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In laboratory inactivation of H5N1 in raw whole milk through milk acidification: results from a pilot study

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ABSTRACT

Avian Influenza virus H5N1 2.3.4.4.b has recently been detected in cattle, with milk from infected animals reported to contain a high viral load, serving as a potential source for shedding and dissemination of this virus. Currently, pasteurization is the only widely recognized method for on-farm inactivation of H5N1 in milk. A current concern is that according to USDA data, less than 50% of large dairy farms pasteurize non-saleable milk, with a much lower percentage occurring in medium and small dairy farms. The objective of this pilot study was to evaluate the effect of milk acidification to a pH of ~4.0 to 4.4 and lactoperoxidase system (LPS) on the inactivation of low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) in raw whole milk. Initial trials in our study used the LPAI H6N2 virus as a surrogate for HPAI H5N1. For the milk acidification trials, citric acid was used to acidify milk. For evaluation of milk acidification and LPS, milk samples were inoculated with LPAI H6N2, with samples collected before and after treatment at various times. Evaluation of virus viability was conducted using specific-pathogen-free (SPF) embryonated chicken eggs and viral quantification using real-time quantitative PCR (RT-qPCR). Three acidification experiments were conducted using milk spiked with LPAI H6N2. Given the positive outcome observed in the inactivation of LPAI with citric acid, a fourth trial was conducted with milk containing a high load of H5N1 originating from actively infected cows. Our findings observed that MILK ACIDIFICATION with a pH between 4.1 and 4.2 resulted in the inactivation of LPAI H6N2 and HPAI H5N1 virus in milk after 6 h of treatment. Milk treatment with LPS was not effective for the inactivation of the H6N2 virus, and no further trials were conducted for this treatment option. This is the first study reporting the effectiveness of MILK ACIDIFICATION for the inactivation of HPAI H5N1 in milk originating from animals infected with H5N1. Milk acidification is an effective, accessible, and easy-to-use alternative to milk pasteurization, and future studies should evaluate the on-farm effectiveness of acidification of non-saleable milk to inactivate H5N1.
Keywords: H5N1, milk acidification, non-saleable milk, viral inactivation

INTRODUCTION

On March 25th, the US Department of Agriculture (USDA) reported the first confirmed detection of Highly Pathogenic Avian Influenza (HPAI) A (H5N1 clade 2.3.4.4b genotype B3.13) virus in dairy cattle and unpasteurized milk samples from cattle in Texas and Kansas in 4 dairy herds (USDA, 2024). Since its first identification, the National Veterinary Services Laboratories (NVSL) has reported the detection of this virus in dairy cattle in multiple states (USDA). Cattle infected with H5N1 may be asymptomatic or present clinical signs that can include apparent systemic illness, reduced milk production, and abundant virus shedding in milk (Burrough et al., 2024, Rodriguez et al., 2024). H5N1 2.3.4.4.b has been shown to possess features that facilitate infection and transmission in mammals, including a receptor-binding specificity for a dual human/avian-type receptor-binding, and a tropism for mammary glands in dairy cattle (Eisfeld et al., 2024). The virus's tropism for the mammary gland leads to high viral load and shedding in milk, representing a concern for the potential for transmission of the virus to animals and humans (Butt et al., 2024).

A study conducted by researchers from the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) has demonstrated that standard US continuous flow high-temperature-short-time (HTST; 72°C for 15s) pasteurization parameters will effectively inactivate H5N1 in milk (Spackman et al., 2024). Furthermore, a study evaluating both low-temperature-long-time (LTLT) (batch pasteurization; 63°C for 30 min) and HTST demonstrated effective in-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

activation of H5N1 clade 2.3.4.4b virus spiked into raw whole milk (>6 log) (Alkie et al., 2025). Together, these studies support pasteurization as an effective method for the treatment of milk containing H5N1, making it a safe product for animal and human consumption.

Although pasteurization is an effective method for deactivation of H5N1 when considering this practice used for the treatment of non-saleable milk i.e., waste milk; (**WM**), the use of this method on dairy farms is variable. As per the USDA National Animal Health Monitoring System (NAHMS) data from dairy farms, only 43.8% of large operations (500 cows or more), 3% of medium operations (100–499 cows), and 1% of small operations (fewer than 100 cows) pasteurize WM before feeding it to calves (USDA, 2016). The potential for WM with a high viral load of H5N1 that is not inactivated before disposal or re-purpose for animal consumption, represents a health risk for animals consuming this milk and humans handling this milk, and a potential source for spread of the virus within and between farms. One study evaluating the pathogenicity of feeding mice with H5N1 virus-containing milk originating from an H5N1-infected cow demonstrated a rapid induction of signs of disease by d 1 postinfection and virus dissemination to respiratory and non-respiratory organs by d 4 postinfection (Eisfeld et al., 2024). In another study, 12 Holstein calves (5 to 6 mo of age) inoculated with US H5N1 bovine isolate genotype B3.13 (H5N1 B3.13) through their nostrils using an atomization device resulted in moderate nasal replication and shedding with no severe clinical signs or transmission to sentinel calves (Halwe et al., 2024). A current concern for which limited data is available is the feeding of raw whole milk containing H5N1 to calves. In their discussion, Halwe et al. (2024) indicated that data from their study should not necessarily represent exposure to H5N1 expected from calves ingesting milk containing H5N1, given that the aerosolized dose used for nostril inoculation of calves in their study would not generate comparable results, representing a much lower exposure dose when compared with milk ingestion. Similar findings were observed in another study that inoculated heifer's aerosol respiratory route with the same H5N1 strain used by Halwe et al. (2024), with mild clinical signs, and infection was confirmed by virus detection, lesions, and seroconversion (Baker et al., 2024). To our knowledge, no study to date has evaluated the pathogenicity and transmission of preweaned calves fed H5N1 virus-containing milk originating from an H5N1-infected cow.

Due to the biological risk of H5N1 on the farm, and the potential challenges and barriers to adoption of pasteurization of waste milk by dairy farms, our project focused on evaluating alternative methods for deactivating H5N1 in raw milk. Two methods were selected for evaluation, namely milk acidification and the lactoperoxidase system (LPS), based on data supporting biological plausibility for effectiveness to inactivate H5N1, low cost of implementation, accessibility, and ease of use. The LPS activates the enzyme lactoperoxidase which is naturally present in milk that has an antimicrobial action and is generally recognized as safe (GRAS) by the FDA for use in milk (FDA, 2016). The LPS is effective as an antiviral against influenza A in cell-free experimental systems, however, no studies have been conducted evaluating the LPS antiviral effect on H5N1 in milk (Patel et al., 2018).

Acidification of milk to a pH between 4.0 to 4.5 and feeding to pre-weaned calves is a practice that has become common in recent years in some regions of the US, intending to lower the milk pH to a point where it is unsuitable for bacterial growth and survival, without undesirable health side effects on calves (Parker et al., 2016, Todd et al., 2018, Garzon et al., 2020). Acidifying milk to a pH between 4.1 and 4.4 has resulted in a reduction in coliform and aerobic bacterial growth in raw bulk tank milk (Chen et al., 2020), colostrum (Collings et al., 2011), and milk replacers (Todd et al., 2016), as well as results in a lower incidence of diarrhea in calves compared with calves fed pasteurized milk (Chen et al., 2020). Poultry research has investigated the capabilities of litter acidification in the inactivation of avian influenza. Results showed the inactivation of low pathogenic H6N2 with a commonly known litter amendment based on sodium bisulfite (Figueroa et al., 2021).

The overarching objective of this pilot study was to evaluate the effect of the LPS and milk acidification on the inactivation of influenza A virus H5N1. Secondary objectives include the evaluation effect of various low pH values on the deactivation of LPAI. Our assumption was that the use of a LPS and milk acidification would result in the inactivation of the influenza A virus in raw whole cow milk after treatment.

