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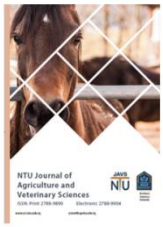

The Effect of Different Concentrations of Hydrogen Peroxide at a Temperature of 35°C and with Different Incubation Times on the numbers of Bacillus Subtilis in Raw milk

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A B S T R A C T

This study was conducted to investigate the potential factors effects on hydrogen peroxide bacterial spores killing severity. This study was achieved in Dairy Science and Technology Department , Food Sciences College, Al-Qasim Green University, Babylon, Iraq, during (2022-2023). Itincluded an experiment evaluation of the effect of 0.01, 0.05, and 0.1% concentrations of hydrogen peroxide at temperature of 35 degrees Celsius during an incubation time of 10, 20, 40, and 80 minutes. After that, the number of bacteria was measured by the plate counting method. The treatments were subjected to heat treatments at temperatures of 85, 90, and 95 degrees Celsius with and without added Hydrogen Peroxide for 20 minutes. The results showed that 0.1% Hydrogen Peroxide concentration eliminated entirely vegetative Bacillus bacteria and its spores. The results also indicated the concentration of 0.05%.and Heat treatment 90 and 95 °C eliminated bacterial spores to the comparison to control treatment 4.5×4^{10} Cfu.ml^{-1} raw milk. The results also show a concentration of 0.01%. with heat treatment at 85, 90, and 95°C reduced the number of bacteria, 45×10^2, 35×10^2 Cfu.ml^{-1} raw milk, and 25×10^2, respectively, compared to the control treatment 4.5×10^4 Cfu.ml^{-1} raw milk.

Introduction

Raw milk has a high nutritional value and a near-neutral pH and high-water activity. So it is a good environment for the growth and reproduction of many microorganisms (Quigley et al., 2013). One of the main reasons for low pH in raw milk is the production of acids by microorganisms due to lactose fermentation and precipitation of calcium and phosphate minerals (Fox et al., 2015). Likewise, the pH of sterilized milk decreased in samples containing lactose that were treated with heat between (65-95) °C (Al-Saadi, 2014). Also, the high moisture content promotes the growth of microbes and reduces milk's shelf life (Ajai et al., 2012). For the microbiological safety of milk, it's heated before use, as heat treatment is an essential step in the dairy industry, it aims to prolong the shelf life of milk and improve its quality by reducing the bacterial present in it, thus reducing the risk of food poisoning (Raikos, 2010).

Many studies have confirmed the benefits of using some microbes for manufacturing purposes, for example in the field of manufacturing dairy products to improve sensory qualities or nutritional value or add more preferable flavors (Jassim et al., 2023; Sawasn & Abd Ali, 2022), or for economic commercial purposes such as extending shelf life or increasing productivity by using certain types of bacteria (AL-janabi & Al-Bedrani, 2022) , as well as enhancing public health through the production of probiotic foods (Rasham et al., , 2023). On the other side, many microbes can lead to commercial catastrophe or health via food spoilage and birth -illness (Patil et al., 2023). Spore-forming bacteria are a source of concern to dairy product manufacturers due to their spores resistance to high temperatures and the nature of their spread, in addition to their resistance to freezing and ultraviolet radiation (Setlow et al., 2016). The structure of bacterial spores differs significantly from that of vegetative cells in several components that are unique to spores. These differences allow them to survive under environmental stresses that may be fatal to vegetative cells. The structure of bacterial spores consists of an Exosporium, a spore layer, an outer membrane, a cortex, an inner membrane, and a spore core (Leggett et al., 2012).

Ultra-heat treatment up to 138°C - 140°C for 2 - 3 seconds effectively eliminates spores, but the disadvantage of this method is changing the sensory properties of the milk. Also, this method is not suitable for the cheese manufacturing process, because the temperatures used in ultra-heat treatment UHT alter the clotting properties of milk (Chapman & Boor, 2001; Chavan et al., 2011).

