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Impact of pH and temperature in dairy processing on the infectivity of H5N1 avian influenza viruses

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ABSTRACT

Highly pathogenic avian influenza viruses of subtype H5N1 (clade 2.3.4.4b) can cause a mastitis-like disease in dairy cows. The presence of high amounts of infectious H5N1 virus in milk has raised significant concerns about the safety of raw milk products. In this study, the effect of temperature and pH on the stability of H5N1 viruses was investigated. We found that both bovine and avian H5N1 viruses remained infectious when incubated in milk at 4 °C for four weeks. When the viruses were incubated in milk at 21 °C, infectivity of avian H5N1 decreased only slightly and of bovine H5N1 moderately. The avian H5N1 virus was stable at 50 °C for 30 min but was inactivated at higher temperatures (55 °C for 10 min, 60 °C for 1 min, or 72 °C for 30 s). Bovine and avian H5N1 viruses were stable at pH levels between 6.0 and 10.0, but were partially inactivated at pH 5.0 and completely inactivated at pH 4.0. Both H5N1 viruses were completely inactivated when incubated with yoghurt at pH 4.2. Incubation of the avian H5N1 virus with soft and semi-hard cheese at pH 5.0–5.3 reduced infectious titers by 5.1 and 3.9 $\log_{10}$, respectively. In contrast, the infectivity of bovine H5N1 was only minimally reduced following incubation with semi-hard cheese. In conclusion, H5N1 viruses are efficiently inactivated by pasteurization and most thermisation procedures. However, in untreated raw milk bovine H5N1 virus may survive cheese-making processes if the production temperature stays below 50 °C.

1. Introduction

In February 2024, a milk drop syndrome was recorded in dairy cows in Texas, USA. The following month, a highly pathogenic avian influenza (HPAI) virus of subtype H5N1 (clade 2.3.4.4b, genotype B3.13) was identified as the etiological agent (Mostafa et al., 2024; Webby and Uyeki, 2024). Although this avian influenza virus did not show mutations characteristic of adaptation to the mammalian host (Hu et al., 2024), it was designated “bovine H5N1” (Eisfeld et al., 2024). The infected animals developed a mastitis-like disease characterized by fever, reduced appetite, decreased milk production, and abnormal milk consistence (Halwe et al., 2025). Animals generally recovered slowly and milk yield remained low for at least four weeks (Caserta et al., 2024).

The virus was found to replicate predominantly in the mammary glands of lactating cows with over 10^8 focus-forming units (FFU) per milliliter shed into the milk (Halwe et al., 2025). The virus was further transmitted between animals by contaminated milking equipment (Le Sage et al., 2024), however, the role of other transmission routes remains unclear. Moreover, virus spillover to cats, mice, and poultry have been reported (Mainenti et al., 2025; Mostafa et al., 2024; Naraharisetti et al., 2025). Several farm workers who had been in contact with infected cows became infected as well. Fortunately, most of them displayed only mild symptoms such as conjunctivitis (Garg et al., 2025; Uyeki et al., 2024). However, infection with H5N1 of genotype D1.1 resulted in critical illness of an adolescent from British Columbia (Jassem et al., 2025), and proved fatal in a young child in Mexico (WHO, 2025).

By March 2025, bovine H5N1 virus has affected approximately 983 herds in 17 US states (USDA, 2025). Recently, genotype D1.1 of H5N1

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HPAI virus (clade 2.3.4.4b) has also been detected in dairy cattle in the United States of America, indicating that that there have been at least two independent incursions of H5N1 viruses into cattle (Pekar et al., 2025). There is evidence that European H5N1 HPAI (clade 2.3.4.4b) virus isolates can cause a similar disease in lactating cows, suggesting that the phenomenon is not unique to the American genotypes B3.13 and D1.1, but may represent a general feature of H5N1 HPAI viruses of clade 2.3.4.4b (Halwe et al., 2025).

Since infected dairy cows were found to shed large amounts of infectious H5N1 virus into the milk, it was not surprising that H5N1 genomic RNA was detected in around 17.4 % of retail dairy products in the USA (Suarez et al., 2025). However, infectious virus was not detected in pasteurized milk (Spackman et al., 2024b), and recent studies confirmed that H5N1 HPAI virus is inactivated by pasteurization (72 °C, 15 s) (Alkie et al., 2025; Guan et al., 2024). In a recent study, the thermal inactivation spectrum of H5N1 viruses in raw milk was investigated showing that H5N1 viruses are rapidly inactivated by pasteurization and by most thermization conditions used in the dairy industry (Nooruzzaman et al., 2025). Another study showed that H5N1 viruses are inactivated by acidification of the contaminated milk (Crossley et al., 2025).

H5N1 viruses were found to be stable in pasteurized or irradiated milk and in unpasteurized milk for several days, in particular at an incubation temperature of 4 °C (Caceres et al., 2024; Kaiser et al., 2025). Feeding of virus-contaminated unpasteurized milk resulted in lethal infection of mice and cats (Burrough et al., 2024; Eisfeld et al., 2024). A recent study in macaques showed that non-human primates reacted differentially, as they did not show severe symptoms following oral infection with bovine H5N1 (Rosenke et al., 2025). Nevertheless, significant concerns over the safety of milk products remain since different types of cheese are produced using untreated raw milk.

The aim of the present study was to evaluate the impact of two important parameters in cheese production, pH and temperature, on the infectivity of H5N1 viruses. Our findings may be useful in assessing the risk of raw milk products, which is an important consideration for consumers.

2. Materials and methods

2.1. Cells

Madin-Darby canine kidney (MDCK) type II cells were kindly provided by Georg Herrler (University of Veterinary Medicine, Hannover, Germany) and maintained with minimum essential medium (MEM, Life Technologies, Zug, Switzerland) supplemented with 5 % of fetal bovine serum (FBS; Pan Biotech, Aidenbach, Germany).

2.2. Viruses

The HPAI virus A/cattle/Texas/063224-24-1/2024 (H5N1) (A/ca/Tex/24), clade 2.3.4.4b, genotype B3.13 (GISAID accession number: EPI_ISL_19155861), was kindly provided by Diego Diel (Cornell University, Ithaca, NY, USA). This virus was originally isolated from the milk of infected dairy cows in Texas, USA (Caserta et al., 2024).

The related A/bovine/Texas/24-029328-02/2024 (H5N1) virus (A/bo/Tex/24) (GenBank accession nos.: PP599470–PP599477) (Burrough et al., 2024) was produced by reverse genetics using synthetic cDNA encoding all eight genomic RNA segments (Genscript Biotech, Piscataway, New Jersey, USA). The cDNA of RNA segment 4 was modified in the way that the HA proteolytic cleavage site P_{337}LREKRRKR↓GLF was changed into the monobasic cleavage site P_{337}LRETR↓GLF. The eight cDNAs were cloned into the pHW2000 plasmid and recovered from transfected cells as previously described (Avanthay et al., 2023). Virus stocks were produced on MDCK cells in the presence of 1 μg/mL of acetylated trypsin (Merck KGaA, Darmstadt, Germany) and stored in aliquots at −70 °C.