MATERIALS AND METHODS

Study Design

A step-by-step approach was used for our pilot study, with trials that initially utilized LPAI H6N2 as a surrogate for HPAI H5N1. Based on the results from these trials, methods were created to evaluate the inactivation of H5N1 virus-containing milk originating from an H5N1-infected cow. The reason for using this approach was practicality and safety. Low pathogenic H6N2 can be used in a BSL2 laboratory and poses a reduced risk to laboratory workers. The first trial was conducted on May 14, 2024, when the virus had been confirmed in only 9 states and a total of 27 dairy farms (USDA).

Viruses

LPAIV endemic isolate A/Chicken/California/2000 (H6N2) was used in all LPAI experiments. The virus was replicated and titrated in 9- to 11-d-old specific-pathogen-free (SPF) embryonated eggs as previously described (L. Dufour-Zavala, 2008). Procedures involving the HPAIVs were performed under the Select Agent Program at the California Animal Health and Food Safety Laboratory. Activities involving LPAIVs were performed at the California Animal Health and Food Safety Laboratory, University of California, Davis.

Influenza A testing

Egg inoculation and reverse transcriptase quantitative PCR (RT-qPCR) testing was conducted at the California Animal Health & Food Safety Lab System (CAHFS, Davis, CA). Virus isolation in embryonated eggs is considered the gold standard for avian influenza virus isolation in samples collected in suspicious cases and was used in our study for both LPAI and HPAI trials (Monne et al., 2008, Zhang and Gauger, 2020). For each sample, collected for egg inoculation viability testing, milk samples were mixed 1:1 with a standard antibiotic cocktail containing, Penicillin (10,000 IU/ml), Streptomycin (6,000 ug/ml), Gentamicin sulfate (1,000 ug/ml), Kanamycin sulfate (650 ug/ml), and Amphotericin B (10 ug/ml). The treated samples were held at room temperature for one hour and then processed for virus isolation using standard egg inoculation techniques (Williams, 2016). Briefly, 2 hundred µL of the antibiotic-treated samples were inoculated into the allantoic cavity of Specific Pathogen Free chicken eggs (AVS Bio) and incubated for up to 5 d or until embryo death was observed. Eggs dying before 24 h of inoculation were considered non-specific mortality. Allantoic fluid harvested from eggs was stored at −70°C for further analysis. The viral particles/µL (RNA copies/µL) were calculated after RT-qPCR, by conducting a standard curve using 5 serial dilutions (10^2 – 10^6) in triplicate of an armored RNA construct (Armored RNA Quant, Asuragen, Austin, TX; Catalog Number #49220).

RT-qPCR testing for the Avian Influenza virus was performed using the protocol provided by the National Animal Health Laboratory Network (NAHLN). The NAHLN protocols targeting the matrix genes and the hemagglutinin gene for the subtypes H5 and H5 GsGd include input from peer-reviewed publications (Spackman et al., 2002, Wise et al., 2004, Spackman and Suarez, 2008, Xing et al., 2008, Van Borm et al., 2010, Spackman, 2020). After each of the RT-qPCRs was done in milk or allantoic fluid a Ct value was recorded. A decrease in the Ct value after egg inoculation and harvest means the virus replicating in the egg and confirms virus viability (Hauck et al., 2021)

Low Pathogenic Avian Influenza (LPAI) Trials

A total of 3 LPAI milk acidification trials (trials 1, 2, and 3) and one LPS trial were conducted. For the LPAI trials, fresh milk samples from cows were provided by a local dairy farm. Milk was kept refrigerated in a cooler with ice during transport from the farm to the laboratory, and the chilled milk was used for inoculation with LPAI and milk acidification, with the rest of the experiment being conducted with the milk exposed to environmental temperature. HPAI virus may be inactivated due to thermal conditions, as was shown in a study that over a period of days, HPAI may have long-term stability at 4°C, but even at temperatures as low as 20°C, a 3-log reduction in the virus titers can occur within 7 d (Nooruzzaman et al., 2024). For our study, a control sample was exposed to the same thermal conditions as that of the milk receiving treatment, accounting for potential inactivation caused by thermal conditions alone.

An aliquot for each raw milk sample was submitted for milk quality parameter testing, including milk, fat, protein, and solids-nonfat (**SNF**) percent (Table 1). For each trial, milk from 2 different cows was used. For all milk acidification trials, the pH of milk was measured using a pH meter (Traceable Calibrated pH/ORP Meter, Cole-Parmer Instrument Company, LLC, Vernon Hills, IL).

LPAI Milk Acidification

Three trials were conducted, with slightly different objectives: Trial 1, with the specific objective of evaluating deactivation of LPAI in milk at a pH of 4.2; Trial 2, with the specific objective of evaluating deactivation of LPAI in milk at a pH of 4.4; and Trial 3, with the specific objective of evaluating deactivation of LPAI in milk at a pH of 4.1. For all trials, LPAI milk acidification was performed by adding citric acid.

For trial 1 had the following groups: **1)** Non-treated control group inoculated with LPAI H6N2; and **2)** Treated group inoculated with LPAI H6N2. A total of 2 replicate trials were conducted, one using milk from cow "A" and the other using milk from cow "B" (Table 1). For inoculation, milk samples (9 mL) were spiked with 1 mL of allantoic fluid containing LPAI/H6N2 (Ct value = 15.5) and inverted repeatedly over 1 min. Before the milk acidification, a pre-treatment sample was collected for egg inoculation and qPCR testing for H6N2. For the milk acidification treatment group, a total of 42 mg of citric acid powder was used, adding approximately 14 mg at a time, and slowly inverting the tube containing the milk 3 to 4 times between additions, to reach a pH of 4.2. Following the milk solution was vortexed for 10 s at 1500 rpm. Upon reaching the desired pH, a time zero (0)

Table 1. Milk quality parameters for raw whole milk collected from individual dairy cows from a dairy farm in California. For each LPAI H6N2 milk acidification trials (1, 2 and 3) and lactoperoxidase system trial (LPS), milk from two cows was used, with half of the replicates using milk from each of these cows (e.g., for the six replicates conducted for trial 2, three used milk from cow "E" and another three used milk from cow "F")

Trials	Cow	Milk Fat (%)	Milk Protein (%)	Lactose (%)	SNF (%)[1]
1 & LPS	A	5.48	3.36	4.50	8.95
1 & LPS	B	5.85	2.90	4.72	8.68
2	C	5.53	3.35	4.92	9.45
2	D	4.30	3.68	4.84	9.70
3	E	4.13	3.43	4.67	9.28
3	F	4.77	3.67	4.89	9.81

[1]SNF, solids-nonfat.

sample was collected, with repeated collection of samples after 1 h (1) and 6 h (6). Samples were collected in parallel for the control group. After time point 0, the tubes were vortexed at 1500 rpm every 30 min to minimize pH variation over time. For each sampling time point, a 500 µL sample was collected for quantification of H6N2, and a 500 µL sample was collected for egg inoculation. Trial 1 replicates occurred on May 14th, 15th, and 16th, 2024.

For the LPAI milk acidification trial 2, the main goal was to evaluate the treatment effect when using a higher pH, which would reduce the amount of citric acid needed to reach the desired pH. A total of 6 replicate trials were used for trial 2, 3 using milk from cow "C" and 3 using milk from cow "D" (Table 1). The protocol followed the same step-by-step as trial 1. For the M milk acidification AC group, a total of 60 mg of citric acid powder was used, adding approximately 20 mg at a time, and slowly inverting the tube containing the milk 3 to 4 times between additions, to reach a pH of 4.4, following methods used for trial 1. For trial 2, the post-LPAI spiked sample was measured for pH again for time points 0, 1, 2, 4, and 6. Trial 2 3 replicates occurred on June 17th, 18th, and 20th, 2024.

