**Research Note**

**Peroxide Test Strips Detect Added Hydrogen Peroxide in Raw Milk at Levels Affecting Bacterial Load**

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**ABSTRACT**

Hydrogen peroxide (H$_2$O$_2$) has a long-established history of use as a preservative in milk worldwide. The use of H$_2$O$_2$ to activate the inherent lactoperoxidase enzyme system has dramatically improved the quality of raw dairy products in areas in which cooling is not widely available. In the United States, however, where refrigeration is widely available, the addition of H$_2$O$_2$ to milk is not permitted, with the exception of certain applications prior to cheesemaking and during the preparation of modified whey. Due to the relatively quick deterioration of H$_2$O$_2$ in fluid milk, the detection of raw milk adulterated with the compound can be challenging. In this study we evaluated (i) total aerobic bacterial counts and (ii) ability of peroxide test strips to detect H$_2$O$_2$ in raw milk with various concentrations (0, 100, 300, 500, 700, and 900 ppm) of added H$_2$O$_2$, incubated at both 6 and 21°C for 0, 24, and 48 h. Results showed that at both 6 and 21°C the H$_2$O$_2$ concentration and time had a significant effect on bacterial loads in raw milk. Additionally, commercially available test strips were able to detect H$_2$O$_2$ in raw milk, with predicted probability of >90%, immediately after addition and after 24 and 48 h for the higher concentrations used, offering a viable method for detecting raw milk adulteration with H$_2$O$_2$.

Hydrogen peroxide (H$_2$O$_2$) has been shown to be an effective bactericidal and sporicidal agent (2, 7, 10, 11, 13). Use of liquid and vapor phase H$_2$O$_2$ is common in the food industry and in the pharmaceutical and medical industries (8) to reduce or eliminate bacterial contamination. Also, the use of H$_2$O$_2$ to control microbial deterioration of food products is common in the fruit and vegetable industry (1, 4, 6). Benefits of using H$_2$O$_2$ include its broad-spectrum activity; also effective against pathogens, and its nontoxic nature following degradation (10).

The global dairy industry also has a long history of H$_2$O$_2$ use. Studies dating back to the 1880s have demonstrated the preservative effects of H$_2$O$_2$ on raw milk (14). In 1957, the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) Expert Panel on Milk Quality recognized the potential for H$_2$O$_2$ as a processing alternative in developing countries where the lack of infrastructure severely limits raw milk quality (11). Concerns over the use of so-called “direct addition” H$_2$O$_2$ as a milk preservative include the relatively large concentrations of H$_2$O$_2$ used, the lack of regulatory control, the potential for the destruction of certain inherent vitamins (8), the alteration of casein and whey proteins, which has been shown to lead to “soft curd” in cheese making (12), and the potential for residual H$_2$O$_2$ to inactivate starter cultures. General acceptance of “direct addition” H$_2$O$_2$ application has, thus, been limited. The only widely recommended method for preserving raw milk in the absence of reliable refrigeration is activation of the inherent lactoperoxidase enzyme system in raw milk through the addition of small concentrations of both H$_2$O$_2$ (typically less than 10 ppm) and thiocyanate, which may not be naturally present in consistently sufficient concentrations (15 ppm; (15)) in raw milk. Alternatively, some lactic acid bacteria are capable of producing H$_2$O$_2$ in quantities sufficient to activate the lactoperoxidase system (5) and have, therefore, been used in place of H$_2$O$_2$ to activate the lactoperoxidase system. Because quantities of thiocyanate, a necessary component of the lactoperoxidase system, vary naturally in raw milk (15), activation of the lactoperoxidase system either through the addition of H$_2$O$_2$ or H$_2$O$_2$-producing lactic acid bacteria must be cautioned against as a method of controlling bacterial growth in raw milk. In the United States, the only approved use of H$_2$O$_2$ in the dairy industry is in certain cheese and cheese by-product applications at a maximum level of 0.05 and 0.04%, respectively, and residual H$_2$O$_2$ is subsequently removed (16); raw milk quality is controlled using good hygiene during milking, proper equipment sanitation, and immediate cooling. Despite the recommendation that direct addition of H$_2$O$_2$ to raw milk should not be used as a raw milk preservative, this practice is still used in some parts of the world and on some farms. Thus, the objective of this study

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was to evaluate the effect of the direct addition of \( \text{H}_2\text{O}_2 \) on bacterial growth in raw milk at refrigerated and ambient temperatures at 24 and 48 h posttreatment and to evaluate the ability of commercially available peroxide test strips to detect residual \( \text{H}_2\text{O}_2 \) at each test point.