The LPAI viruses A/duck/Hokkaido/Vac-1/2004 (H5N1) (A/du/Hok/04) (Soda et al., 2008) and A/duck/Potsdam/1402/1986 (A/du/Pot/86) (H5N2) (GenBank accession numbers: CY00577–CY005783, CY014642) were kindly provided by Yoshihiro Sakoda (Hokkaido University, Sapporo, Japan) and Timm Harder (Friedrich-Loeffler-Institute, Greifswald — Insel Riems, Germany), respectively. Both viruses were propagated for 2 days at 37 °C in the allantoic cavities of 10-day-old embryonated specific pathogen-free (SPF) chicken eggs. Alternatively, the viruses were propagated on MDCK cells in the presence of acetylated trypsin.

The chimeric vesicular stomatitis virus VSVΔG(HA:NA:GFP) encoding the HA and NA glycoproteins of the HPAI virus A/Pelican/Bern/1/2022 (H5N1), clade 2.3.4.4b (GISAID accession numbers EPI3526757 and EPI3526758), along with green fluorescent protein (GFP) was generated according to a previously reported method (Thompson et al., 2023). The virus was propagated on MDCK cells and aliquots stored at −70 °C.

2.3. Virus titration

Infectious virus titers of HPAI A/ca/Tex/24 (H5N1) were determined on MDCK cells by limiting dilution. To this end, MDCK cell monolayers in 96-well tissue culture plates were incubated in quadruplicates with serially diluted virus (100 μL/well) for 2 days at 37 °C. The cells were washed once with PBS (200 μL/well) and fixed for 1 h at 21 °C with 4 % of buffered formalin containing 0.1 % (w/v) crystal violet. The plates were washed with tap water and dried. Virus titer was calculated using the Spearman-Kärber method and expressed as tissue infectious dose 50 % ($TCID_{50}$) (Ramakrishnan, 2016). Based on the Poisson distribution, the theoretical relationship between $TCID_{50}$ and focus-forming units (FFU) is approximately 0.69 FFU = 1 $TCID_{50}$. The virus stock used in the present study had a titer of 10^8 $TCID_{50}$/mL corresponding to 6.9×10^7 FFU/mL.

For determination of infectious LPAI virus titers, MDCK cells grown in 96-well tissue culture plates were inoculated in duplicate with 40 μL per well of serial 10-fold virus dilutions for 1 h at 37 °C. Thereafter, 160 μL of MEM containing 1 % methylcellulose was added to each well, and the cells incubated for 24 h at 37 °C. The cells were fixed for 30 min with 4 % formalin in PBS, permeabilized with 0.25 % (v/v) of Triton X-100, and incubated for 60 min with a monoclonal antibody directed to the influenza nucleoprotein (American Type Culture Collection, Manassas, Virginia, USA, ATCC HB-65, clone H16-L10-4R5), diluted 1:50 with PBS, and subsequently for 60 min with goat anti-mouse IgG conjugated with Alexa Fluor-488 (diluted 1:500 in PBS; Life Technologies, Zug, Switzerland). Infected cells were detected by fluorescence microscopy (Observer Z1 microscope, Zeiss, Feldbach, Switzerland), and infectious virus titers were calculated and expressed as focus-forming units per milliliter (FFU/mL). The virus stocks of both A/du/Hok/04 (H5N1) and A/du/Pot/86 (H5N2) had a titer of 5×10^8 FFU/mL. VSVΔG(HA:NA:GFP) was titrated in an analogous manner using the GFP reporter for detection and enumeration of infected cells.

2.4. Thermal stability of H5N1 avian influenza viruses

Fresh raw milk was obtained from a regional dairy farm and heated for 10 min at 90 °C to prevent bacterial growth during long-term incubation experiments (Haas et al., 2025). The heat-inactivated milk (450 μL) was spiked with 50 μL of either A/ca/Tex/24 (H5N1) or A/du/Hok/04 (H5N1) and incubated for up to 28 days at either 4 °C, 21 °C, or 37 °C. Every 7 days, three samples per temperature of incubation were frozen at −70 °C. Finally, infectious titers were determined by virus titration on MDCK cells (see Section 2.2).

For treatment of A/du/Hok/04 (H5N1) virus at temperatures ≥37 °C, 80 μL of either heat-inactivated milk or 80 μL of MEM were spiked with 20 μL of virus stock and incubated in thin-walled 0.2 mL PCR tubes (Eppendorf SE, Hamburg, Germany) for the indicated times

and temperatures using a T100 Thermal Cycler (BioRad, Cressier, Switzerland) with the lid heated to 105 °C. Infectious titers were determined by virus titration on MDCK cells (see Section 2.2).

2.5. Polykaryon formation assay

MDCK cells were grown on cover slips (12-mm in diameter) and infected with VSVΔG(H5:N1:GFP) using a multiplicity of infection (MOI) of 0.01 FFU/cell. At 20 h post infection, the cells were briefly rinsed with MES buffer (50 mM 4-morpholine ethane sulfonic acid, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$), adjusted to pH 5.0–6.2, and incubated in the respective buffer for 10 min at 37 °C. The cells were washed once with MEM and subsequently incubated for 2 h at 37 °C in MEM supplemented with 5 % FBS. Thereafter, the cells were washed once with PBS and fixed for 30 min at 21 °C with PBS containing 4 % formalin. For staining of nuclei, the cells were incubated for 5 min at 37 °C with 0.1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI, Merck KGaA, Darmstadt, Germany) and mounted in ProLong Gold Antifade Mountant (Thermo Fisher). Image acquisition was performed using the LCI Plan-NEOFLUAR 63×/1.3 water immersion objective of an Observer Z1 fluorescence microscope (Zeiss).

2.6. Analysis of virus stability at different pH values

For the treatment of viruses at different pH values, the following buffer systems were used: 50 mM Na_2HPO_4/citric acid, 150 mM NaCl, pH 2.0–5.0; 50 mM MES, 150 mM NaCl, pH 5.0–7.0; 50 mM Tris/HCl, 150 mM NaCl, pH 7.0–10.0. To 450 μL of the respective buffer 50 μL of either A/ca/Tex/24 (H5N1), A/du/Hok/04 (H5N1) or A/du/Pot/86 (H5N2) were added and incubated for 30 min at 21 °C. To replace the buffer with cell culture medium, each incubation (500 μL) was loaded onto a Sephadex G-25 column (PD MiniTrap G-25, Cytiva Europe, Freiburg, Germany) that has been equilibrated with MEM containing 5 % FBS and penicillin/streptomycin (Life Technologies, Zug, Switzerland). The virus was eluted from the column by adding 1 mL of MEM containing 5 % of FBS and penicillin/streptomycin, and was subsequently titrated on MDCK cells (see Section 2.2).