For the LPAI milk acidification trial 3, the main goal was to re-evaluate a lower pH for the treatment of milk, given poor outcomes observed for a higher pH of 4.4. A total of 6 replicate trials were used for trial 3, 3 using milk from cow "E" and 3 using milk from cow "F" (Table 1). The protocol followed the same step-by-step as trial 1. For the milk acidification treatment group, a total of 87 mg of citric acid powder was used, adding approximately 29 mg at a time, and slowly inverting the tube containing the milk 3 to 4 times between additions, to reach a pH of 4.1. Furthermore, more sampling points were included in trial 3 to obtain a higher data granularity and including samples at time zero ("0 hr"), 1 h (1), 2 h (2), 4 h (4), and 6 h (6) (**Figure 1**). For trial 3, the post-LPAI spiked sample was measured for pH again for time points 0, 1, 2, 4, and 6. Trial 3 3 replicates occurred on August 19th, 20th, and 21st, 2024.

HPAI H5N1 Milk Acidification

For the HPAI milk acidification trial, an H5N1 virus-containing milk originating from an H5N1-infected cow was used. Milk was kept refrigerated at 4°C before being used for the study, and the chilled milk was used for inoculation milk acidification, with the rest of the experiment being conducted with the milk exposed to environmental temperature. Two treatment groups were evaluated: **1)** the control group not treated for acidification; and **2)** the acidification treated group. Before the acidification of the milk, a pre-treatment sample was collected for egg inoculation and RT-qPCR testing for H5N1. A 10 mL milk aliquot for the control and milk acidification group was created. For the milk acidification treatment group, a sterile water solution containing citric acid at 0.5 g/ml was used to slowly reach a pH of 4.1. A solution with citric acid was used to facilitate the treatment of milk, given the small volume. After the pH of 4.1 was reached, the milk solution was vortexed for 10 s at 1500 rpm. If a pH of roughly 4.1 was not reached more citric acid solution was added until the desired pH value was obtained. Upon reaching the desired pH, samples were collected following at time 2 h (2), 4 h (4), 6 h (6), 8 h (8), and 10 h (10). Samples were collected in parallel for the non-acidified control group. After "0hr" sample collection, the tubes were vortexed at 1500 rpm every 30 min to minimize pH variation over time. For each sampling time point, a 500 µL sample was collected for RT-qPCR quantification of H5N1, and a 500 µL sample was collected for egg inoculation. For the H5N1 milk acidification trial, the post-LPAI spiked sample was measured again for pH for time points 0, 1, 2, 4, 6, 8, and 10. For the H5N1 milk acidification trial, 3 replicates occurred on October 10th, 11th, and 14th, 2024.

LPAI Milk LPS Treatment

The milk used for the LPS milk trials was kept refrigerated in a cooler with ice during transport from the farm to the laboratory, and the chilled milk was used for in-

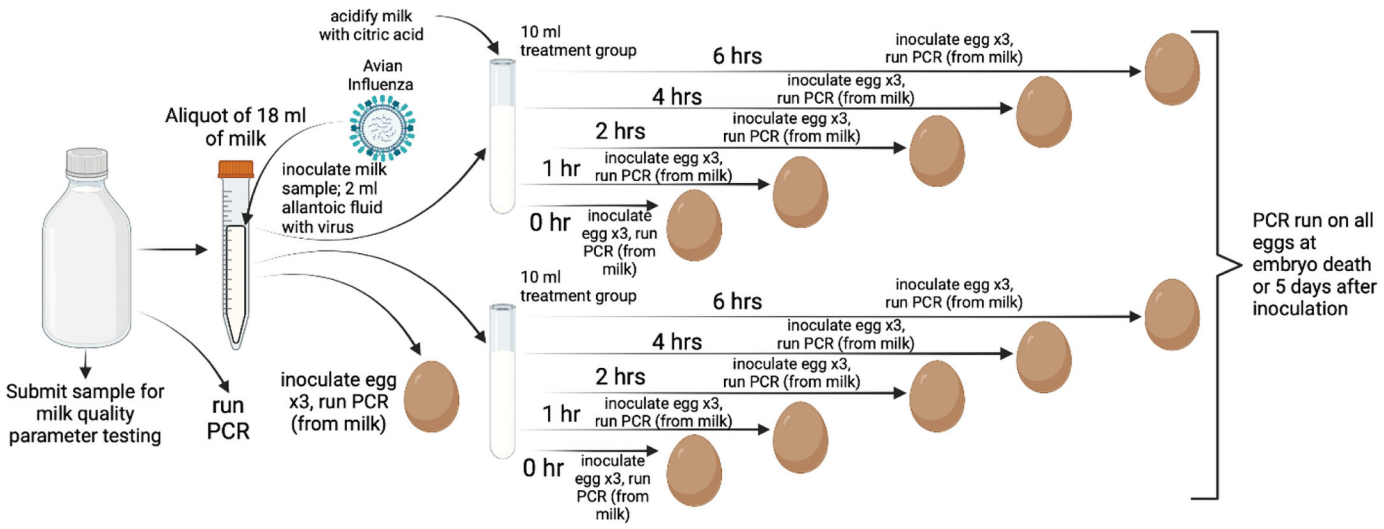

Figure 1. Descriptive outline of LPAI milk acidification for Trial 3, including time points when samples were collected, and specific testing conducted for each sample, including RT-qPCR (PCR), and Specific Pathogen Free (SPF) chicken eggs inoculation. A control sample was collected before the milk sample was aliquoted into the treatment and control group, with that sample being inoculated into 3 SPF eggs. Following, for each of the 5 time points (0, 1, 2, 4, and 6hrs after treatment), milk samples were collected and inoculated into 3 SPF eggs, resulting in a total of 15 SPF eggs being inoculated per treatment group, and a total of 30 SPF eggs being inoculated for both treatment and control group.

oculation with LPAI and LPS treatment, with the rest of the experiment being conducted with the milk exposed to environmental temperature. For the LPS milk treatment, there were 2 groups: **1)** the control group inoculated with LPAI H6N2 but not treated with LPS, and **2)** LPS treated group inoculated with LPAI H6N2. A total of 2 replicates were used for trial 1, one using milk from cow "A" and one using milk from cow "B" (Table 1). For inoculation, milk samples were spiked with 1mL of allantoic fluid containing LPAI/H6N2 (Ct value = 15.5), and the tube containing the milk was inverted repeatedly for 1 min to ensure dilution of the virus throughout the sample. Before LPS treatment of the milk, a pre-treatment sample was collected for egg inoculation and RT-qPCR testing for H6N2. Replicate H6N2 spiked milk samples were separated into 2 94 mL aliquots for the control and LPS treatment group. For the LPS, activation occurs by the addition of thiocyanate as sodium thiocyanate and hydrogen peroxide in the form of sodium percarbonate (FAO/WHO, 1991, Awol et al., 2023). Following that approach, for the LPS treatment group, a milk sample of 94 mL was treated with 0.0019g of sodium thiocyanate powder and stirred for 1 min. Following, 0.0030g of sodium percarbonate powder was added, and milk was stirred for 3 min. Following, the solution was not handled for 5 min to allow the enzymatic reaction to take place, after which time zero (0) samples were collected, with repeated collection samples after 30 min (30) and 1 h (1) (**Figure 2**). LPS protocol and timeline for sample collection were based on prior studies on the activation of LPS in milk (FAO/WHO, 1991, FDA, 2016). Samples were collected in parallel for the non-LPS H6N2 inoculated control group. Lactoperoxidase activity was not measured and represents a limitation of this pilot study. For each sampling time point, a 500 µL sample was collected for qPCR quantification of H6N2, and a 500 µL sample was collected for egg inoculation. The LPS trial had 3 replicates that occurred on May 14th, 15th, and 16th, 2024.