Some researchers have indicated the ability of some bacteria spores, such as Bacillus spore-thermograms, to withstand UHT (ultra-high temperature 138-140°C for 4 seconds) in milk processing (Scheldeman et al., 2006; Cattani et al., 2013; Esteban et al., 2013).

Despite their extreme resistance, bacterial spores can be killed by several mechanisms, such as DNA damage (dry heat, radiation, formaldehyde, or nitrous acid), damage to the inner membrane (oxidizing agents), damage to essential enzymes (moist heat, small oxidizing agents such as hydrogen peroxide), and damage to organ components. Spore germination (sodium hydroxide) and penetration of all the barriers of spores (strong acids) as well as mechanisms unknown so far (high pressure, gas dynamic heating, plasma, supercritical fluids (Setlow, 2014).

Hydrogen peroxide possesses a broad spectrum of antimicrobial activity in that it is effective against bacteria, yeasts, fungi, viruses, and spores (Tschernjawskaja and Belowa, 1990; Brudzynski, 2006). As one of the reactive oxygen species, hydrogen peroxide causes oxidative damage (Jones and Joshi, 2021). This oxidative damage is mainly produced by reactive hydroxyl radicals (OH●) (Collin, 2019). Hydrogen peroxide (H2O2) produces hydroxyl (HO●) and (HOO●) radicals. Both components attack cell walls and often destroy cell walls by causing them to collapse. With most other disinfectants, spores' bacteria require higher concentrations of hydrogen peroxide. A longer contact time than for vegetative bacteria to eliminate those (Boateng et al., 2011).

In a study conducted by Abed Saleh et al . (2020) where they added hydrogen peroxide at a concentration of 0.1% to milk samples that showed coagulant when heated to 121°C for 15 minutes with pH values of 6.2, 6.3, 6.4, and 6.6 showed that hydrogen peroxide was not a good enhancer of the thermal stability of the milk, and it is not Heat resistant. A study conducted by (Al-Bedrani et al., 2012) on extracting garlic juice and producing it commercially indicated that refrigerated preservation at room temperature of 5°C was superior to preservation at 25°C with the use of some preservatives such as natamycin and hydrogen peroxide through sensory evaluation of the color and odor characteristics of the juice product. All treatments compared with the control treatment.

Research aims:
A_ Detection of spore bacteria in raw milk for the purpose of obtaining evidence of bacterial species contamination of raw milk.
B_ Study of the effects of hydrogen peroxide on spore-forming bacteria.