2.7. Production of yoghurt using LPAI H5N1-spiked raw milk

For production of yoghurt, the Jog BL1 starter culture (Liebefeld Kulturen AG, Switzerland), consisting of a mixture of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus delbrueckii* ssp. *lactis*, was prepared according to the instructions of the manufacturer. Briefly, 1.2 % of the BL1 culture was incubated for 3.5 h at 42 °C in ultra-heated skimmed milk (Cremo, Switzerland) and then stored at 4 °C until further use. Yoghurt was produced using 5 mL aliquots of fresh raw milk with 3 % skimmed milk powder (Rapilait, Migros) with or without 1 % of the prepared BL1 culture and 0.5 mL of A/du/Hok/04 (H5N1). The virus-spiked yoghurt was incubated in a water bath at 42 °C until the pH fell within the range of 4.0–4.6 (usually after 5 h of incubation). The fermentation process was terminated by incubating the yoghurt overnight at 4 °C. The yoghurt (5 mL) was mixed with 5 mL of MEM and centrifuged (3000 ×*g*) for 15 min at 4 °C to pellet precipitated protein. Finally, the supernatant was passed through a 0.2 μm pore size filter and stored at −70 °C prior to virus titration (see Section 2.2) or RT-qPCR analysis (see Section 2.9).

In some experiments, H5N1 viruses were incubated with retail yoghurt (Leger yoghurt Nature, Migros). The yoghurt was diluted 1:5 with sterile water and 4.5 mL of the diluted yoghurt incubated for 72 h at 4 °C with 0.5 mL of either A/duck/Hok/24 (H5N1) or A/bo/Tex/24-HA_{mb} (H5N1). Precipitated proteins were removed by centrifugation (see above), and 0.5 mL of the supernatant was loaded onto a Sephadex G-25 column, eluted with 1 mL of MEM supplemented with 5 % FBS and penicillin/streptomycin, and subsequently titrated on MDCK cells (see Section 2.2).

2.8. Incubation of H5N1 viruses with freshly manufactured raw milk cheese

For soft mini-cheese production, the BAMOS starter culture (Liebefeld Kulturen AG, Switzerland), consisting of a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, was prepared according to the instructions of the manufacturer. Briefly, 0.6 % of the BAMOS culture was incubated for 4 h at 40 °C in ultra-heated skimmed milk (Cremo, Switzerland) and stored at 4 °C until further use. Soft cheese was produced by incubating 30 mL of fresh raw milk with 0.5 % of starter culture for 30 min at 35 °C. Thereafter, 0.025 % of beef rennet (Winkler GR orange, Switzerland) was added and incubated in a water bath at 35 °C for 30 min. The curd was cut into pieces of roughly 15 mm^3 and incubated at 35 °C for 40 min with 6 mL of tap water. Subsequently, the milk was centrifuged (3000 ×*g*) for 20 min at 21 °C, the supernatant (whey) was aspirated, and the precipitated material was incubated at 35 °C until the pH was in the range pH 5.0–pH 5.3. Finally, the soft cheese was refrigerated at 4 °C for 7 days.

For semi-hard mini-cheese production, the RMK 302 starter culture (Liebefeld Kulturen AG, Switzerland), consisting of a mixture of *Lactobacillus delbrueckii* ssp. *lactis* and *Streptococcus thermophilus* was prepared according to manufacturer's instructions. Briefly, 0.2 % of the RMK 302 starter culture was incubated in ultra-heated skimmed milk (Cremo, Switzerland) for 6 to18 h at 38 °C and stored at 4 °C until further use. Semi-hard cheese was produced using 30 mL-aliquots of fresh raw milk mixed with 0.5 % of the starter culture and incubated for 30 min at 35 °C. Thereafter, 0.025 % of beef rennet (Winkler GR orange, Switzerland) was added and incubated for 30 min at 35 °C. The curd was cut into pieces of 4–8 mm^3 to which 4.5 mL of tap water were added. The temperature was increased from 35 °C to 45 °C within 15 min and the temperature was held at 45 °C for another 15 min. Precipitated proteins were pelleted by centrifugation (3000 ×*g*, 20 min) and incubated at 40 °C until the pH dropped to 5.0–5.3. The semi-hard cheese was stored refrigerated at 4 °C.

For incubation of H5N1 viruses with 7 days-old soft cheese or semi-hard cheese, about 0.6 g of the respective cheese was cut into small pieces (approx. 0.5 mm^3) and suspended in 0.9 mL of sterile water. Measurement of the proton concentration showed that the pH was in the range of 5.0–5.3. The cheese was mixed with 0.1 mL of virus stock and incubated for 24 h or 72 h at 4 °C. Subsequently, 0.5 mL of the incubated virus suspension was loaded onto a Sephadex G-25 column and eluted in 1 mL of MEM supplemented with 5 % FBS and penicillin/streptomycin.

2.9. Detection of viral RNA by RT-qPCR

RNA was extracted from 200 μL of spiked raw milk using the NucleoMag Vet kit (Macherey-Nagel AG, Oensingen, Switzerland according to the manufacturer's protocol. Reverse transcription from RNA to cDNA and real-time quantitative PCR (qPCR) were performed on the QuantStudio 5 real-time PCR system (Thermo Fisher Scientific) using the AgPath-ID One-Step RT-PCR kit (Life Technologies, Zug, Switzerland) and segment 7-specific oligonucleotide primers and probe (Hofmann et al., 2008; Spackman et al., 2002). Data were acquired and analyzed using the Design and Analysis Software v1.5.2 (Thermo Fisher Scientific).

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 10, version 10.1.2 (GraphPad Software, Boston, Massachusetts, USA). Unless noted otherwise, the results are expressed as mean ± standard deviation (SD). Specific statistical tests such as the one-way or two-way ANOVA test were used to assess significant differences in infectious titers as indicated in the figure legends. P values < 0.05 were considered significant. The thermal virus inactivation kinetics were calculated based on the time required at a specific temperature to achieve a 1 $\log_{10}$ reduction in

viral titer. *D*-values were calculated by linear regression analysis of the decimal logarithm of virus titer plotted against time and defined as the negative inverse of the slope.

3. Results

3.1. Impact of long-term storage on H5N1 stability in bovine milk

First, we investigated the effect of long-term storage of bovine milk spiked with H5N1 avian influenza viruses on infectious virus titers. We incubated either HPAI A/cattle/Texas/063224-24-1/2024 (H5N1) (A/ca/Tex/24) or LPAI A/duck/Hokkaido/Vac-1/2004 (H5N1) (A/du/Hok/04) with heat-inactivated bovine milk for four weeks at either 4 °C, 21 °C or 37 °C. Aliquots of the virus-spiked milk were taken at weekly intervals and infectious virus titers were determined on MDCK cells by endpoint titration (Fig. 1). Both A/ca/Tex/24 and A/du/Hok/04 were stable when incubated for four weeks at 4 °C and showed no significant loss of infectivity during this time. However, when the viruses were incubated with milk at 21 °C, their infectivity decreased over time. While A/du/Hok/04 showed a tenfold reduced infectious titer after four weeks of incubation, the infectious titer of A/ca/Tex/24 was reduced by about 2.5 $\log_{10}$ at the end. Incubation of the viruses at 37 °C had a stronger effect on virus infectivity. While the infectious titer of A/ca/Tex/24 fell below the detection limit after three weeks of incubation, infectious A/du/Hok/04 was still detectable after four weeks of incubation, albeit at significantly reduced levels. These findings suggest that virus stability may differ in a strain-specific manner. However, at 4 °C, the infectious titers of both virus strains remained high with no signs of significant inactivation.