Statistical Analysis

Following common practice for RNA data presentation and analysis, the data was log-transformed using based 10 (Log_{10}) in JMP. Descriptive data analysis was conducted in JMP Pro 17.2 (SAS Institute, Cary, NC). The assumption of normality for the log_{10} viral RNA copies/µL data was tested using the Shapiro-Wilk W test, and the assumption of homogeneity of variance was tested using Levene's test using JMP Pro 17.2. Because the log_{10} viral RNA copies/µL data for all trials rejected assumptions of normality using the Shapiro-Wilk W test, a non-parametric test was used to analyze the data. For the log_{10} viral RNA copies/µL data, the Kruskal-Wallis non-parametric test was used for pair-wise comparison between treatment groups within each time point, and Dunn All Pairs for joint ranks was used for pair-wise comparison between 2 times points within a treatment group. For all analyses, a P-value of <0.05 was the threshold of significance.

RESULTS

Milk quality parameters for the LPAI milk acidification and LPS trials and each milk replicas are presented in Table 1. Means and standard error (μ ± 95% SD) for each milk quality parameter were: 5.01 ± 0.71 for milk fat percent, 3.40 ± 0.28 for milk protein percent, 4.75 ± 0.16 for lactose percent, and 9.31 ± 0.43 for solids-nonfat percent.

LPAI Milk LPS Treatment

The least squares means (LSM) for $\log_{10}$ viral RNA copies/μL for LPAI H6N2 in the allantoic fluid at each time point for the LPAI milk acidification trials are presented in Figure 2. Data for the detection of live avian influenza A virus in milk for the milk acidification study is presented in Table 2. For all positive control groups in the trials, live virus was detected at all time points tested.

For LPAI milk acidification trial 1, a significantly lower $\log_{10}$ viral RNA copies/μL for RT-qPCR was observed in the milk acidified group at time point 6 compared with the control group (**Figure 3**). Results for the detection of live LPAI H6N2 consistently found no live virus detected for both replicates at 6 h for the milk acidification group (**Table 2**). The mean value and standard deviation for the milk pH for the milk acidification group for LPAI milk acidification trial 1 at time point 0 was 4.1 ± 0.05 (**Figure 4**).

For LPAI milk acidification trial 2, a significant difference was not observed for any time point between milk acidification and the control group for $\log_{10}$ viral RNA copies/μL for RT-qPCR (**Figure 3**). Although a significant difference between milk acidification and the control group was not observed at 6 h, or between 2 times points within a treatment group for RNA copies/μL, a noticeable numeral difference was observed, with a decrease observed for milk acidification over time, with no relevant variation observed for the control group. For the results for detection of live LPAI H6N2, half of the replicates had no live virus detected at time point 6 h for the milk acidification group, while the other half had a live virus detected by that time point. The split in these findings coincided with the milk sample used, with all samples with a no live virus detection being from the trial that used milk from cow C, and all samples with a no live virus detection being from the trial that used milk from cow D (**Table 1**). A noticeable difference between these cows was a numerically higher milk fat percent for cow C, at 5.53%, when compared with cow D, at 4.30%. The mean and standard deviation values for the milk pH for the milk acidification group for all replicates and time points was 4.32 ± 0.06, and the mean values of pH for each time point for LPAI milk acidification trial 2 are outlined in Figure 4.

For LPAI milk acidification trial 3, a significant difference was observed at time point 0 between milk acidification and the control group, with significantly higher $\log_{10}$ viral RNA copies/μL for milk acidification when compared with the control group. Furthermore, in trial 3, significantly lower $\log_{10}$ viral RNA copies/μL was observed for milk acidification when compared with the control group at time point 6 (**Figure 3**). For trial 3,

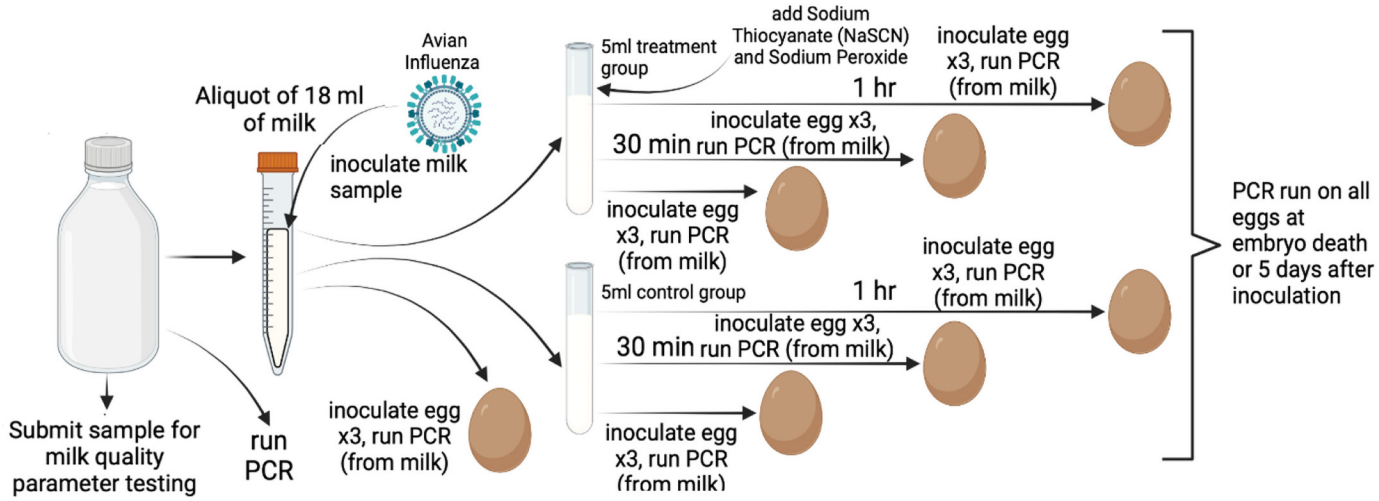

Figure 2. Descriptive outline of LPAI lactoperoxidase system (LPS) trial, including time points when samples were collected, and specific testing conducted for each sample, including RT-qPCR (PCR), and Specific Pathogen Free chicken eggs inoculation. A control sample was collected before the milk sample was aliquoted into the treatment and control group, with that sample being inoculated into 3 SPF eggs. Following, for each of the 3 time points (0, 30 min and 1 h after treatment), milk samples were collected and inoculated into 3 SPF eggs, resulting in a total of 9 SPF eggs being inoculated per treatment group, and a total of 18 SPF eggs being inoculated for both treatment and control group.

Table 2. Detection of live avian influenza A virus in milk in the milk acidification study by viral isolation in embryonated SPF eggs. For trials where samples for specific time points for hours after milk acidification were not available (n/a) were indicated in the table. For all control groups in the trials, a live virus was detected for all time points tested

Hours after	LPAI H6N2[2]			HPAI H5N1[2]
Milk acidification treatment[1]	Trial 1[3]	Trial 2[4]	Trial 3[5]	Trial
0	+[6]/+	+/+/+/+/+/+	+/+/+/+/+/+	NA
1	+/+	+/+/+/+/+/+	+/+/+/+/+/+	NA
2	NA	+/+/+/+/+/+	+/+/–/+/–/+	+/+/+
4	NA	+/+/+/+/+/+	+/–/-/+/–/-	-/+/+
6	-[7]/-	–/–/-/+/+/+	–/–/–/–/–/–	–/–/-
8	NA	NA	NA	–/–/-
10	NA	NA	NA	–/–/-

[1]Time in hours after milk acidification treatment reached the desired pH for each trial.

[2]Results for detection of live virus for each trial at various time points for highly pathogenic avian influenza virus (HPAI) and low pathogenic avian influenza virus (LPAI). Each result for each trial and time point combination represents test results from replicates. Not available (n/a) indicates that no samples were collected for that time point for the referred trial.

[3]In order, the first virus viability represents replicates using cow A, and the second using cow B (Table 1).

[4]In order, the first three virus viability results represent replicates using cow C, and the following three virus viability results represent replicates using cow D (Table 1).

[5]In order, the first three virus viability results represent replicates using cow E, and the following three virus viability results represent replicates using cow F (Table 1).