Materials and Methods

Experiment location
A laboratory experiment was achieved in Food Sciences Collage - Al-Qasim Green University, collected 200 samples of raw milk from several areas of Babil Governorate and gave the following symbols (T1, T2, T3, T4, T5, T6, T7), samples were stored in sealed, refrigerated, and sterile containers then transported to the laboratory, Cultural the sample carried out on the media to detect the types and numbers of bacteria that form spores. Hydrogen peroxide was added to the treatments at 0.1, 0.05, and 0.01% concentrations . After that, hydrogen peroxide was detected. The samples to which the concentrations of peroxide were added have been incubated at 4, 25, and 35 degrees Celsius for 10, 20, 40, and 80 minutes . After that, several spore bacteria were detected. The added models with and without peroxide were subjected to heat sterilization at 85, 90, and 95 degrees Celsius for 20 minutes. Then, the samples were cultured to detect the number of spore bacteria.
Isolation of Bacillus Subtilis from Raw Milk.
From different districts of Babil Governorate, 200 samples of raw milk were randomly taken to detect the bacteria that form spores were placed in a water bath at a temperature of 80°C for 10 minutes to kill the vegetative cells and leave only the spores (Rahimi et al., 2013). A decimal dilution process was conducted for each milk sample. Where 1 ml of each sample was taken and transferred to sterile, tightly closed tubes containing 9 ml of distilled water to obtain a 10-1 dilution. Shake the tube, then take 1 ml of the previous dilution with another sterile pipette and add it to another tube to obtain a 10-2 dilution (10- 1 -10-2). They were cultured in a Petri dish containing the selective differentiation medium Bacillus Agar. The dishes were incubated at 30°C for 48 hours (Mansour et al., 2015). Three replicates were cultured for each sample. The initial diagnosis of the growing bacteria was made according to the morphological appearance. The colonies were cultured once. Others were plated , individually on a nutrient agar medium and the plates were coded and incubated at 37°C for 24 hours. Then microscopic examination, staining with gram stain, and the following biochemical tests were performed (catalase test, gelatin test, methyl red stain test, motility test, Voges–Proskuer test) to ensure the purity of the isolates.
Count the number of colonies Cfu.ml-1 raw milk.
Then the total number of colonies was calculated by multiplying the number by the reciprocal of the dilution (Clark, 1965), and then the rate of bacterial colonies present in one dish was extracted from the following equation.
Number of bacteria in 1 ml = (number of growing colonies x reciprocal of dilution)
Detection of hydrogen peroxide in raw milk.
Use potassium iodide and starch indicator
Reagents
A. Potassium iodide solution: 20 g of potassium iodide was weighed and dissolved in distilled water to obtain a 100 ml solution.
B. Starch solution: We take 1 gram of starch powder and dissolve it in distilled water by heating and bringing the volume to 100 ml.
C. Potassium iodide reagent - starch: Mix equal amounts of 20% potassium iodide solution and 1% starch solution
We take 1 ml of the milk sample, put it in a test tube, then add 1 ml of starch reagent Potassium iodide mix well, and monitor the color of the solution in the tube. The color will developing to blue if hydrogen peroxide is present, while the pure milk sample remains white (Sharma et al., 2012).
statistical analysis
The statistical program Statistical Analysis System - SAS (2018) was used to analyze the data to study the effect of different parameters on the studied traits according to a complete random design (CRD), and the significant differences between the means were compared with the least significant difference test (LSD-Least Significant Difference).

RESULTS AND DISCUSSION

The Effect of adding Hydrogen peroxide at a concentration of 0.01% to milk at different incubation times and pasteurization temperatures.
Table (1) shows the effect of hydrogen peroxide at a concentration of 0.01% on spore bacteria incubated at 35°C for 10 minutes. The numbers of bacteria decreased after adding hydrogen peroxide, which gave a rate of 70×102 Cfu.ml-1 raw milk compared to the control treatment of 4.5×104 Cfu.ml-1 raw milk. The results also

indicated that there are significant differences between the incubation times (10, 20, 40, 80) minutes, as the 35°C treatment and the 80-minute time recorded the lowest average number of spore bacteria, which amounted to 50 × 102 Cfu.ml-1 raw milk, which indicates that it is the best treatment by reducing the number of spores bacteria. The results of the heat treatments to which the spore bacteria were exposed (85, 90, 95°C) with and without hydrogen peroxide indicated that there were significant differences in the incubation times, which were (10, 20, 40, and 80) minutes, as the lowest rate of bacterial numbers was recorded. Spores in the heat treatment at 95°C with hydrogen peroxide at an incubation time of 80 minutes gave number of 25× 102 Cfu.ml-1 raw milk compared to the comparison treatment of 4.5×104 Cfu.ml-1 raw milk and a comparison with the peroxide treatment that gave a rate of 70×102 Cfu.ml-1 raw milk Spore resistance is due to several factors related to the spore structure, such as the characteristics of the spore layer, the impermeability of the inner spore membrane, low core hydration, and high levels of DPA and SASPs. Both are common components of the DNA protection and repair mechanisms of spores. Spores from different strains, species, and genera can differ greatly . In their resistance, saturation of spore DNA with SASPs is the main mechanism that protects spores from dry heat. although DNA repair by spore enzymes during growth and mineralization of the core using DPA and divalent cations also play a role. (Setlow, 2014). Over time, exposure to hydrogen peroxide will damage the skin and organs due to its strong antioxidant capacity (Adly, 2010). A concentration range of 0.25-3% H2O2 can be utilized. The effectiveness of hydrogen peroxide depends on many factors, for example, concentration, pH, temperature, contact time, and use with physical agents (Feuerstein et al., 2006). The effects of hydrogen peroxide, which is considered to have a low molecular weight, can be due to it being able to pass through the pores of bacterial cell wall and the membranes of microorganisms, thus damaging the cell wall and its contents, and this is consistent with what was mentioned (Feng et al., 2020).