3.2. Thermal stability of A/du/Hok/04 (H5N1) at elevated temperatures

To investigate viral stability at higher temperatures, A/du/Hok/04 (H5N1) was diluted in MEM or bovine milk and incubated for 30 min at 4 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C or 60 °C. Subsequently, the infectious virus titer was determined on MDCK cells by endpoint titration (Fig. 2a). We observed that A/du/Hok/04 (H5N1) remained stable for 30 min at 4 °C and 37 °C. However, a 2-fold and 3.5-fold decrease in the infectious virus titer was detected at 40 °C and 45 °C, respectively. On the other hand, a more pronounced reduction in viral titer was found when the virus was incubated at 50 °C (35-fold reduction). Notably, no infectious virus was detectable when the virus was incubated for 30 min at either 55 °C or 60 °C. A very similar inactivation pattern was found when A/du/Hok/04 (H5N1) was incubated in MEM (Fig. 2a). However, the infectious virus titers remained at generally higher levels compared to virus incubated in milk, suggesting that A/du/Hok/04 is less stable in bovine milk. To determine the minimum incubation period leading to complete virus inactivation, we incubated A/du/Hok/04 in bovine milk at 55 °C or 60 °C for shorter periods. We observed that infectious virus titers fell below the detection limit when the virus was treated for 10 min at 55 °C (Fig. 2b) or for 1 min at 60 °C (Fig. 2c). Likewise, the virus was completely inactivated when incubated for 30 s at 72 °C (Fig. 2c). When the virus was incubated in bovine milk at 72 °C for 15 s, a reduction of infectious titer by 4.5 $\log_{10}$ was achieved, in line with previous reports which showed that pasteurization can efficiently inactivate bovine H5N1 virus (Alkie et al., 2025; Cui et al., 2024; Nooruzzaman et al., 2025; Spackman et al., 2024a).

3.3. The HA of H5N1 HPAI clade 2.3.4.4b initiates membrane fusion at mildly acidic conditions

Influenza A viruses enter cells by receptor-mediated endocytosis and subsequent fusion of the viral envelope with the endosomal membrane. Membrane fusion is mediated by hemagglutinin (HA), the major viral envelope glycoprotein, which is usually in a metastable conformation. The acidification of the endosomal lumen triggers a conformational change in HA which initiates the membrane fusion process. As this conformational change is irreversible, a premature exposure of influenza A viruses to low pH can result in HA denaturation and complete loss of infectivity. The HA proteins of different influenza A virus strains can vary considerably in their acid stability or pH values at which irreversible conformational changes are induced (Russell, 2014; Russell et al., 2018). To determine the pH threshold at which the HA of H5N1 HPAI initiates membrane fusion, we performed a polykaryon formation assay. To this end, we infected MDCK cells with a recombinant vesicular stomatitis virus vector encoding the HA and NA antigens of HPAI A/Pelican/Bern/1/2022 (H5N1) (clade 2.3.4.4b) along with the green fluorescent protein (GFP) reporter. At 16 h post infection, the cells were briefly incubated with buffers adjusted to different pH values and subsequently incubated for 2 h at 37 °C in cell culture medium. The cells were fixed and analyzed for the formation of polykaryons (syncytia) (Fig. 3a). The results indicate that mildly acidic conditions (pH of 6.0–6.1) are sufficient to trigger the conformational change in the HA of this H5N1 (clade 2.3.4.4b) virus.

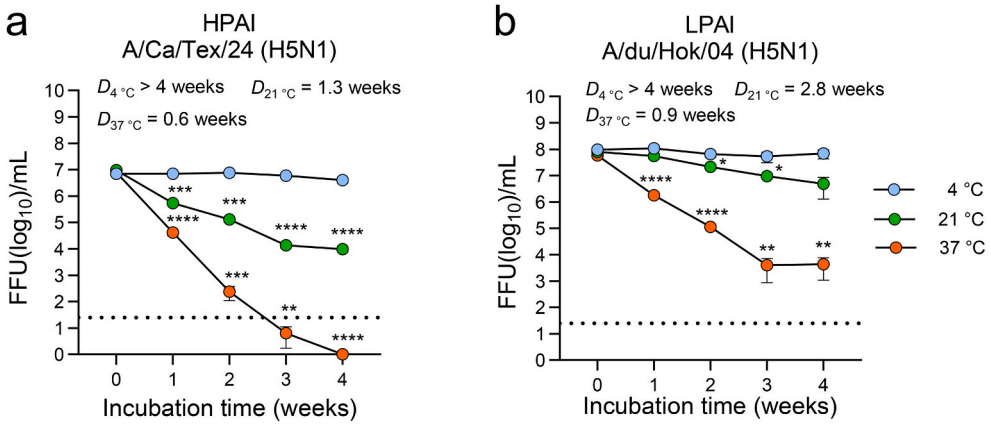

Fig. 1. Impact of long-term storage of H5N1 viruses in bovine milk. Raw milk was heated for 10 min at 95 °C, spiked with either HPAI A/cattle/Texas/063224-24-1/2024 (H5N1) (A/ca/Tex/24) (a) or LPAI A/duck/Hokkaido/Vac-1/2004 (H5N1) (A/du/Hok/04) (b), and incubated for up to 4 weeks at either 4 °C, 21 °C, or 37 °C. At the indicated time points, the samples were frozen at −70 °C. After the last sample was collected, all samples were thawed and infectious virus titers determined on MDCK cells by end point dilution. Mean titers and standard deviations of three incubation experiments are shown. The two-way ANOVA with Tukey's multiple comparison test was used to assess significantly different infectious titers (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). The time to achieve 1 $\log_{10}$ reduction in viral titer (*D*-value) is indicated for each of the incubation temperatures used.

