International Journal of Food Microbiology 151 (2011) 93–97

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

In vitro influence of D/L-lactic acid, sodium chloride and sodium nitrite on the infectivity of feline calicivirus and of ECHO virus as potential surrogates for foodborne viruses

J. Straube [a], T. Albert [b,*], J. Manteufel [a], J. Heinze [b], K. Fehlhaber [b], U. Truyen [a]

[a] Institute of Animal Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, Leipzig University, An den Tierkliniken 1, 04103 Leipzig, Germany
[b] Institute of Food Hygiene, Faculty of Veterinary Medicine, Leipzig University, An den Tierkliniken 1, 04103 Leipzig, Germany

ARTICLE INFO

Article history:
Received 17 January 2011
Received in revised form 14 July 2011
Accepted 13 August 2011
Available online 24 August 2011

Keywords:
Foodborne viruses
Virus inactivation
Food preservation
D/L-lactic acid
Sodium chloride
Sodium nitrite

ABSTRACT

The importance of foodborne viruses is increasingly recognized. Thus, the effect of commonly used food preservation methods on the infectivity of viruses is questioned. In this context, we investigated the antiviral properties of D,L-lactic acid, sodium chloride and sodium nitrite by in vitro studies. Two model viruses, Feline Calicivirus (FCV) and Enteric Cytophatic Human Orphan (ECHO) virus, were chosen for this study simulating important foodborne viruses (human noroviruses (NoV) and human enteroviruses, resp.). The model viruses were exposed to different solutions of D,L-lactic acid (0.1–0.4% w/w, pH 6.0–3.2), of sodium chloride (2–20%, w/v) and of sodium nitrite (100, 150 and 200 ppm) at 4 and 20 °C for a maximum of 7 days. Different results were obtained for the two viruses. ECHO virus was highly stable against D,L-lactic acid and sodium chloride when tested under all conditions. On the contrary, FCV showed less stability but was not effectively inactivated when exposed to low acid and high salt conditions at refrigeration temperatures (4 °C). FCV titers decreased more markedly at 20 °C than 4 °C in all experiments. Sodium nitrite did not show any effect on the inactivation of both viruses. The results indicate that acidification, salting or curing maybe insufficient for effective inactivation of foodborne viruses such as NoV or human enteroviruses during food processing. Thus, application of higher temperature during fermentation and ripening processes maybe more effective toward the inactivation kinetics of less stable viruses. Nevertheless, more studies are needed to examine the antiviral properties of these preserving agents on virus survival and inactivation kinetics in the complex food matrix.

1. Introduction

Viral transmission due to consumption of contaminated food is an important public health issue. Mainly human noroviruses (NoV), rotaviruses, hepatitis A virus (HAV) and certain human enteroviruses are foodborne pathogens of significant concern (Cliver, 1997; Koopmans and Duizer, 2004). Foodborne viruses are environmentally stable and are transmitted through fecal–oral route. Infected individuals shed high numbers of virus particles in their stool and it is known that a low infectious dose is needed to establish illness (Koopmans and Duizer, 2004).

Raw or minimally processed food products such as shellfish, vegetables, salads, fruits, raw meat products, as well as, raw milk are considered to pose a higher risk for consumers (Koopmans et al., 2002; Koopmans and Duizer, 2004; Carter, 2005; Kerbo et al., 2005).

Regarding foodborne viruses, questions emerged about the effect of preservation methods upon these pathogens during processing and storage of food. Consequently, various studies on the survival and inactivation of foodborne viruses were conducted. Examinations mainly focused on the impact of heat treatment, chilling, freezing, acidification, reduced water activity or high hydrostatic pressure. Results of different studies were recently reviewed by Cliver (2010) and by Baert et al. (2009). It seems that apart from heat treatment, measures like acidification, chilling or freezing are not sufficient enough to effectively inactivate most foodborne viruses.