[6]Live virus detected.

[7]Lives virus not detected.

Abbreviations: HPAI, highly pathogenic avian influenza virus; LPAI, low pathogenic avian. influenza virus; SPF, specific-pathogen-free.

within the milk acidification group, a significantly lower $\log_{10}$ viral RNA copies/μL for RT-qPCR was observed for time point 6 when compared with time point 0. Similar to LPAI milk acidification trial 1, results for detection of live LPAI H6N2 consistently found no live virus detected for all replicates at time point 6 h for the milk acidification group (**Table 2**). At time point 4, 4 replicates had no live virus detected, and 2 replicates had live virus detected, and these were equally distributed for the 3 replicates conducted using milk from cows E and F, with 2 replicates with no live virus, and one with live virus detected at time point 4. The mean value and standard deviation for the milk pH for the milk acidification group for all replicates and time point for LPAI milk acidification trial 3 was 4.08 ± 0.05, and mean values of pH for each time point are outlined in Figure 4.

HPAI H5N1 Milk Acidification

For the HPAI H5N1 trial, milk originating from an H5N1-infected cow, significantly lower $\log_{10}$ viral RNA copies/μL for RT-qPCR were observed for the milk acidification group when compared with the control group. Similar to LPAI milk acidification trial 1 and 3 using LPAI H6N2, results for detection of live LPAI H6N2 consistently found no live virus detected for all replicates at time point 6 h for the milk acidification group (**Table 2**). At time point 4, one replicate had no live virus detected, and 2 replicates had live virus detected. The mean value and standard deviation for the milk pH for the milk acidification group for all replicates and time point for trial 3 was 4.16 ± 0.03, and mean values of pH for each time point are outlined in Figure 4.

LPAI Milk LPS Treatment

For the trial evaluating the lactoperoxidase system (**LPS**), no significant difference was observed between LPS and the control group, or within time points for a treatment group, for $\log_{10}$ viral RNA copies/μL for RT-qPCR, indicating no significant treatment effect of LPS for reduction of LPAI H6N2 virus in milk under the treatment protocols evaluated (**Figure 3**). Milk cows from cows A and B were used for trial 3 (**Table 1**).

DISCUSSION

Our findings suggest milk acidification as an effective approach for completely inactivating HPAI H5N1 in milk originating from cows infected with H5N1. After 6 h of treatment with milk acidification, H5N1 was not observed in either the LPAI H6N2 trials or in the HPAI H5N1 trial (**Table 2**). To our knowledge, this is the first report for the complete inactivation of HPAI H5N1 in whole milk using milk acidification. For the H5N1 milk

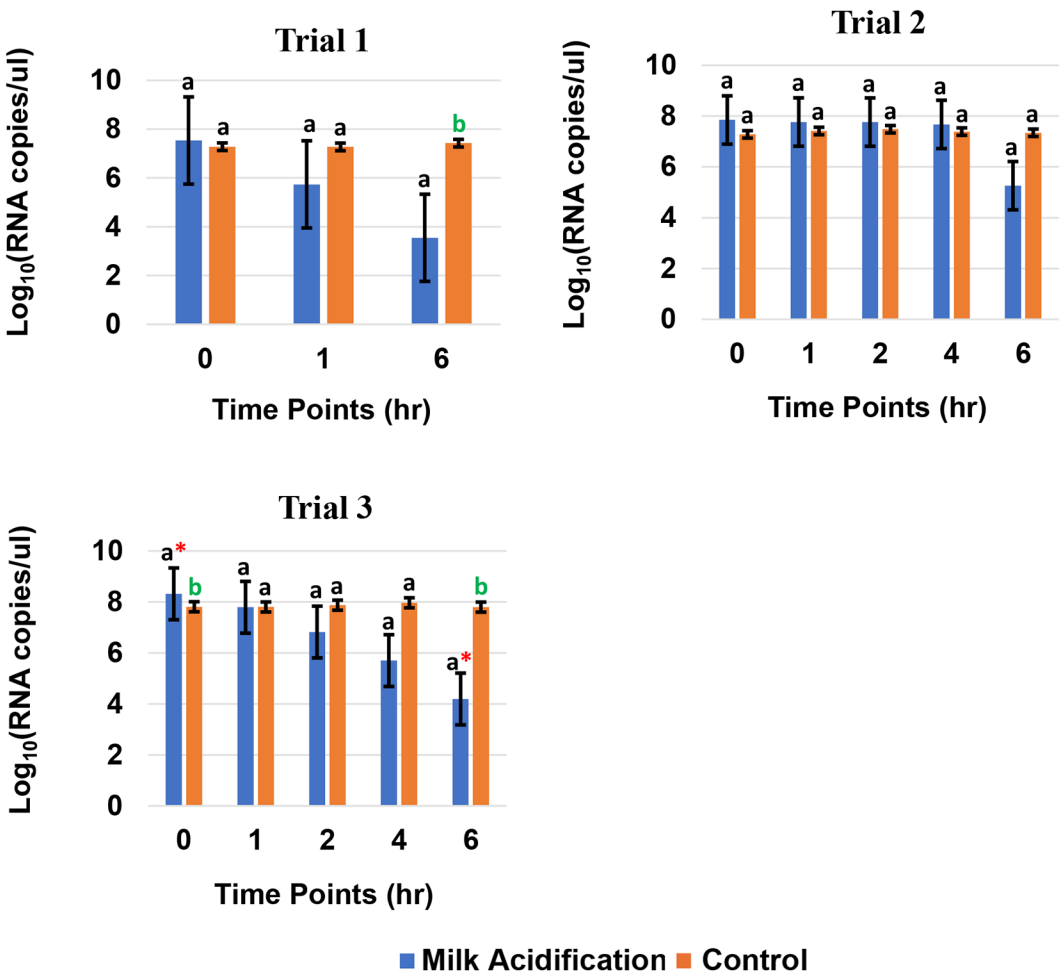

Figure 3. Least squares means of RNA copies/µL in allantoic fluid of eggs inoculated with milk with LPAI H6N2 after milk received treatment with acidification. Respective control groups for each trial are reported. Data is presented for each of the 3 trials conducted, with target milk acidification pH of 4.2, 4.4, and 4.1 for Trials 1, 2, and 3, respectively. Error bars represent the 95% confidence interval of the LSM. Different letters indicate a significant difference between treatment groups within each time point when using the Kruskal-Wallis non-parametric test. The red asterisk (*) indicates a significant difference between 2 times points within a treatment group when using Dunn All Pairs for joint ranks.

acidification trial, we observed that milk acidification resulting in a significant reduction in viral load.

Similar findings on the inactivation of influenza A virus have been observed for studies conducted on substrates other than milk. One study evaluating the effect of an acidifier amendment on the inactivation of LPAI in litter observed that acidifier amendment immediately inactivated LPAI virus when directly mixed with the virus and in the presence of broiler litter (Figueroa et al., 2021). Different from our study, the study by Figueroa et al. (2021) used sodium bisulfate for acidification, and a much lower pH of ~1, which may explain the quicker inactivation effect observed. Because the long-term goal of evaluating acidification of milk in our study was for both safe disposal of milk without active H5N1 virus and potential use for feeding preweaned calves on the farm, maintaining a pH within the range evaluated is important to reduce potential safety, nutritional, and palatability as evaluated by previous studies, and which could limit the use of acidified milk as a feed source for calves (Todd et al., 2017, Denholm, 2022). The use of citric acid instead of other available acids is considered, with thoughts focused on the palatability and products approved and classified as generally recognized as safe by the FDA (, Anderson, 2009).