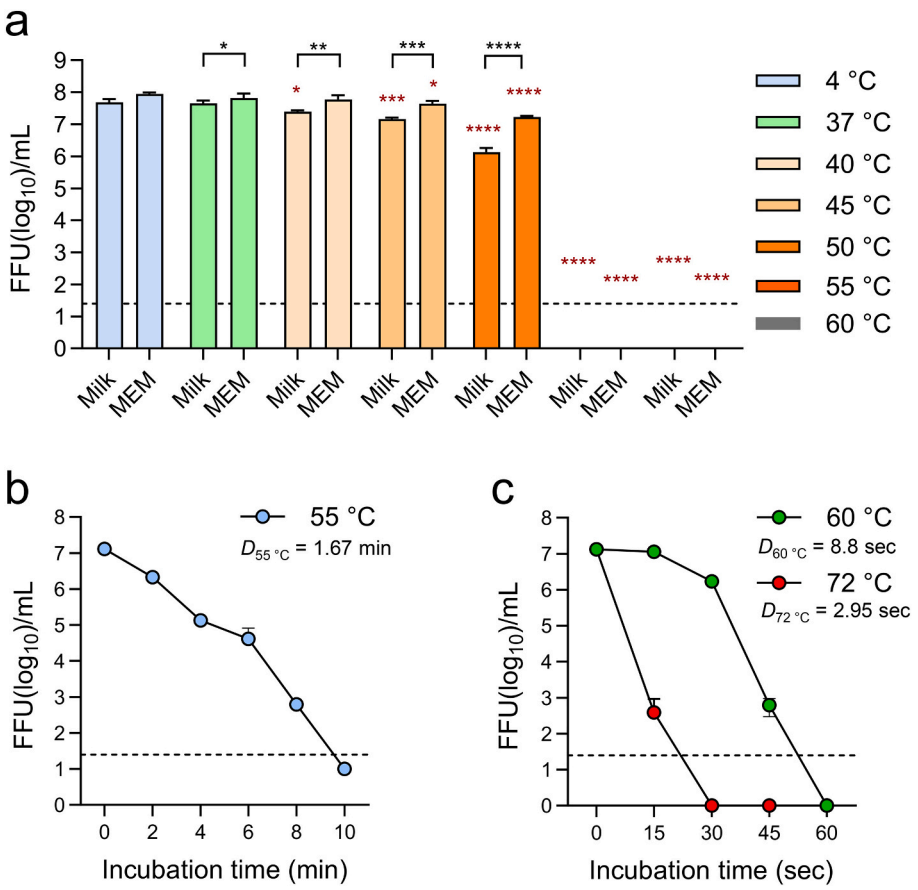

Fig. 2. Thermal stability of A/du/Hok/04 (H5N1). (a) Bovine milk or MEM were spiked with A/du/Hok/04 (H5N1) and incubated for 30 min at the indicated temperatures. (b–c) Bovine milk was spiked with A/du/Hok/04 (H5N1) and incubated for the indicated times at either 55 °C (b), 60 °C or 72 °C (c). Infectious virus titers were determined on MDCK cells by end point titration. The interrupted line indicates the limit of detection of infectious virus. Mean values and standard deviations of three inactivation experiments are shown. Significantly differences in infectious virus titers were calculated using the two-way ANOVA test. Significant differences between spiked milk and MEM are indicated by black asterisks (Sidak's multiple comparisons) and significant differences between elevated temperatures and 4 °C are indicated by red asterisks (Tukey's multiple comparisons); $*p < 0.05$; $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. The time to achieve 1 $\log_{10}$ reduction in viral titer (*D*-value) is indicated for each of the incubation temperatures used.

3.4. Inactivation of H5N1 viruses in a pH-dependent manner

To investigate the stability of H5N1 viruses at different acid and alkaline conditions, the virus was incubated for 30 min at 21 °C with buffers adjusted to defined pH values. The buffer was subsequently replaced by cell culture medium using Sephadex-G25 gel filtration columns, and infectious virus titers were determined on MDCK cells by end point dilution. Using the infectious titer at pH 7.0 as reference, we found that the infectivity of A/ca/Tex/24 (H5N1) was not affected when the virus was exposed to pH values in the alkaline range (pH 8–10) or when the pH was slightly acidic (pH 6.0) (Fig. 3b). A gradual reduction of the infectious titer, however, was observed when the pH was lowered stepwise from pH 6.0 to 4.0, with no infectivity left at pH 4.0. Similarly, A/du/Hok/04 (H5N1) was stable in the pH range 6–10, showed a significant drop of infectious titer at pH 5.0, and a complete loss of infectivity at pH 4.0 (Fig. 3c). However, when the experiment was performed with A/du/Pot/86 (H5N2), some residual infectivity was still observed at pH 4.0 (Fig. 3d), suggesting that some avian influenza virus strains might be more resistant to low pH-mediated inactivation.

3.5. Inactivation of H5N1 viruses by incubation with raw milk cheese and yoghurt

To investigate the stability of H5N1 viruses in fermented milk products, we produced laboratory-scale soft and semi-hard cheeses using raw milk, cut the cheeses into small pieces that were suspended in water and incubated them for 24 h at 4 °C with A/du/Hok/04 (H5N1). In parallel, the virus was incubated for 24 h at 4 °C with buffer adjusted to either pH 7.0, pH 6.0, pH 5.0 or pH 4.0. Afterwards, the buffer was replaced by MEM by size exclusion chromatography and infectious virus titer determined on MDCK cells. When the virus was incubated with buffers adjusted to pH 7.0 or pH 6.0, the virus titer remained at the same level (Fig. 4a). Incubation of the virus for 24 h with buffer adjusted to pH 5.0 resulted in a drop of infectivity by 3.3 $\log_{10}$, while incubation at pH 4.0 almost completely inactivated the virus. When A/du/Hok/04 (H5N1) was incubated with soft cheese at pH 5.0 or with semi-hard cheese at pH 5.3, the infectious titer was reduced by 5.1 $\log_{10}$ and 3.9 $\log_{10}$, respectively. Interestingly, the incubation with both kinds of cheese resulted in a more pronounced reduction of virus titer compared to incubation with pH 5.0 buffer alone, indicating that factors other than pH might have contributed to this loss in infectivity. To assess whether incubation with cheese or buffers of different pH would result in degradation of viral RNA, total RNA was extracted from the samples and analyzed by RT-qPCR for the presence of the viral RNA genome segment 7. Compared to virus incubated at pH 7.0, we observed a decline in the 45-Ct values when A/du/Hok/04 (H5N1) was incubated at acidic conditions or with one of the cheese types (Fig. 4b), suggesting that virus integrity was affected and some viral RNA degraded. We compared the inactivation of A/du/Hok/04 (H5N1) propagated on embryonated chicken eggs with virus produced on MDCK cells and found that the incubation with raw milk cheese resulted in significant inactivation of the virus regardless of the cell substrate on which it has been produced