However, little is known about how common preservation methods like salting, curing and microbial fermentation have an effect on virus inactivation. These methods are widely used for the processing of raw meat products or different dairy products.

Microbial fermentation, salting or curing processes are the crucial steps to obtain microbiological safety as they prevent multiplication of pathogenic microorganisms but until now little information has been published about the antiviral effect of these food preserving methods.

Therefore, the objective of this study was to determine the impact of different D,L-lactic acid, sodium chloride, as well as, sodium nitrite concentrations on the infectivity of two different non-enveloped viruses, Feline Calicivirus (FCV) and Enteric Cytopathic Human Orphan (ECHO) virus, by in vitro experiments. The two viruses, FCV

* Corresponding author. Tel.: +49 341 9738217; fax: +49 341 9738249.
E-mail address: albert@vetmed.uni-leipzig.de (T. Albert).

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.08.010

and ECHO viruses, were chosen for this study. The former was used as a widely accepted surrogate for the non-cultivable NoV. ECHO virus is an environmentally stable representative for the human enteroviruses within the *Picornaviridae* family. Enteroviruses can be transmitted via contaminated food and water and cause various diseases in humans, including gastroenteritis and therefore considered as foodborne viruses.

2. Material and methods

2.1. Viruses and cells

The FCV strain KS20 and the ECHO virus strain Farouk were used for the study. FCV and ECHO virus were propagated in Crandell Reese Feline Kidney (CRFK) cells and Buffalo Green Monkey (BGM) cells, respectively. Cells were grown in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum. Virus stocks used for the inactivation experiments were obtained by inoculation of young cells (2 to 24 h after seeding). When the cytopathogenic effect (cpe), had reached about 80–90%, viruses and cells were harvested by 1 cycle of freezing and thawing. The suspension was clarified by centrifugation (10 min, 1.800 × *g*, 4 °C) and virus stocks were stored in aliquots at −80 °C until use. Calculation of virus titers for the stocks and for the virus suspensions after exposure was done according to the 50% endpoint method of Spearman (1908) and Karber (1931) and expressed as 50% Tissue Culture Infectious Dose ($TCID_{50}$)/ml. Titration was performed in the 96-well format.

2.2. Experimental design

Phosphate buffered saline (PBS, pH 7.4) was supplemented with 90% D,L-lactic acid (Roth, Germany) to obtain D,L-lactic acid concentrations of 0.1%, 0.15%, 0.2%, 0.3% and 0.4% (w/w). Lactic acid was used as a sterile filtrated solution containing a racemate in equal amounts of D- and L-lactic acid.

In analog, sodium chloride (Roth, Germany) was added to final concentrations of 2%, 6%, 12% and 20% (w/v). In addition, another 2% sodium chloride solution was supplemented with sodium nitrite (Merck, Germany) to the amounts of 100, 150, and 200 ppm. pH values of 7.20–7.69 were used to exclude antiviral effect due to low pH values like they are common for salted products. The solutions were sterilized by autoclaving before use.

For the experiments, a total of 4.5 ml of each sample solution was transferred into a sterile tube and 0.5 ml of virus stock was added. Concentrations of D,L-lactic acid and sodium chloride were calculated in consideration of this additional dilution step.

The tubes were gently mixed and stored at 4 °C and 20 °C, respectively. The pH value was determined for each tested solution. Samples were taken after 3 h and after 7 days. The long-time exposure was tested taking into consideration common processing times in food industry (e.g. raw cured products). Short-time exposure of 3 h was chosen for screening subsequent antiviral effects.

After exposure samples were subjected to sterile filtration, using a 0.22 µm syringe filter and, a serial 10-fold dilution was prepared. To test for virus infectivity, 100 µl of each dilution step was inoculated into four replicate wells of a 96-well microtiter plate containing confluent cell monolayers of CRFK and BGM cells, respectively. The plates were checked daily by microscopy and the virus titer was estimated from cytopathogenicity after a maximum of 4 days. The number of wells showing CPE in each row was then recorded and then the viral titer was quantified as tissue culture infective dose ($TCID_{50}$/ml). Each single experiment was carried out as 5 independent trials. For FCV virus, stocks used in the inactivation experiments ranged from 6.25 to 8.75 $TCID_{50}$/ml. For ECHO virus, stocks with a titer of 8 log $TCID_{50}$/ml were used.