Table 1. The effects of hydrogen peroxide at a concentration of 0.01%, at incubation temperature of 35°C, different incubation times, and different pasteurization temperatures on the numbers of spore bacteria

.Numbers of spores bacteria Cfu.ml^{-1} raw milk	Peroxide treatment		Bacteria numbers after treatment with H_20_2 Cfu.ml^{-1} raw milk	Heat treatments for 20 minutes						
				85°C		90°C		95°C		
	Incubation temperature °C	Incubation timeMinut		Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide Cfu.ml^{-1} raw milk	Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide Cfu.ml^{-1} raw milk	Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide Cfu.ml^{-1} raw milk	LSD
4.5×^{4}10	35	10	70×10^2	95×10^2	60×10^2	90×10^2	50×10^2	90×10^2	45×10^2	10.18 *
4.5×^{4}10	35	20	65×10^2	85×10^2	55×10^2	86×10^2	45×10^2	85×10^2	30×10^2	8.96 *
4.5×^{4}10	35	40	35×10^2	80×10^2	50×10^2	80×10^2	40×10^2	80×10^2	28×10^2	8.41 *
4.5×^{4}10	35	80	50×10^2	75×10^2	45×10^2	70×10^2	35×10^2	70×10^2	25×10^2	9.84 *
	LSD		9.02 *	8.75 *	8.12 *	8.69 *	7.16 *	8.66 *	7.01 *	---
* (0.05≥ P)										

The Effect of adding Hydrogen peroxide at a concentration of 0.05% to milk at different incubation times and pasteurization temperatures .

Table (2) shows the effect of 0.05% hydrogen peroxide on spore bacteria incubated at 35°C for 10 minutes. The number of bacteria decreased after adding hydrogen peroxide , which gave 55×10^2 Cfu.ml^{-1} raw milk compared to the control treatment of 4.5×^{4}10 Cfu.ml^{-1} raw milk.

The results also observed Significant differences between the incubation times (10, 20, 40, 80) minutes, where the 35°C treatment and the 80-minute treatment recorded the lowest rate of spore bacteria numbers, which amounted to 40×10^2 Cfu.ml^{-1} raw milk Without hydrogen peroxide, there are significant differences in all incubation times, which are (10, 20, 40, 80) minutes. The reason for this is that the concentration of 0.05% hydrogen peroxide is low at a temperature of 85 °C, so it cannot kill spores of bacteria permanently. The results of the heat treatments to which the spore bacteria were exposed (90, 95°C) with hydrogen peroxide also indicated that there were no significant differences in all incubation times, which were (10, 20, 40, 80) minutes, while the heat treatments were recorded without hydrogen peroxide. Significant differences, as the 95°C treatment at an incubation time of 80 minutes recorded the lowest rate in the number of spore bacteria, which gave a rate of 40×10^2

Cfu.ml^{-1} raw milk compared to the comparison treatment 4.5×^{4}10 Cfu.ml^{-1} raw milk. The reason for the elimination of spore bacteria and their failure to grow under high temperatures and concentrations of hydrogen peroxide is due to the presence of free hydroxyl radicals (OH•), which can oxidize and destroy cell components such as enzymes, proteins, fats, and DNA. Block (1991) stated that it works to target exposed sulfhydryl groups and double bonds specifically.