**MATERIALS AND METHODS**

Raw whole milk (\(\sim 3.78 \text{ liters} \)) was obtained on three separate occasions from the Cornell Teaching and Research farm (Harford, NY) between November 2011 and February 2012 and was transported to the Milk Quality Improvement Program laboratory (Cornell University, Ithaca, NY) at or below 6°C. For each of the three independent trials, raw milk was thoroughly mixed according to *Standard Methods for the Examination of Dairy Products* (9) and was brought to the final incubation temperature (6 or 21°C) prior to preparing 100 ml of raw milk aliquots with initial concentrations of either 100, 300, 500, 700, or 900 ppm of \( \text{H}_2\text{O}_2 \) as well as untreated raw milk controls (in 4-oz [\(118.3-\text{ml}\)] sterile vials). All treated and control samples were spiral plated in duplicate for enumeration of total bacterial count on standard plate count agar (Difco, BD, Sparks, MD) and were tested for residual \( \text{H}_2\text{O}_2 \) using commercially available peroxide test strips (EMD Chemicals, Inc., Gibbstown, NJ) after 0, 24, and 48 h of incubation at either 6 or 21°C. Peroxide test strips were used according to the manufacturer’s instructions; briefly, the strip was fully immersed in the treated and untreated milk samples for 1 s, and then, after allowing residual milk to drip off the strip, the result was determined after 30 s. Bacterial colonies on each plate were enumerated after 48 h of incubation at 32°C. All bacterial count data were log transformed prior to statistical analysis, which was performed in R (R Foundation for Statistical Computing, http://www.r-project.org/) using the following R packages: (i) `brglm` (http://www.ucl.ac.uk/~ucakiko/software.html), (ii) `plyr` (http://www.jstatsoft.org/v40/i01/), and (iii) `ggplot2` (17).

**RESULTS AND DISCUSSION**

\( \text{H}_2\text{O}_2 \) affects bacterial load in raw milk at refrigerated and ambient temperatures. Overall, our results indicate that both \( \text{H}_2\text{O}_2 \) concentration (0, 100, 300, 500, 700, and 900 ppm) and incubation time after \( \text{H}_2\text{O}_2 \) addition (0, 24, and 48 h), and their interaction, had a significant effect \( (P < 0.05) \) on bacterial load in raw milk incubated at 6 and 21°C after \( \text{H}_2\text{O}_2 \) addition. At refrigerated storage temperatures (6°C), bacterial counts in untreated raw milk and raw milk treated with 100 and 300 ppm of \( \text{H}_2\text{O}_2 \) did not change significantly from 0 to 48 h \( (P = 0.48, 0.59, \text{and} \ 0.69) \), respectively; Fig. 1A). Whereas bacterial counts decreased, on average, more than 1 log CFU/ml in milk treated with higher \( \text{H}_2\text{O}_2 \) concentrations, the observed decrease in bacterial numbers observed over 48 h in milk treated with 500, 700, and 900 ppm and incubated at refrigeration temperatures was not significant \( (P = 0.23, 0.16, \text{and} \ 0.13) \), respectively; Fig. 1A). Similarly, when bacterial loads of the treated samples and the control were compared after 48 h of refrigerated storage, the only treatment that was even marginally significantly different \( (P = 0.09) \) from the control was 900 ppm; again, despite a mean difference of more than 2.5 log CFU/ml. The remainder of the treatments (100, 300, 500, and 700 ppm) were not significantly different \( (P = 0.86, 0.91, 0.23, \text{and} \ 0.12) \), respectively; Fig. 1A) from the control at 48 h. Using multiple linear regression analysis, we observed that, for every additional hour (at refrigeration temperature) that raw milk is exposed to 900 ppm of \( \text{H}_2\text{O}_2 \), there is a reduction of 0.04 log CFU/ml in the total bacterial count; at 500 ppm, every additional hour results in a reduction of only 0.02 log CFU/ml (Fig. 2A and Table 1). For every 100 ppm of \( \text{H}_2\text{O}_2 \) increase, there was a decrease of 0.17 and 0.29 log CFU/ml in the total bacterial count at 24 and 48 h, respectively, compared with the untreated control (Fig. 2A and Table 1). In other words, the longer raw milk is exposed to \( \text{H}_2\text{O}_2 \) at low temperatures, the more effect the \( \text{H}_2\text{O}_2 \) will have on the bacterial load; and, similarly, the higher the concentration of \( \text{H}_2\text{O}_2 \) raw milk is exposed to, the more dramatic the effect on bacterial load over time.