In a brief report, the inactivation of H5N1 in lactose using acidification was evaluated. For this study, lactose was inoculated with HPAI H5N1 clade 2.3.4.4b, and various concentrations of citric acid between 0.1 and 0.6% were evaluated for inactivation of H5N1(Kwon et al., 2024). In their study, they observed that citric acid at 0.6% was effective at inactivating H5N1 inoculated in lactose at 2.15×10^5 50% Tissue Culture Infectious Dose/ml (TCID50/ml) after 20 min. Although this study

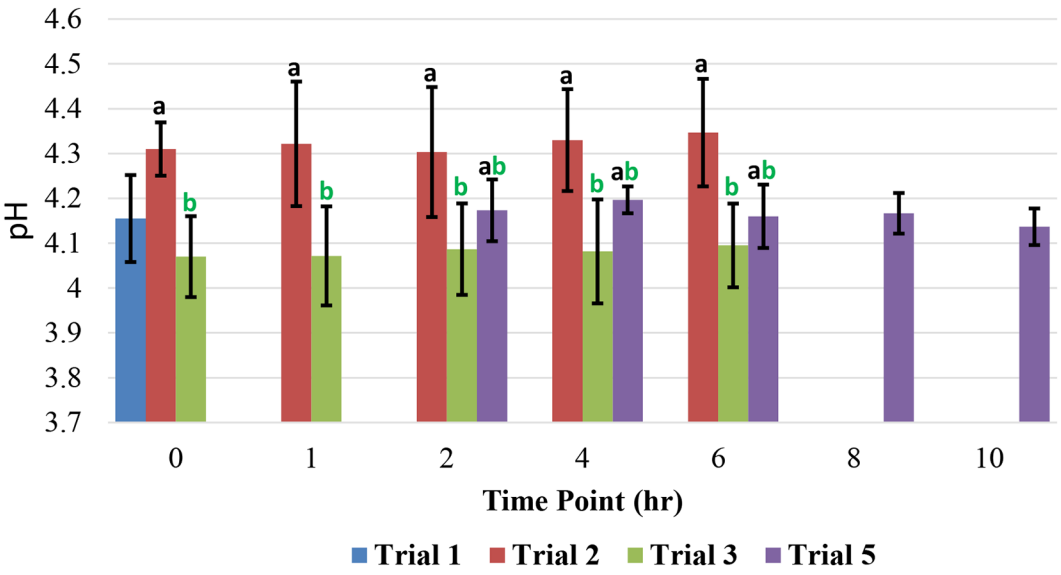

Figure 4. Mean values for pH measures for milk acidified treatment group for each trial by time point. Error bars represent the 95% confidence interval of the mean. For trial 1, pH was measured only for time point zero. Different letters indicate a significant difference in pH between trials 2, 3, and 5 when the same time points were available using Dunn All Pairs for joint ranks. Trials 1, 2, and 3 evaluated milk acidifications on inactivation of LPAI H6N2, and trial 5 evaluated milk acidification on inactivation of HPAI. Initial target pH for milk acidification groups for each of the trials 1, 2, 3, and 5 was of approximately 4.2, 4.4, 4.1, and 4.1, respectively.

observed similar findings as ours, a quicker inactivation of HPAI was observed at 20 min, versus 6 h in our study. These differences could be due to the major difference in substrate used, with our study using whole milk, and the use of milk with H5N1originating from animals infected with H5N1. A comparison between pH values for the study by Kwon et al. (2024) and our study cannot be evaluated because the pH of acidified lactose was not provided in their study, and further comparisons considering pH limits for utilization of post-acidified substrate cannot be considered.

An interesting finding in our study was for LPAI milk acidification trial 2, where viral inactivation was only observed in replicates for milk from cow C when compared with those from cow D (**Table 2**). This finding raises the potential hypothesis for a greater virucidal effect in milk with a higher fat content when compared with milk with a lower fat content, a major difference in milk composition between cows C and D (Table 1). Our study does not have a sufficient sample size to evaluate this hypothesis, and further studies with milk acidification for the inactivation of HPAI should consider evaluating the effect of milk fat. Previous studies have demonstrated an antimicrobial activity, which may explain the observed effect of higher fat with a more effective inactivation of the HPAI virus. In one study, digestion products of bovine milk triglycerides and membrane lipids were tested in vitro and demonstrated effective bactericidal effects against many common enteric bacterial pathogens, including due to the effect of fatty acids ($C_{10:0}$ and $C_{12:0}$), lysosphingolipids, and sphingolipids. In another study, enveloped viruses, such as HPAI, were shown to be lipid-sensitive, being affected by fatty acid present in milk (Thormar et al., 1987). Further studies designed to specifically evaluate the effect of milk fat components' virucidal effect against HPAI are needed to fully evaluate this potential effect.

Another main difference in trial 2 when compared with trial 3, for which effective inactivation of LPAI was observed for all replicates at time point 6, was a significantly lower pH (Figure 4), with mean and standard deviation values of 4.08 ± 0.05 and 4.32 ± 0.06, respectively. The pH for the H5N1 milk acidification trial, which resulted in similar outcomes as that of trial 3, also used a lower pH, at 4.16 ± 0.03. Another relevant finding for our study, independent of the initial pH, was a very low variability in the pH once milk acidification occurred throughout the following time point. From these findings, our recommendations would be to acidify milk at a pH between 4.05 and 4.2, with an ideal target pH as close to 4.1 as possible. The low variability in pH of milk acidification supports the practical use of this practice, as it does not require adjustments of pH after initial milk acidification occurs and emphasizes the importance of correctly conducting this initial step to secure effective treatment outcomes that result in H5N1 inactivation.

The lactoperoxidase system (LPS) was not observed to be an effective method for the inactivation of LPAI H6N2 in milk (Figure 6). In one in vitro study where a cell-free experimental system was used to characterize the virucidal effect of substrate of LPS (namely thiocya-

nate or iodide, and the hydrogen peroxide) against various influenza viruses, LPS substrates were effective at inactivating H1N1, H1N2, and H3N2 after exposure for 1 h (Patel et al., 2018). The study by Patel et al., (2018), differed from our study by not testing this effect in milk, and by directly testing LPS substrates, and not natural activation of the LPS system and following generation of substrates. Our study considered specific conditions for evaluating LPS, and future research considering the use of this method may consider increasing the time points for evaluation of LPS activity, as well as considering the use of different concentrations of LPS in milk.

A main limitation of this pilot study was the small sample size. The in vitro testing conducted was designed to maximize the accuracy in the evaluation of the main treatment effect being evaluated, including through sequential time point sampling with comparison within a treatment group, and between the treatment group and a control group, for which the only difference was the application of the treatment proposed. Through this approach, we aimed to retain scientific rigor that secures a robust and unbiased experimental design, methodology, analysis, interpretation, and reporting of results. Future studies should expand the evaluation of the effect of milk acidification on H5N1 2.3.4.4.b under field conditions, including through using a larger sample size, and a wider representation of milk with various compositions, including fat, to consider the potential synergistic effect of acidification of milk with other components, that together could affect inactivation of the virus in milk.

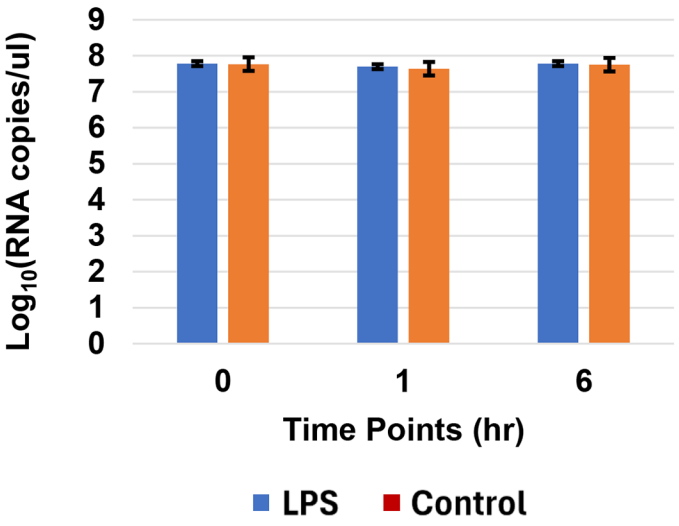

Figure 5. Least squares means (LSM) of RNA copies/µL in allantoic fluid of inoculated eggs for the LPAI H6N2 lactoperoxidase system (LPS) trial for each time point (hrs). Error bars represent the 95% confidence interval of the LSM. Using a non-parametric test, no significant difference was observed between the treatment group within each time point, or between time points within the same treatment group.