Table 2. The effect of hydrogen peroxide at a concentration of 0.05%, an incubation temperature of 35°C, different incubation times, and different temperatures on the numbers of spore bacteria

Numbers of spores bacteria Cfu.ml^{-1} raw milk	Peroxide treatment		Bacteria numbers after treatment with H_20_2 Cfu.ml^{-1} raw milk	Heat treatments for 20 minutes						LSD
				85°C		90°C		95°C		
	Incubation temperature °C	Incubation time Minutes		Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide Cfu.ml^{-1} raw milk	Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide Cfu.ml^{-1} raw milk	Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide Cfu.ml^{-1} raw milk	
4.5×^{4}10	35	10	55×10^{2}	75×10^{2}	40×10^{2}	70×10^{2}	Nil	65×10^{2}	Nil	9.82 *
4.5×^{4}10	35	20	50×10^{2}	70×10^{2}	25×10^{2}	65×10^{2}	Nil	60×10^{2}	Nil	9.05 *
4.5×^{4}10	35	40	45×10^{2}	65×10^{2}	20×10^{2}	60×10^{2}	Nil	55×10^{2}	Nil	8.66 *
4.5×^{4}10	35	80	40×10^{2}	55×10^{2}	15×10^{2}	50×10^{2}	Nil	4×10^{2}	Nil	8.59 *
	LSD		7.42 *	8.68 *	7.95 *	9.02 *	NS 0.00	8.56 *	NS 0.00	---

* (0.05≥ P)

The Effect of adding Hydrogen peroxide at a concentration of 0. 1% to milk at different incubation times and pasteurization temperatures . Table (3) shows the effects of 0.1% hydrogen peroxide on spores of bacteria incubated at 35°C for 10 minutes. The number of bacteria decreased after added H2O2, which gave 0 Cfu.ml-1 raw milk, compared to the control treatment 4.5×410 Cfu.ml-1 raw milk. The results also no significant differences in the incubation times (10, 20, 40, 80) minutes, indicating the role of peroxide concentration in reducing the number of spore bacteria. The results of the heat treatments to which the spores of bacteria were exposed, (85, 90, and 95°C) without hydrogen peroxide, indicated that there were significant differences in all incubation times, which were (10, 20, 40, and 80) minutes, as the 95°C treatment was recorded at the incubation time. 80 minutes the lowest rate in the number of bacteria spores, , which gave a rate of 50×102 Cfu.ml-1 raw milk. compared to the control treatment of 4.5×410 Cfu.ml-1 raw milk.

Table 3: The effect of hydrogen peroxide at a concentration of 0.1%, an incubation temperature of 35°C, different incubation times, and different temperatures on the numbers of spore bacteria

Numbers of spores bacteria Cfu.ml^{-1} raw milk	Peroxide treatment		Bacteria numbers after treatment with H_20_2	Heat treatments for 20 minutes						LSD
				85°C		90°C		95°C		
	Incubation temperature °C	Incubation time minutes		Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide	Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide	Without hydrogen peroxide Cfu.ml^{-1} raw milk	With hydrogen peroxide	
4.5×^{4}10	35	10	Nil	75×10^{2}	Nil	70×10^{2}	Nil	65×10^{2}	Nil	12.35 *
4.5×^{4}10	35	20	Nil	70×10^{2}	Nil	65×10^{2}	Nil	60×10^{2}	Nil	10.82 *
4.5×^{4}10	35	40	Nil	65×10^{2}	Nil	60×10^{2}	Nil	55×10^{2}	Nil	10.07 *
4.5×^{4}10	35	80	Nil	60×10^{2}	Nil	55×10^{2}	Nil	50×10^{2}	Nil	9.58 *
	LSD		NS 0.00	8.71	NS 0.00	9.37 *	NS 0.00	7.62 *	NS 0.00	---

* (0.05≥ P)

Conclusion:

This study shows the factors that affects the severity of hydrogen peroxide on the killing of bacterial spores (hydrogen peroxide concentration, incubation time, and degree Celsius of heat treatment). The number of spores is inversely proportional to the concentration of peroxide, degree of heat treatment and incubation time, in addition, pasteurization treatment has a synergetic effect with peroxide to kill bacterial spores. Also, the heat treatment was utilized for facilitating the hydrogen peroxide degradation in order to eliminate it in the final product .

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