At ambient storage temperatures (21°C), total bacterial counts in untreated raw milk and raw milk treated with 100 and 300 ppm of \( \text{H}_2\text{O}_2 \) showed a significant increase over 48 h \( (P < 0.05 \text{ and Fig. 1B}) \). No significant difference was found for raw milk samples treated with 500 and 700 ppm
and held at ambient temperatures for 48 h ($P = 0.90$ and 0.26, respectively), despite the nearly 2-log decrease in bacterial load in the sample treated with 700 ppm of $H_2O_2$ over the 48-h test period (Fig. 1B). Finally, the raw milk sample treated with 900 ppm of $H_2O_2$ showed a significant decrease in bacterial numbers (at 48 h versus 0 h; $P < 0.05$), despite being held at ambient temperatures for 48 h (Fig. 1B). When the bacterial loads of the treated samples were compared with the control after 48 h of storage at ambient temperatures, the 700- and 900-ppm treatments were found to be significantly different from the control ($P < 0.05$), whereas the remaining treatments (100, 300, and 500 ppm) were not significantly different ($P = 1.0$, 0.98, and 0.29, respectively; Fig. 1B) from the control. Exploring the interaction of time and $H_2O_2$ concentration at ambient temperatures using multiple linear regression reveals effects similar to those seen at refrigerated temperatures: the longer raw milk is exposed to $H_2O_2$, the greater the effect on the bacterial load (Fig. 2B). Conversely to results from samples held at refrigeration temperatures, bacterial numbers in samples exposed to 100, 300, and 500 ppm of $H_2O_2$ and held at ambient temperatures increased by 0.09, 0.05, and 0.02 log CFU/ml for each hour of storage, respectively (Fig. 2B and Table 1). At higher concentrations, however, there was a decrease in bacterial numbers for every hour of ambient temperature storage of 0.01 and 0.05 log CFU/ml in milk treated with 700 and 900 ppm, respectively (Fig. 2B and Table 1). Further, for each additional 100 ppm of $H_2O_2$ used, there was a decrease in bacterial load of 0.56 and 1.00 log CFU/ml at 24 and 48 h, respectively, relative to the untreated control (Fig. 2B and Table 1).

The effects of varying concentrations of $H_2O_2$ on the bacterial load of raw milk held at refrigerated and ambient temperatures are not unexpected. The bacteriostatic and bactericidal activity of $H_2O_2$ has long been known (10). Recommended concentrations for effective raw milk preservation described in 1957 by the FAO/WHO Expert Committee on Food Additives ranged from 0.10 to 0.40 g of $H_2O_2$ per liter of raw milk (equivalent to 100 to 400 ppm) (11). These recommendations have since been rescinded, primarily because of the introduction of the use of activation of the inherent lactoperoxidase system in raw milk, which provides a sufficient extension of raw milk shelf life for producers in developing countries, especially those in warm climates, to market their product (3). Activation of the inherent lactoperoxidase system should only be considered in the most extreme cases, in which the lack of infrastructure precludes adequate cooling; this preservation method is not a substitution for cooling and/or pasteurization.

**TABLE 1. Estimate of effects of time and $H_2O_2$ concentration in raw milk stored at both 6°C and 21°C for 48 h**

<table>
<thead>
<tr>
<th>$H_2O_2$ at:</th>
<th>6°C</th>
<th>21°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ppm</td>
<td>0.0029</td>
<td>0.1141</td>
</tr>
<tr>
<td>100 ppm</td>
<td>-0.0021</td>
<td>0.0956</td>
</tr>
<tr>
<td>300 ppm</td>
<td>-0.0122</td>
<td>0.0584</td>
</tr>
<tr>
<td>500 ppm</td>
<td>-0.0222</td>
<td>0.0213</td>
</tr>
<tr>
<td>700 ppm</td>
<td>-0.0323</td>
<td>-0.0159</td>
</tr>
<tr>
<td>900 ppm</td>
<td>-0.0423</td>
<td>-0.0530</td>
</tr>
<tr>
<td>Reduction in Log bacterial count/additional 100 ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>-0.0005</td>
<td>-0.0012</td>
</tr>
<tr>
<td>24 h</td>
<td>-0.0017</td>
<td>-0.0056</td>
</tr>
<tr>
<td>48 h</td>
<td>-0.0029</td>
<td>-0.0101</td>
</tr>
</tbody>
</table>
have historically been used. Bacteriocins of lactic acid bacteria, which inhibit the growth of indicator bacteria, have been shown to have bacteriocidal activity over time is most readily observable in raw milk after 24 h of storage at both temperatures was quite low at most concentrations (Fig. 3A and 3B); this is not surprising because of the difficulty in regulating the purity and quantity of the compound used, the potential effects on milk quality, and the negative impact on the image of product wholesomeness. Despite the effectiveness of bactericidal and bacteriostatic agents such as direct addition of H$_2$O$_2$, activation of the inherent lactoperoxidase system is ultimately the only recommended method of maintaining the keeping quality of raw milk for short periods of time in locations with no access to cooling.

In areas of the world where rapid on-farm cooling of raw milk, by far the best practice for extending the keeping quality of raw milk, cannot be easily implemented, preservatives such as H$_2$O$_2$ have historically been used. The addition of any chemical preservative to milk is widely discouraged and is prohibited in most areas of the world because of the difficulty in regulating the purity and quantity of the compound used, the potential effects on milk quality, and the negative impact on the image of product wholesomeness. Despite the effectiveness of bactericidal and bacteriostatic agents such as direct addition of H$_2$O$_2$, activation of the inherent lactoperoxidase system is ultimately the only recommended method of maintaining the keeping quality of raw milk for short periods of time in locations with no access to cooling.

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REFERENCES


