Emerging Microbes & Infections
2024, VOL. 13, 2420731 (4 pages)
https://doi.org/10.1080/22221751.2024.2420731

RESEARCH LETTER

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Survivability of H5N1 avian influenza virus in homemade yogurt, cheese and whey

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ABSTRACT
Influenza virus is typically associated with respiratory infections, but H5N1 in US dairy cows raises public health concerns about milk by-products. We show that simple home recipes can inactivate H5N1 in cheese, yogurt, and whey. While viral RNA was present, no viable virus was found, ensuring food safety.

ARTICLE HISTORY Received 24 July 2024; Revised 16 October 2024; Accepted 20 October 2024

KEYWORDS Influenza A virus; H5N1; clade 2.3.4.4b; cattle; yogurt; cheese

Avian influenza virus (AIV), primarily known for causing respiratory infections, has typically not been associated with foodborne transmission. Similarly, cattle were once thought to be resilient to AIV infections [1]. However, recent outbreaks of H5N1 AIV in dairy cows in the United States and the detection of the virus in fresh milk [2] have raised new public health concerns about milk and dairy by-products as potential sources for human infections. To date, four dairy farm workers in Texas, Michigan, and Colorado have been confirmed to be infected with the virus [3]. Additionally, the consumption of unpasteurized H5N1-contaminated milk has caused the deaths of numerous cats near dairy farms [4], and laboratory mice [5]. Recent studies have shown the rapid inactivation of naturally contaminated or spiked milk at pasteurization temperatures (e.g. 63 and 72°C) [5–7]. However, little is known about the virus's ability to remain infectious in milk by-products, particularly in non-controlled, home-like conditions. Here we investigated the inactivation of H5N1 in whey, cheese and yogurt made from H5N1 spiked whole milk using simple home recipes and laboratory equipment (Figure 1(A)).

Preparation and analysis of H5N1-infected dairy products. Two H5N1 viruses were utilized in this study: A/swan/Germany/R65/2006 (clade 2.2.2, designated H5N1-06) and A/chicken/Germany/AI04286/2022 (clade 2.3.4.4b, designated H5N1-22). All experiments were conducted in biosafety level 3 laboratory facilities at the FLI. Commercial organic whole milk (3.8%) and yogurt as a starter were purchased from a supermarket. The milk was spiked with each virus to a final concentration of 10^4 - $10^{4.5}$ plaque-forming units per millilitre (pfu/ml), similar to the viral load detected in milk obtained from naturally H5N1-infected cows in various states [8]. Cheese was prepared by heating 1 litre of milk to 80°C–85°C using a heat-block (Fig. 1). The milk was stirred continuously for 30 minutes before intermittently adding 50 ml of concentrated bio-lemon juice until curds formed. The curds were left to settle for 20 minutes at room temperature (20°–22°C) and then strained through bamboo cloth and a sieve, with whey collected in a separate beaker. Yogurt was prepared by adding 3 spoonsful of commercial yogurt (about 45 gm) as a starter to 0.5 litres of H5N1-spiked milk and incubating it at 42°C for 8 hours. As controls, we incubated spiked concentrated yogurt starter culture, and spiked milk without starter culture at 42°C for 8 h.

Samples obtained from freshly spiked milk, cheese, yogurt and whey (as by-product from prepared cheese and yogurt) were evaluated for infectivity through infection of embryonated chicken eggs and plaque assays. Milk and whey samples were directly inoculated into eggs and onto MDCKII cells. Yogurt and

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/22221751.2024.2420731.