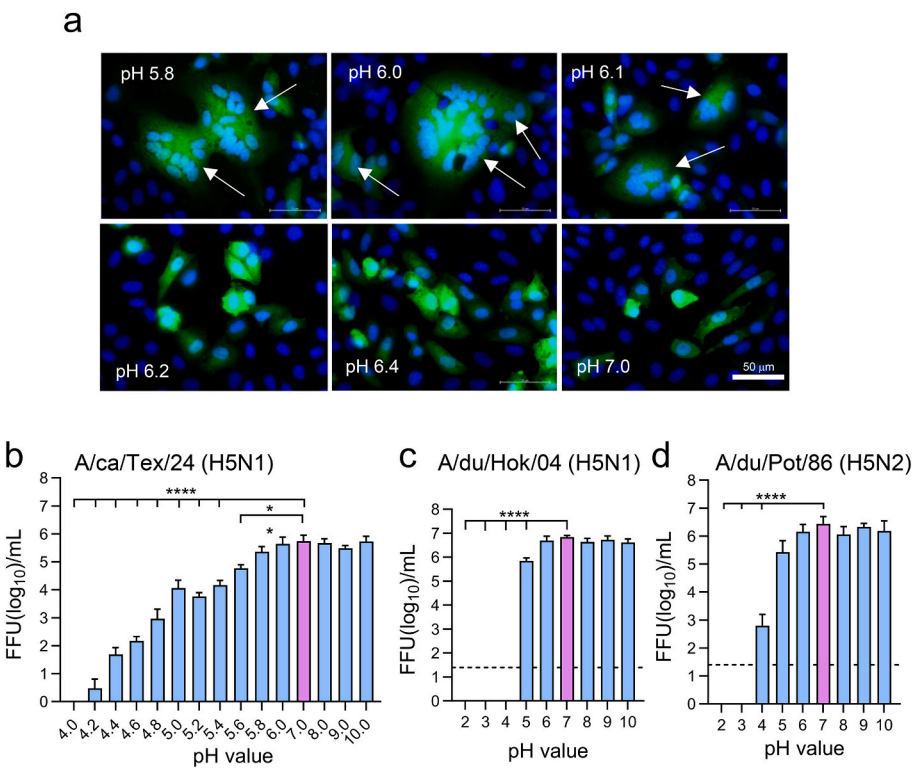

Fig. 3. Acid stability of H5Nx influenza viruses. (a) Determination of the pH threshold triggering HA-mediated membrane fusion. Vero cells were infected with VSVΔG(HA:NA:GFP) encoding the HA and NA glycoproteins of A/Pelican/Bern/1/2022 (H5N1) as well as green fluorescent protein (GFP). At 18 h post infection, the cells were exposed for 10 min at 37 °C to buffers adjusted to the indicated pH values and subsequently incubated for 2 h at 37 °C in MEM medium. The cells were fixed and nuclei stained with DAPI. The cells were visualized by fluorescence microscopy (nuclei: blue fluorescence; GFP: green fluorescence). Bar size = 50 µm. Arrows point to syncytia formed. (b–d) Analysis of pH-dependent inactivation of H5Nx influenza viruses. HPAI A/ca/Tex/24 (H5N1) (b), LPAI A/du/Hok/04 (H5N1) (c), and LPAI A/du/Pot/86 (H5N2) (d) were incubated for 30 min at 21 °C with buffers adjusted to the indicated pH values. The buffers were subsequently replaced by MEM using Sephadex G25 size exclusion chromatography, and infectious virus titers determined on MDCK cells. Mean values and standard deviations of three inactivation experiments are shown. Significantly different titers compared to the treatment at pH 7.0 were calculated using the one-way ANOVA test with SIDAK's multiple comparisons (**$p < 0.01$, ****$p < 0.0001$).

(Fig. 4c, d).

Next, we compared the inactivation of A/du/Hok/04 (H5N1) and A/ca/Tex/24 (H5N1) and incubated both viruses for 72 h at 4 °C with semi-hard cheese at pH 5.05–5.15. We found that the infectious titer of A/du/Hok/04 (H5N1) decreased by 4.9 $\log_{10}$ (Fig. 4e), while the amount of viral RNA decreased only slightly (Fig. 4f). Surprisingly, the infectivity of A/ca/Tex/24 (H5N1) was not significantly affected when the virus was incubated under the same conditions. The infectious titer of the recombinant A/bo/Tex/24-HA_{mb} (H5N1) virus encoding an HA with a monobasic proteolytic cleavage site was reduced by 1.2 $\log_{10}$ under these conditions, however, this drop was small compared to the loss of infectivity observed with A/du/Hok/04 (H5N1) (Fig. 4e). Interestingly, incubation of A/bo/Tex/24-HA_{mb} with buffer adjusted to pH 5.0 reduced infectious titer by 2.5 $\log_{10}$ (Fig. 4g, h), indicating that the virus is only partially inactivated at this pH value.

Finally, we produced yoghurt by incubating A/du/Hok/04-spiked raw milk with or without starter culture for about 5 h at 42 °C. When the milk with starter culture added reached a pH of 4.2 to 4.6, the fermentation process was stopped by incubating the yoghurt overnight at 4 °C. Milk without starter culture added stayed at pH 6.5. Finally, the samples were mixed with MEM, sterile-filtrated, and infectious virus titer determined on MDCK cells. Using this approach, we detected significant titers of infectious virus in the preparation without starter culture, whereas no infectious virus was found in the samples to which starter culture had been added (Fig. 4i). RT-qPCR analysis of the samples revealed a significant drop in viral RNA in milk with starter culture (Fig. 4j). This drop most likely reflects the loss of virus particles due to low pH-triggered aggregation and precipitation of the virus particles and their retention on the Sephadex G25 gel filtration columns. We also investigated the stability of A/bo/Tex/24-HA_{mb} by incubating the virus with retail yoghurt at pH 4.2. Under these conditions, A/bo/Tex/24-HA_{mb} was inactivated as efficiently as A/du/Hok/04 (Fig. 4i, j).

4. Discussion

Raw milk or raw milk products from cows, goats or sheep may be contaminated with bacterial pathogens such as *Campylobacter*, *Listeria*, *Brucella*, Shiga toxin-producing *Escherichia coli* and *Salmonella*, and viral pathogens such as tick-borne encephalitis virus (TBE) and hepatitis E virus (Gonzalez et al., 2022; Holzhauer and Wennink, 2023; Huang et al., 2016; Idland et al., 2022; Williams et al., 2023; Zahmanova et al., 2024). Following the detection of H5N1 highly pathogenic avian influenza (HPAI) virus in milk from dairy cows in the United States of America in 2024, this virus must be added to the list of pathogens of concern. Pasteurization of milk has been shown to inactivate most of these pathogens (Alkie et al., 2025; Cui et al., 2024; Oliver et al., 2005; Schafers et al., 2025; Spackman et al., 2024a). However, several types of cheese are made from untreated raw milk. In the present study we investigated whether H5N1 viruses would "survive" the cheese-making process.