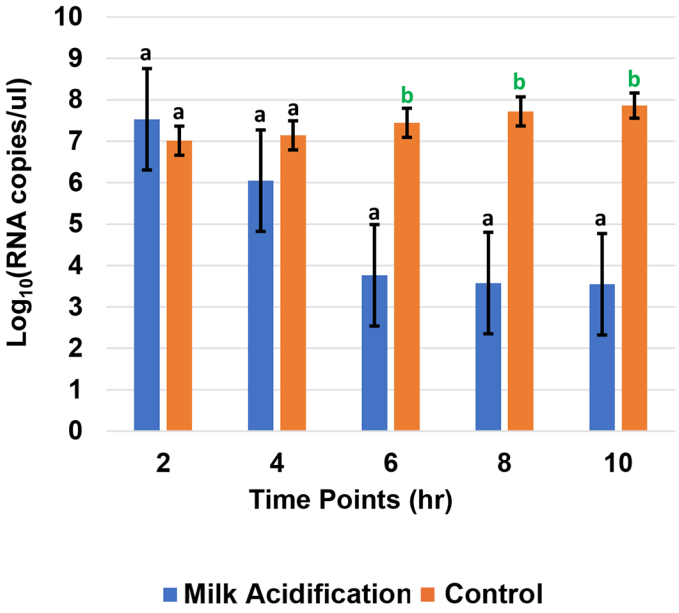

Figure 6. Least squares means (LSM) of RNA copies/µL in allantoic fluid of inoculated eggs for the H5N1 milk acidification trial for each time point (hrs). Error bars represent the 95% confidence interval of the LSM. Different letters indicate a significant difference between treatment groups within each time point when using the Kruskal-Wallis non-parametric test. No significant difference between 2 times points within a treatment group when using Dunn All Pairs for joint ranks was observed.

CONCLUSIONS

Our pilot study findings suggest milk acidification as an effective approach for completely inactivating HPAI H5N1 in milk originating from cows infected with H5N1 under laboratory conditions. After 6 h of treatment with milk acidification, whole raw milk containing H5N1 was not observed in either the LPAI H6N2 trials or in the HPAI H5N1 trial. Our findings support an ideal mean target pH for milk acidification of 4.1, avoiding a pH above 4.2 due to the potential reduced effect on H5N1 inactivation in milk. Our study observed that milk with a higher fat percent may affect milk acidification treatment effect, enhancing LPAI H6N2 inactivation when compared with milk with a lower pH; however further studies are needed to confirm this finding in our study. Together, our study supports the effectiveness of milk acidification for the inactivation of H5N1 in milk, serving as a potential alternative to pasteurization for the inactivation of HPAI H5N1. Further studies evaluating HPAI H5N1 inactivation using milk acidification in a herd infected with H5N1 are needed to evaluate our findings under farm conditions.

NOTES

Credit Authorship Contribution Statement B. Crossley Conceptualization, Methodology, Team Directing, Data curation, BSL3 laboratory oversight, Writing -original draft, Writing- review & editing. **C. Miramontes:** Conceptualization, Methodology, Field Sampling, Data curation, Writing -original draft, Writing- review & editing. **D. Rejmanek:** Conceptualization, Methodology, Data curation, Writing -original draft, Writing- review & editing. **R. Gallardo:** Conceptualization, Methodology, Writing -original draft, Writing- review & editing. **R. Pereira:** Conceptualization, Methodology, Team Directing, Field Sampling, Data curation, Writing -original draft, Writing- review & editing.

Declaration of Competing Interest None.

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REFERENCES

Alkie, T. N., N. Nasheri, P. Romero-Barrios, A. Catford, J. Krishnan, L. Pama, K. Hooper-McGrevy, C. Nfon, T. Cutts, and Y. Berhane. 2025. Effectiveness of pasteurization for the inactivation of H5N1 influenza virus in raw whole milk. Food Microbiol. 125:104653. https://doi.org/10.1016/j.fm.2024.104653.

Anderson, N. 2009. Acidifying Whole Milk and Milk Replacer with Citric Acid. in Ceptor Animal Health New. OMAFRA.

Awol, K., M. Taye, and B. Kassa. 2023. Activation of lactoperoxidase system and its potential for microbial inhibition and preservation of milk in the Great African Rift Valley climate. Cogent Food Agric. 9:2247691. https://doi.org/10.1080/23311932.2023.2247691.

Baker, A., B. Arruda, M. Palmer, P. Boggiatto, K. Sarlo Davila, A. Buckley, G. Zanella, C. Snyder, T. Anderson, C. Hutter, T.-Q. Nguyen, A. Markin, K. Lantz, E. Posey, M. Torchetti, S. Robbe-Austerman, D. Magstadt, and P. Gorden. 2024. Experimental reproduction of viral replication and disease in dairy calves and lactating cows inoculated with highly pathogenic avian influenza H5N1 clade 2.3.4.4b.

Burrough, E. R., D. R. Magstadt, B. Petersen, S. J. Timmermans, P. C. Gauger, J. Zhang, C. Siepker, M. Mainenti, G. Li, A. C. Thompson, P. J. Gorden, P. J. Plummer, and R. Main. 2024. Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus Infection in Domestic Dairy Cattle and Cats, United States, 2024. Emerg Infect Dis. 2024; 30(7):1335–1343 30(7).

Butt, S. L., M. Nooruzzaman, L. M. Covaleda, and D. G. Diel. 2024. Hot topic: Influenza A H5N1 virus exhibits a broad host range, including dairy cows. JDS Commun. 5:S13–S19. https://doi.org/10.3168/jdsc.2024-0638.

Chen, Y., Y. Gao, S. Yin, S. Zhang, L. Wang, and Y. Qu. 2020. Effect of acidified milk feeding on the intake, average daily gain and fecal microbiological diversity of Holstein dairy calves. Asian-Australas. J. Anim. Sci. 33:1265–1272. https://doi.org/10.5713/ajas.19.0412.

Collings, L. K. M., K. L. Proudfoot, and D. M. Veira. 2011. The effects of feeding untreated and formic acid-treated colostrum ad libitum on intake and immunoglobulin levels in dairy calves. Can. J. Anim. Sci. 91:55–59. https://doi.org/10.4141/CJAS10043.

Denholm, K. 2022. A review of bovine colostrum preservation techniques. J. Dairy Res. 89:345–354. https://doi.org/10.1017/S0022029922000711.

Dufour-Zavala, L. J. G., M. W. Jackwood, J. E. Pearson, W. M. Reed, and P. Woolcock. 2008. Titration of biological suspensions. In: A laboratory manual for the isolation, identification, and characterization of avian pathogens. Association of Avian Pathologists.

Eisfeld, A. J., A. Biswas, L. Guan, C. Gu, T. Maemura, S. Trifkovic, T. Wang, L. Babujee, R. Dahn, P. J. Halfmann, T. Barnhardt, G. Neumann, Y. Suzuki, A. Thompson, A. K. Swinford, K. M. Dimitrov, K. Poulsen, and Y. Kawaoka. 2024. Pathogenicity and transmissibility of bovine H5N1 influenza virus. Nature 633:426–432. https://doi.org/10.1038/s41586-024-07766-6.

FAO/WHO. 1991. Guidelines for the preservation of raw milk by use of the lactoperoxidase system.

FDA. 2016. Generally Recognized as Safe (GRAS) Notification for the Lactoperoxidase System. GRAS Notice (GRN) No. 665.