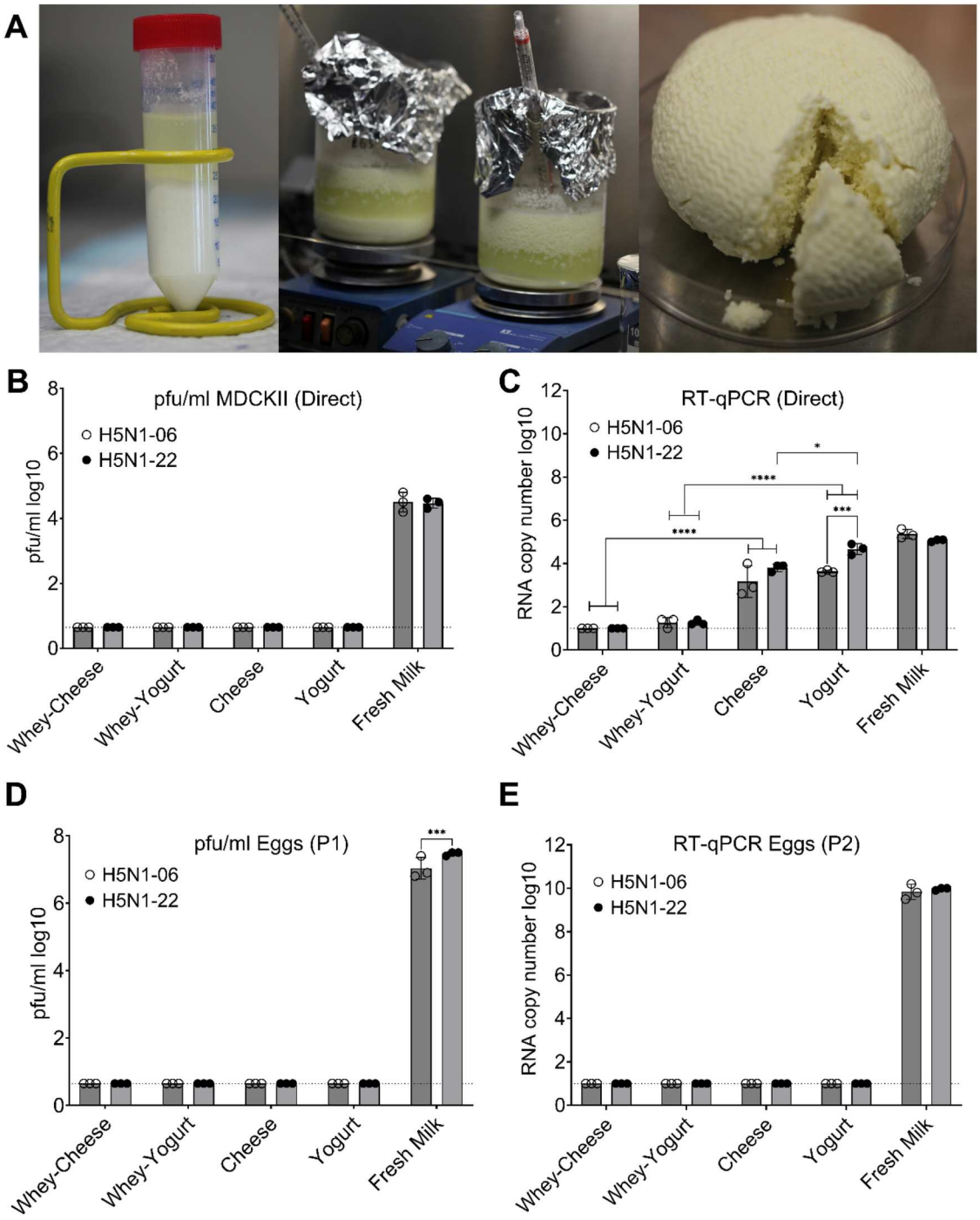

Figure 1. Viral RNA levels and viable virus titres in H5N1-spiked fresh milk and prepared whey, yogurt, and cheese. (A) Milk by-products produced under laboratory conditions in this study. (B) Viable virus titers in freshly prepared samples (1 hour after preparation) using plaque assay in MDCKII cells, expressed as plaque-forming units per ml (pfu/ml). (C) RNA levels detected by quantitative RT-qPCR, expressed as copy numbers in freshly prepared spiked milk and by-products. (D) Viable virus titers in eggs inoculated with freshly prepared spiked milk and by-products (P1 = passage 1) were determined using plaque assay in MDCKII cells (pfu/ml). (E) RNA levels expressed as copy numbers in allantoic fluids of eggs after the second passage (P2 = passage 2). Negative samples were assigned the values of the detection limits of the RT-qPCR and plaque assay. The experiments in panels B to E were performed using 3 samples from each substrate. Statistical significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

cheese samples were first dissolved in PBS (pH 7.2) to prepare 10% suspensions for detection of virus [9]. Approximately 400 µl of dissolved samples were inoculated into three eggs each. Eggs were monitored twice daily for 4 days. RNA was extracted from all samples using Trizol (Th. Geyer GmbH, Germany) and the RNeasy kit according to manufacturers' guidelines (QIAGEN, Germany). Real-time reverse transcription quantitative PCR (RT-qPCR) targeting the M1 gene was performed. RNA copy numbers were calculated using standard curves generated with pHW-M1-plasmids [10]. The pH was measured by pH-

indicator strips (Merck, Germany). Statistical analysis was conducted using one-way ANOVA by GraphPad Prism, version 9.2.0 (La Jolla, California, USA; www.graphpad.com). Statistical significance was considered at $p \leq 0.05$.

