincubation for 24 h at 4 °C and for 2 h at 4 °C, 25 °C and 30 °C in pasteurized milk (3.6% fat, inoculation level: (2.0–3.5) × 10^8 CFU/ml) prior to *in vitro* virulence assays. Storage in DPBS under the same conditions was used as a control.

To evaluate the effect of milk fat the type strain EGDe was incubated for 2 h at 4 °C in pasteurized milk containing 0.9, 1.6 and 3.6% fat (inoculation level: (2.0–3.5) × 10^8 CFU/ml). In addition *L. monocytogenes* EGDe was stored for 2 h at 4 °C in pasteurized milk (0.9% fat content), whey (produced from pasteurized milk), DPBS and in DPBS 5% (w/v) lactose (Merck) to determine the effect of casein and lactose on the virulence (inoculation level: (2.0–3.5) × 10^8 CFU/ml). For the production of whey casein was precipitated with 1% HCl and pH was adjusted to pH 6.8 with 1 M NaOH (both from Sigma-Aldrich, St. Louis, USA).

In addition, the growth of *L. monocytogenes* stored under the different conditions was determined by serial plating on TSA-Y plates.

2.3. In vitro virulence assay

Human intestinal epithelial Caco-2 cells were cultivated in Eagle's minimum essential medium (MEM) 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% nonessential amino acids (all from PAA) at 37 °C in a humidified atmosphere (96% relative humidity) containing 5% CO_2. The cells were seeded 2 days prior the experiment and starved 24 h prior to experiments in MEM containing 0.1% bovine serum albumin (BSA; PAA).

The virulence potential of *L. monocytogenes* was determined according to Conte et al. (Conte et al., 1994). Briefly, three wells of Caco-2 were infected for 1 h at 37 °C with *L. monocytogenes* incubated at different conditions at a multiplicity of infection (MOI) of 25. The cells were washed twice with DPBS and either lysed with cold 0.1% Triton X-100 (Merck), for adhesion, or incubated in MEM with 0.1% BSA containing 100 μg/ml gentamicin (PAA) for 45 min (invasion) and 4 h (cytosolic intracellular proliferation). Cells were again washed twice with DPBS and lysed with 1 ml of cold 0.1% Triton X-100 (Merck). Adherent, and intracellular bacteria were determined by serial plating on TSA-Y plates and colony forming units (CFU) were counted 24 h after incubation at 37 °C. Values were calculated as CFU/ml per 10^5 cells at MOI of 25. Each experiment was performed at least 3 times.

2.4. Statistical analysis

Microsoft Excel® 2007 and SPSS.20 software (SPSS Inc., Chicago, USA) were used for statistical analysis. To compare the effect of three different treatments, univariate analysis was used to calculate the mean values and standard deviations of CFU counts of at least three biological replicates and values were compared statistically using ANOVA nested analysis (Bonferroni post-hoc-test). To study the effect of two different

Table 1
Composition of milk.

Per 100 ml	Past. milk (0.9% fat)	Past. milk (1.6% fat)	Past. milk (3.6% fat)	Raw milk
Carbohydrates [g]	5.1	4.9	4.9	4.7
Protein [g]	3.5	3.4	3.4	3.6
Fat [g]	0.9	1.6	3.6	3.4
Fibers [g]	0	0	0	n.d.
Sodium [g]	0.05	0.05	0.05	n.d.
Calcium [mg]	125	125	125	n.d.

n.d.: not determined.