To assess whether cheese made from raw milk containing infectious H5N1 influenza viruses would pose a food safety risk we first analyzed whether temperature along with prolonged storage time affects virus infectivity. Incubation of A/ca/Tex/24 (H5N1) and A/du/Hok/04 (H5N1) with milk at 4 °C showed that the infectious titer of these viruses remained stable for at least four weeks. Incubation in milk at 21 °C

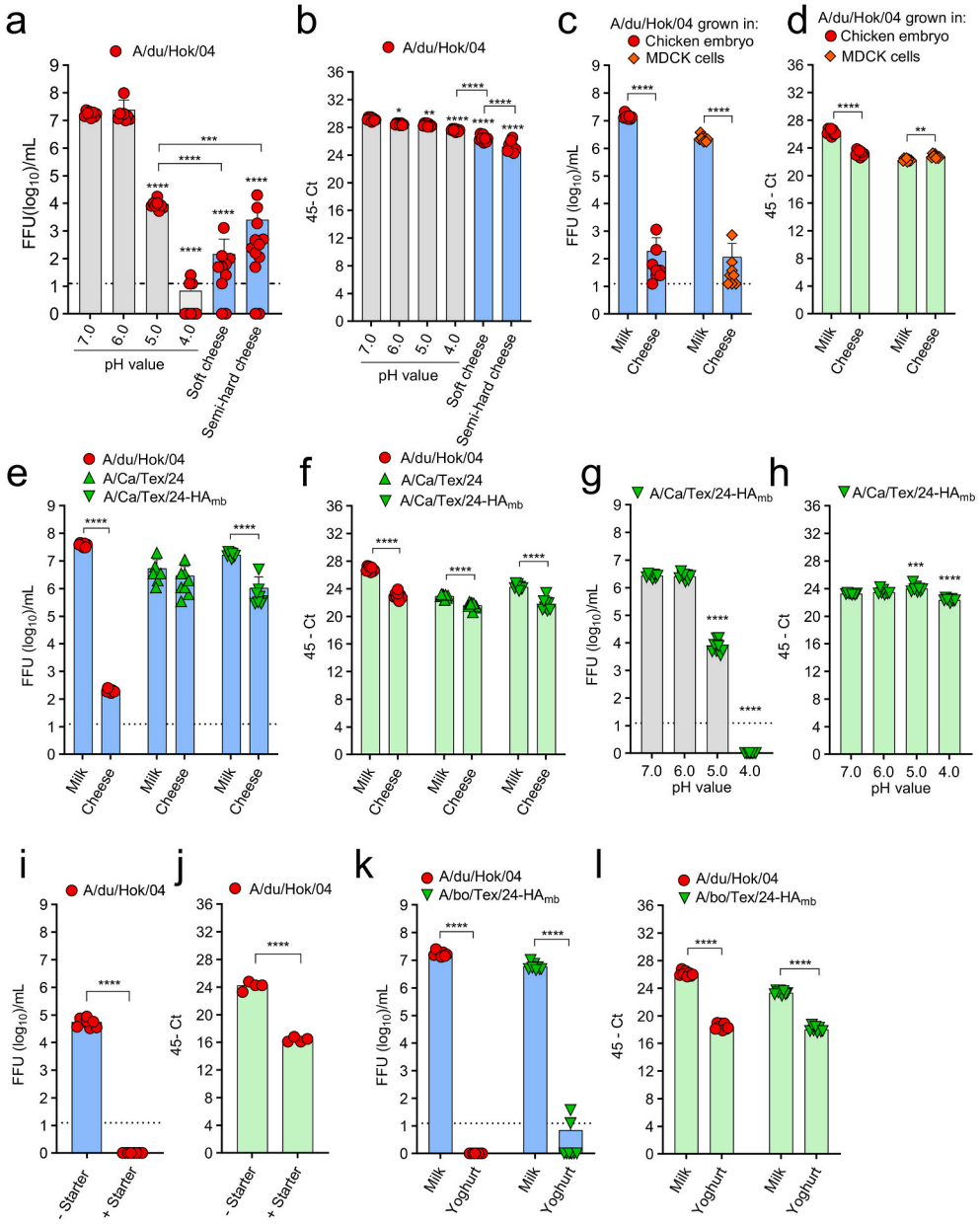

Fig. 4. Effect of lactic acid fermentation on H5N1 infectivity. (a–d) Analysis of the stability of A/du/Hok/04 (H5N1). (a, b) A/du/Hok/04 (H5N1) was propagated on embryonated chicken eggs and incubated for 24 h at 4 °C with buffers of the indicated pH or with soft cheese or semi-hard cheese. (c, d) A/du/Hok/04 was propagated on either embryonated chicken eggs or MDCK and incubated for 24 h at 4 °C with either heat-inactivated milk (pH 6.5) or semi-hard cheese (pH 5.2). The incubation medium was replaced afterwards by MEM using size exclusion chromatography. (a, c) Infectious virus titers were determined on MDCK cells. (b, d) Detection of viral RNA by RT-qPCR. The maximum amplification cycles minus the cycle threshold number (45-Ct) are plotted. (e–h) Analysis of the stability of the bovine H5N1 viruses A/ca/Tex/24 (H5N1) and A/bo/Tex/24-HA$_{mb}$. LPAI A/du/Hok/04, HPAI A/ca/Tex/24, and the recombinant A/bo/Tex/24-HA$_{mb}$ were incubated for 72 h at 4 °C with either heat-inactivated milk (pH 6.5) or semi-hard cheese (pH ranging from 5.05 to 5.15). (e) Determination of infectious titers on MDCK cells. (f) Determination of virus content by RT-qPCR. (g, h) Incubation (72 h, 4 °C) of A/bo/Tex/24-HA$_{mb}$ in buffers adjusted to the indicated pH values. The buffers were replaced by MEM by size exclusion chromatography before infectious titers (g) and viral RNA load (h) were determined. (i, j) Inactivation of A/du/Hok/04 (H5N1) during the production of yoghurt. Raw milk was spiked with virus and incubated at 42 °C with or without starter culture. When the pH of the yoghurt fell in the range of pH 4.2 to 4.6, the yoghurt was stored overnight at 4 °C. The yoghurt was diluted with MEM, sterile-filtrated, and infectious virus titers (i) and viral RNA load (j) determined. (k–l) Incubation (72 h, 4 °C) of A/du/Hok/04 and A/bo/Tex/24-HA$_{mb}$ with either heat-inactivated milk (pH 6.5) or retail yoghurt (pH 4.2). (k) Determination of infectious titers. (l) Determination of viral RNA content by RT-qPCR. Mean values and standard deviations of 8 incubation experiments are shown for each condition, except one experiment where 4 samples were analyzed by RT-qPCR (j). (a, b, g, h) Significant differences were assessed by the one-way ANOVA test with Tukey's or Dunnett's multiple comparisons (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$). (e–f, k, l) Significant differences in virus titer or viral RNA content were assessed using the unpaired two-tailed Student *t*-test (****$p < 0.0001$).

resulted in a moderate decrease in infectivity over time. This decrease was more pronounced when the virus was incubated at 37 °C. We also observed strain-specific differences, as A/du/Hok/04 (H5N1) was more stable than A/ca/Tex/24 (H5N1) during long-term storage at these temperatures (Caceres et al., 2024; Nooruzzaman et al., 2025). It should be emphasized that the experiments were carried out with heat-inactivated milk to prevent bacterial growth and fermentation. Therefore, our experiments only provide insights into the influence of the parameter "temperature", but do not exclude that bacteria or other factors that may be present in untreated raw milk could have an

influence on virus infectivity. A recent study has shown that bovine H5N1 virus is stable in unpasteurized milk for four days at both 4 °C and room temperature (Caceres et al., 2024). In contrast, another study reported that the infectivity of bovine H5N1 decreases over time when incubated in untreated raw milk at 4 °C (Nooruzzaman et al., 2025).