Figueroa, A., T. Derksen, S. Biswas, A. Nazmi, D. Rejmanek, B. Crossley, P. Pandey, and R. A. Gallardo. 2021. Persistence of low and highly pathogenic avian influenza virus in reused poultry litter, effects of litter amendment use, and composting temperatures. J. Appl. Poult. Res. 30:100096. https://doi.org/10.1016/j.japr.2020.09.011.

Garzon, A., P. Pandey, L. Tell, S. S. Aly, R. Poppenga, and R. Pereira. 2020. Evaluation of Heat and pH Treatments on Degradation of Ceftiofur in Whole Milk. Front. Vet. Sci. 7:288. https://doi.org/10.3389/fvets.2020.00288.

Halwe, N. J., K. Cool, A. Breithaupt, J. Schön, J. D. Trujillo, M. Nooruzzaman, T. Kwon, A. K. Ahrens, T. Britzke, C. D. McDowell, R. Piesche, G. Singh, V. Pinho Dos Reis, S. Kafle, A. Pohlmann, N. N. Gaudreault, B. Corleis, F. M. Ferreyra, M. Carossino, U. B. R. Balasuriya, L. Hensley, I. Morozov, L. M. Covaleda, D. Diel, L. Ulrich, D. Hoffmann, M. Beer, and J. A. Richt. 2024. H5N1 clade 2.3.4.4b dynamics in experimentally infected calves and cows. Nature. https://doi.org/10.1038/s41586-024-08063-y.

Kwon, T., J. T. Gebhardt, E. L. Lyoo, M. Nooruzzaman, N. N. Gaudreault, I. Morozov, D. G. Diel, and J. A. Richt. 2024. Bovine Highly Pathogenic Avian Influenza Virus Stability and Inactivation in the Milk Byproduct Lactose. Viruses 16:1451. https://doi.org/10.3390/v16091451.

Monne, I., S. Ormelli, A. Salviato, C. De Battisti, F. Bettini, A. Salomoni, A. Drago, B. Zecchin, I. Capua, and G. Cattoli. 2008. Development and Validation of a One-Step Real-Time PCR Assay for Simultaneous Detection of Subtype H5, H7, and H9 Avian Influenza Viruses. J. Clin. Microbiol. 46:1769–1773. https://doi.org/10.1128/JCM.02204-07.

Nooruzzaman, M., L. M. Covaleda, N. H. Martin, K. Koebel, R. Ivanek, S. D. Alcaine, and D. G. Diel. 2024. Thermal inactivation spectrum of influenza A H5N1 virus in raw milk. bioRxiv:2024.2009.2021.614205.

Parker, A. M., J. K. House, M. S. Hazelton, K. L. Bosward, V. L. Mohler, F. P. Maunsell, and P. A. Sheehy. 2016. Milk acidification to control the growth of Mycoplasma bovis and Salmonella Dublin in contaminated milk. J. Dairy Sci. 99:9875–9884. https://doi.org/10.3168/jds.2016-11537.

Patel, U., A. Gingerich, L. Widman, D. Sarr, R. A. Tripp, and B. Rada. 2018. Susceptibility of influenza viruses to hypothiocyanite and hypoiodite produced by lactoperoxidase in a cell-free system. PLoS One 13:e0199167. https://doi.org/10.1371/journal.pone.0199167.

Rodriguez, Z., C. Picasso-Risso, A. O'Connor, and P. L. Ruegg. 2024. Hot topic: Epidemiological and clinical aspects of highly pathogenic avian influenza H5N1 in dairy cattle. JDS Commun. 5:S8–S12. https://doi.org/10.3168/jdsc.2024-0650.

Spackman, E. 2020. Avian Influenza Virus Detection and Quantitation by Real-Time RT-PCR. Methods Mol. Biol. 2123:137–148. https://doi.org/10.1007/978-1-0716-0346-8_11.

Spackman, E., N. Anderson, S. Walker, D. L. Suarez, D. R. Jones, A. McCoig, T. Colonius, T. Roddy, and N. J. Chaplinski. 2024. Inactivation of Highly Pathogenic Avian Influenza Virus with High-temperature Short Time Continuous Flow Pasteurization and Virus Detection in Bulk Milk Tanks. J. Food Prot. 87:100349. https://doi.org/10.1016/j.jfp.2024.100349.

Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256–3260. https://doi.org/10.1128/JCM.40.9.3256-3260.2002.

Spackman, E., and D. L. Suarez. 2008. Detection and identification of the H5 hemagglutinin subtype by real-time RT-PCR. Methods Mol. Biol. 436:27–33. https://doi.org/10.1007/978-1-59745-279-3_5.

Thormar, H., C. E. Isaacs, H. R. Brown, M. R. Barshatzky, and T. Pessolano. 1987. Inactivation of enveloped viruses and killing of cells by fatty acids and monoglycerides. Antimicrob. Agents Chemother. 31:27–31. https://doi.org/10.1128/AAC.31.1.27.

Todd, C., K. Leslie, S. Millman, J. Sargeant, H. Migdal, K. Shore, N. Anderson, and T. Devries. 2016. Milk Replacer Acidification for Free-Access Feeding: Effects on the Performance and Health of Veal Calves. Open J. Anim. Sci. 06:234–246. https://doi.org/10.4236/ojas.2016.63029.

Todd, C. G., K. E. Leslie, S. T. Millman, V. Bielmann, N. G. Anderson, J. M. Sargeant, and T. J. DeVries. 2017. Clinical trial on the effects of a free-access acidified milk replacer feeding program on the health and growth of dairy replacement heifers and veal calves. J. Dairy Sci. 100:713–725. https://doi.org/10.3168/jds.2016-11401.

Todd, C. G., S. T. Millman, K. E. Leslie, N. G. Anderson, J. M. Sargeant, and T. J. DeVries. 2018. Effects of milk replacer acidification and free-access feeding on early life feeding, oral, and lying behavior of dairy calves. J. Dairy Sci. 101:8236–8247. https://doi.org/10.3168/jds.2018-14487.

USDA. 2016. Dairy Cattle Management Practices in the United States, 2014. Fort Collins, CO. USDA–APHIS–VS–CEAH–NAHMS, ed.

USDA. 2024. USDA Confirms Highly Pathogenic Avian Influenza in Dairy Herd in New Mexico.

USDA. Highly Pathogenic Avian Influenza (HPAI) Detections in Livestock.

Van Borm, S., D. L. Suarez, M. Boschmans, O. Ozhelvaci, S. Marché, and T. P. van den Berg. 2010. Rapid detection of Eurasian and American H7 subtype influenza A viruses using a single TaqManMGB real-time RT-PCR. Avian Dis. 54(Suppl):632–638. https://doi.org/10.1637/8734-032509-ResNote.1.

Williams, S. M. 2016. A laboratory manual for the isolation, identification and characterization of avian pathogens. Sixth edition. ed. Isolation, identification, and characterization of avian pathogens. American Association of Avian Pathologists, Jacksonville, FL.

Wise, M. G., D. L. Suarez, B. S. Seal, J. C. Pedersen, D. A. Senne, D. J. King, D. R. Kapczynski, and E. Spackman. 2004. Development of a real-time reverse-transcription PCR for detection of newcastle disease virus RNA in clinical samples. J. Clin. Microbiol. 42:329–338. https://doi.org/10.1128/JCM.42.1.329-338.2004.

Xing, Z., C. Cardona, P. Dao, B. Crossley, S. Hietala, and W. Boyce. 2008. Inability of real-time reverse transcriptase PCR assay to detect subtype H7 avian influenza viruses isolated from wild birds. J. Clin. Microbiol. 46:1844–1846. https://doi.org/10.1128/JCM.02426-07.

Zhang, J., and P. C. Gauger. 2020. Isolation of Swine Influenza A Virus in Cell Cultures and Embryonated Chicken Eggs. Pages 281–294 in Animal Influenza Virus: Methods and Protocols. E. Spackman, ed. Springer US, New York, NY.

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