Absence of Viable H5N1 Virus in Dairy Products Despite RNA Presence. No viable virus was detected in cheese and yogurt, although viral RNA was detectable in these samples but not in whey. Eggs inoculated with samples from yogurt, cheese, or whey showed no signs of mortality. Allantoic fluids showed no hemagglutination (HA) activity, and viral titres in cell culture were below the detection limit of the plaque assay (Figure 1; Supplementary Figure S1). Similarly, no viable virus was found in the spiked concentrated starter yogurt culture. Conversely, all eggs inoculated with freshly-spiked milk died within 36 hours post-inoculation, with determined viral titres of 6.8 to 7.5 log10 pfu/ml (Figure 1) and HA titres ranging from 16 to 256 in the allantoic fluid. Similarly, approximately 4.0 log10 pfu/ml of virus was detected in spiked milk incubated at 42°C without yogurt starter culture. The pH values were 6.5 for fresh milk and 4.4 for the yogurt starter culture. When the starter culture was added, the pH of the milk before incubation at 42°C dropped to 5.8. Likewise, the pH of lemon juice was 4.0, and the pH of spiked milk dropped to 5.0 after adding the lemon juice.

Comparable levels of viral RNA were detected in yogurt, and cheese samples. Whey samples from yogurt were negative for viral RNA, whereas whey samples from cheese exhibited significantly lower titres compared to the cheese itself ($p < 0.05$). After passaging of samples in eggs twice, neither viral RNA nor infectious viral titres were determined in the allantoic fluids of eggs inoculated with any by-product.

Discussion

Our findings clearly demonstrate that H5N1 lost infectivity in cheese, yogurt and whey, although viral RNA was detectable. Similar results have recently been observed in retail dairy products in the US [8]. Both H5N1 viruses retained infectivity in spiked milk incubated at 42°C for 8 hours confirming our previous results on the heat stability of these viruses [11]. It appears that the acidic pH (i.e. that of the starter yogurt culture, and lemon) was the main factor responsible for eliminating the virus infectivity in cheese and yogurt. Interestingly, unlike whey, it seems that the virus binds to milk proteins (e.g. casein), which constitute 80% of milk and contain sialic acid, a major receptor for influenza viruses [12, 13]. Minimal to no viral RNA was detected in whey. It is known that whey contains components (e.g. lactoferrin, lactalbumin, lysozymes) with antimicrobial and antiviral properties [14, 15] and other components (e.g. lactogenin) with high ribonucleolytic (RNase) activity [16], which possibly degraded viral RNA.

Our findings provide valuable insights into survivability of AIV in cheese, yogurt and whey prepared from spiked bovine milk using simple home recipes. These methods are commonly practiced in various countries, particularly by families and students to make their own cheese and yogurt. However, these results may not directly reflect conditions in large-scale industrial production or in naturally contaminated milk from infected cows. Nevertheless, this study demonstrates that uncontrolled homemade cheese, yogurt, and whey do not retain the infectivity of H5N1 virus. Therefore, the risk of virus transmission to animals and humans through these dairy products can be considered negligible or low. Further studies are underway to explore the specific inhibitory mechanisms of milk components on viral infectivity.

Acknowledgements

Conceptualisation: EMA; data curation: EMA, JL; formal analysis: EMA, JL, MK; funding acquisition: EMA; investigation: DH, JL, MK, EMA; methodology: DH, JL, MK, EMA; project administration: EMA, resources: EMA; software: DH, JL, MK, EMA; supervision: EMA, validation: EMA, JL, DH; visualization: DH, JL, MK, EMA; writing – original draft: EMA, JL; and writing – review & editing: DH, JL, MK, EMA.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The work in this study was partially funded by ICRAD, an ERA-NET co-funded under the European Union's Horizon 2020 research and innovation programme (https://ec.europa.eu/programmes/horizon2020/en), under Grant Agreement n° 862605 (Flu-Switch) to E.M. Abdelwhab.

Data availability

All data are available within the manuscript and supplementary materials.

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