2.3. Statistical analysis

Statistical analysis was performed with the software SPSS, version 15.0.

The significance of differences between the mean virus titers and mean virus-titer reductions after the various treatments was calculated by the Student's *t* test with a significance level of $p<0.05$. All data of virus titers were $\log_{10}$ transformed and mean virus titers were compared for the different test setups in the studies with ECHO virus. For FCV, the reduction in the mean virus titers was calculated due to the different initial virus titers. Analyses were therefore based on reduction of mean virus titers.

Graphs were prepared with Sigma Plot (version 11.0, 2008).

3. Results and discussion

3.1. Inactivation by D/L-lactic acid

Lactic acid is used in food processing as a pH regulator, a flavoring agent, or a preservative and is also naturally present in food when produced by lactic acid bacteria. It is beneficial due to its ability in preventing growth of various bacterial pathogens and food spoilage organisms. However, little is known about the virus inactivation properties. Therefore, this study investigated the survival of FCV and ECHO virus under various concentration of D,L-lactic acid at pH values representing a variety of available foods.

ECHO virus was observed to be highly stable towards D,L-lactic acid when exposed to low pH values during long-time exposure at 20 °C (Table 1). A significant reduction of virus titer could only be detected in solutions with 0.4% acid ($p<0.05$) after a 7-day exposure at 20 °C. Although virus titers in solutions stored at 20 °C were slightly lower than in solutions exposed at 4 °C, differences were not significant ($p\geq0.05$). The results obtained for ECHO virus are in agreement with previous findings. It was found that enteroviruses are resistant to pH 3 and hepatitis A to even lower values (Green, 2007; Hollinger and Emerson, 2007). In studies with different human enteroviruses – coxsackievirus A9 and B1, enterovirus 9 and poliovirus – infectivity was lost after exposure for 2 h to a pH of 1 at room temperature (Scholz et al., 1989). Thus, it can be suggested that pH values not commonly present in foods are necessary for the inactivation of the viruses mentioned.

This study indicated that D,L-lactic acid has inactivating properties against FCV. The extent of reduction differed between the conditions applied (Figs. 1 and 2). At 4 °C, FCV was stable after long-time exposure to low pH values (Fig. 2). A significant titer reduction was only seen in solutions with 0.3% and 0.4% D,L-lactic acid ($p<0.05$). In comparison, virus-titer reduction was more marked at 20 °C. A final loss of detectable infectivity was seen under conditions with 0.3% and 0.4% acid

Table 1
Survival of ECHO virus after a 7-day-exposure to solutions with different concentrations of D,L-lactic acid. Significant differences ($p<0.05$) were tested between the initial virus titer of 8.00 $\log_{10}$ $TCID_{50}$/ml and the virus titer obtained after exposure and are marked (*).

D,L-lactic acid [%]	Temperature [°C]	Virus titer [$\log_{10}$ $TCID_{50}$/ml ± SEM]
0.10	4	8.34 ± 0.066
(pH 5.9–6.0)	20	8.20 ± 0.122
0.15	4	8.20 ± 0.122
(pH 4.4–4.6)	20	7.80 ± 0.146
0.20	4	8.35 ± 0.170
(pH 3.8–3.9)	20	8.05 ± 0.122
0.30	4	8.20 ± 0.146
(pH3.4–3.5)	20	8.00 ± 0.079
0.40	4	7.95 ± 0.146
(pH 3.2–3.3)	20	7.75 ± 0.079*

$TCID_{50}$: Tissue culture infectious dose.
SEM: Standard error of the mean.