Thermal treatment of raw milk is widely used in the dairy sector to guarantee product safety and longer shelf-life. It includes treatments such as thermisation (57–68 °C/30 min–15 s), low-temperature-long-time (LTLT) pasteurization (63 °C/30–40 min), high-temperature-short-time (HTST) pasteurization (72 °C–80 °C/15–30 s), and ultra-high temperature (UHT) treatment (135 °C–150 °C/10–1 s) (IDF, 2022). In line with previous reports (Alkie et al., 2025; Nooruzzaman et al., 2025; Spackman et al., 2024a), we found that HTST pasteurization of raw milk at 72 °C for 15 s reduced infectious titers of H5N1 virus efficiently ($D_{72°C} = 3$ s). Moreover, inactivation of H5N1 was also achieved if the virus-spiked milk was treated for 1 min at 60 °C ($D_{60°C} = 8.8$ s) or for 10 min at 55 °C ($D_{55°C} = 1.67$ min), which is in line with previously published data (Nooruzzaman et al., 2025). According to this latter report, H5N1 viruses are also efficiently inactivated at other thermisation conditions including treatment for 22 s at 63 °C or 69 °C (Nooruzzaman et al., 2025). Some cheese types are made from raw milk which is neither treated by pasteurization nor thermisation (Bachmann et al., 2011). Nevertheless, the production of some types of cheese involves heating the curd at 50 °C–55 °C for 30–60 min. It is possible that under these conditions the infectious titers decrease significantly, however, future studies must show how efficiently H5N1 viruses are inactivated by this procedure. We would like to point out that some raw milk cheeses are produced at temperatures below 50 °C. As incubation of virus-spiked milk at 50 °C for 30 min had only a small effect on infectious titers (see Fig. 2a), temperature alone may not result in sufficient inactivation of H5N1 virus. Therefore, we investigated whether other factors such as the drop in pH during the lactic acid fermentation would result in virus inactivation.

Exposure of A/ca/Tex/24 (H5N1) or A/du/Hok/04 (H5N1) to buffers adjusted to different pH values showed that the viruses were stable in the alkaline range (up to pH 10.0), but were inactivated in the acidic range, especially when the pH dropped below pH 5.0. One explanation for this acid lability is probably the role of protons in the life cycle of influenza A viruses. The dominant glycoprotein in the viral envelope, hemagglutinin (HA), exhibits membrane fusion activity in pH-dependent manner. HA is synthesized as a precursor protein that is cleaved into two subunits by cellular proteases (Bottcher-Friebertshauser et al., 2014). This post-translational modification causes HA to adopt a metastable and fusion-competent conformation. Following uptake of the virus by receptor-mediated endocytosis acidification of the endosomes below a certain pH threshold triggers a conformational change in HA, which then initiates the fusion process (Russell, 2014; Russell et al., 2018). The pH-induced HA conformational change is thought to be irreversible (Benhaim et al., 2020; Benton et al., 2020). Therefore, exposure of a virus particle to acidic medium prior to cell entry would result in premature denaturation of the HA protein and loss of infectivity.

The pH threshold triggering this conformational change of the HA of a H5N1 (clade 2.3.4.4b) virus was found to occur at mildly acidic conditions (pH 6.0–6.1). This is consistent with recent data showing that membrane fusion is triggered by various isolates of H5N1 (clade 2.3.4.4b) at a pH value of 5.9 (Yang et al., 2025), which is characteristic for most avian influenza viruses (Russell et al., 2018). Nevertheless, complete inactivation of HPAI A/ca/Tex/24 (H5N1) was only observed when the virus was incubated with buffer adjusted to a pH of 4.0. When the virus was incubated at a pH of 5.0, a considerable amount of infectious virus was maintained (see Fig. 3b, c, d). This finding is consistent with the notion that membrane fusion by influenza virus may involve conformational changes of only a small HA fraction (Remeta et al., 2002). Another fraction of HA may not completely unfold but may stay at transition stages which are still able to fold back into the metastable conformation when the pH is raised again.

The incubation of A/du/Hok/04 (H5N1) with laboratory-scale soft cheese (pH 5.0) or semi-hard mini-cheese (pH 5.3) had a stronger inactivating effect on this virus than the incubation with pH 5.0 buffer alone. The reason for this phenomenon is not yet understood but may be related to the previous notion that the low pH-induced conformational change of HA renders the molecule more susceptible to proteolytic degradation (Puri et al., 1990). We therefore hypothesize that some A/du/Hok/04 (H5N1) virions remain infectious in raw milk even after the pH has dropped to pH 5.0, but loose infectivity during the ripening processes of the cheese which involve bacterial proteases (Kok, 1991; Law and Kolstad, 1983).

In contrast to A/du/Hok/04 (H5N1), incubation of A/ca/Tex/24 (H5N1) with semi-hard cheese (pH 5.1–5.2) did not significantly reduce infectious titers (see Fig. 4e). Likewise, infectious titers of A/bo/Tex/24-HA_{mb} (H5N1) dropped only by 1 $\log_{10}$ when the virus was incubated with semi-hard cheese. As the infectivity of the viruses was more efficiently reduced when the viruses were incubated with buffers adjusted to pH 5.0, we hypothesize that the viruses had been stabilized by binding to a yet unknown factor in the cheese. However, more work is required to identify this factor and to determine the viral molecular determinants that were responsible for this phenotype.

In conclusion, our data show that both temperature and pH are important parameters in cheese production that can contribute significantly to food safety. In particular, pasteurization and most thermisation procedures are capable of efficiently inactivating infectious H5N1 viruses in milk. However, it is important to note that if cheese is produced with untreated raw milk at temperatures less than or equal to 50 °C, it may still contain infectious virus. Other parameters such as the cheese ripening temperature and time, the salt concentration, and the water content might have an impact on H5N1 virus stability and should be investigated in further studies for a comprehensive risk analysis. Nevertheless, the data presented here make a valuable contribution to our general understanding of H5N1 viruses in milk and dairy products.

CRediT authorship contribution statement

Nicole Lenz-Ajuh: Writing – review & editing, Investigation, Conceptualization. **Leonie Rau:** Visualization, Investigation, Formal analysis, Data curation. **Lisa Butticaz:** Investigation, Formal analysis, Data curation. **Étori Aguiar Moreira:** Investigation, Methodology. **Bettina Zimmer:** Methodology, Investigation, Data curation. **Vincent Beuret:** Resources, Methodology. **Florian Loosli:** Resources, Methodology. **Jan-Erik Ingenhoff:** Conceptualization. **Barbara Wieland:** Writing – review & editing, Funding acquisition, Conceptualization. **Gert Zimmer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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