Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2017; 5(5): 930-934

Received: 01-07-2017
Accepted: 02-08-2017

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The efficacy of ozone treatment on the microbiological quality of raw milk at different storage temperatures

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Abstract
In the present study, thirty raw milk samples were collected randomly from different stores and retailer markets in Baghdad province to evaluate the effect of ozone treatment at a concentration of 0.5 ppm on the bacteriological quality (Total aerobic bacteria, Coliforms and *Staphylococcus aureus* counts). The current study was also planned to investigate the effects of ozonation treatment on the shelf life of raw milk after 24hrs of storage at both refrigeration (4 °C) and the ambient temperatures (30 °C). Data revealed that there were significant (P<0.0001) differences in the percentage of microorganisms that affect the raw milk quality, where the highest prevalence were found in the total aerobic bacteria and Coliforms (100%) respectively followed by the *Staph. aureus* (60%). The microorganisms that affect the raw milk quality (aerobic bacteria, Coliform, and *Staph. aureus*) showed a different sensitivity to the ozone treatment at 0.5 ppm for 10, 15 and 20 minutes at different storage temperatures (ambient and refrigeration temperatures). The highest microbial counts that were found in the control milk samples indicated that most of these samples were produced under very poor hygienic conditions. The bacterial counts reduction in raw milk samples after exposure to ozonation treatment for 10, 15 and 20 minutes was not enough for improving both the quality and the shelf life of raw milk.

Keywords: Ozonation treatment, microbiological quality, storage temperatures, raw milk

1. Introduction
Milk is secreted as a sterile fluid from the animal's udder. However, the microbial contamination may occur within the udder and during the different stages of milking process, handling and storage, besides that the animals feed, soil and feces are regarded as a possible sources of contamination [1]. The raw milk that meant for human consumption should be free from any hazard pathogenic-microorganisms [2]. Raw milk is considered as an ideal medium for the microbial growth and multiplication such as aerobic bacteria, *Lactococcus*, Coliforms, Streptococcus and yeasts and moulds [1]. Detection of coliform bacteria in the raw milk can be used as an indicator of raw milk contamination with pathogenic bacteria [3]. Currently there are many attempts toward using methods to produce safe food and food products as milk and dairy products, which are less in the additives and higher in the quality " natural foods" [4]. Ozone applications is the Food and Drug Administration and United States Department of Agriculture validated as antibacterial agent that can be used in the different kinds of food products [5]. Ozone is a safe substance in the natural atmosphere and regarded as one of the most potent sanitizers against a wide range of microorganisms with a strong capacity of both disinfection and sterilization process for many kinds of food [6], therefor the Ozonation process is considered as a one of the emerging method that applied to decrease the initial microbial load in the food products [7], Different studies were reported during the ozone treatment many of pathogenic and non pathogenic microorganisms such as viruses, bacteria fungi and protozoa were destroyed [8], The increasing demand for lightly processed food products with best keeping quality than those foods treated by the commercial traditional chemical preservatives has nudged the researchers to focus on the new ways for extending and prolonging the shelf life of fresh food produce [9], The main aims of this study are enhancing the safety of ozonized raw milk at different storage temperatures (4 °C and ambient temperature) and assessment the ability of used ozone treatment for reducing the microbial contamination and extending the shelf life of raw milk.

2. Materials and Methods

2.1 Collection of raw milk samples

This study was carried out during the period began from 1/12/ 2016 till the 1/5/ 2017. A total of 30 raw milk samples (3 liters each) were collected at weekly intervals (2 samples/week) in a sterile glass bottles from different local retail markets in Baghdad province.All raw milk samples were immediately kept in a cool -box and transported to the laboratory of Veterinary Public Health department for the microbiological quality tests.

2.2 Culturing, preparation of dilutions, and enumeration the bacteriological quality microorganisms

Before and after each of ozonation treatment, the total aerobic bacterial counts, Coliform and *Staph. aureus* counts were determined by the standard plating technique for total aerobic bacteria [10], coliform counts were determined by using the pour plating method on the Violet red bile agar then all the plates were incubated at 37 °C for 24 hrs [11], and *Staphylococcus aureus* isolates that obtained by plating on the Chromogenic agar were further tested for Biochemical identifications tests as Gram stain, Catalase activity, Coagulase and DNase tests with and without toluidine stain were performed. DNase tests were performed for detection the DNase production and the appearance of yellow golden zone surrounding the bacterial colonies was regarded as a positive results. Further rapid biochemical tests were used such as, Dry spot staphytect plus (DR0100) for detection of *Staph. aureus* coagulation and Electronic Rapid TM Staph. plus system with standard colors chart with specific ATCC codes online. ERIC Rapid system was used for the rapid biochemical test for identification of Staphylococcus species in just (4 hours). The ERIC system was based upon special detection by different indication materials. and serological teat as Masta TM Staph Latex agglutination test kit was a rapid Latex test for the identification of Staphylococcal isolates particularly *Staphylococcus aureus* [12].

2.3 Ozonation equipments and operating conditions

2.3.1 Ozonation equipment's

Ozone concentrations can be determined by using the Ozone CHE Ozone concentrations Mets® Kit (concentrations limit were 0-0.6 and 0.6-3ppm).The kit was consisting of activator solution, comparators, (25 mL) plastic sample cup and the manual instructions. The Ozonation equipment which used in the current study was designed in the Veterinary Public Health / Food Hygiene department, University of Baghdad, as shown in Figure 1. This apparatus was consisted from ozone generator (A2Z) which was employed by means of a diffuser at a rate of 600 mg/h with the dose of 0.6 ppm of ozone and the range of ozone generation was at 0.05 - 3 ppm (50 - 3000 ppb) with the minimum detection limit of 0.025 ppm (25 ppb). The calculation of ozone concentration out put (ppm) of the ozone generator was done by using CHE-Mets-Kit. The highest ozone concentration that used in this current study at both 15 and 20 minutes, was (0.5 ppm) respectively. The aeration stone was inserted into the plastic container and the ozonation treatment was carried out at the pH (6.5), at the both refrigeration (4 °C) and ambient (30 °C) temperatures. The Sterilized reaction chamber was consisted of a rectangular plastic box with a plastic transfer tube contacted with a plastic container of a capacity of (500ml) and each milk sample was transferred to this plastic container (30 cm in height and 20 cm in diameter) and before each milk transfer, the plastic container was flushed with fresh distilled water and then sanitized by bubbling ozone gas for 5 minutes.

The ozonated milk was stirred by glass rod during the ozonation process for 10, 15 and 20 min contact time at the both refrigeration and ambient (30 ºC) temperature

Fig 1: Ozonation equipment

2.4 Statistical analysis

Statistical analysis of data was preformed by using SAS (Statistical analysis system-version 9.1).Two ways ANOVA and the least significant differences (LSD),post hoc test were preformed to assess any significant differences among means ($P<0.05$) [13].

3. Results and Discussion

3.1 The cultural, biochemical and serological properties of the microorganisms that affect the raw milk quality

Results of cultural properties of total aerobic bacteria, coliforms and the both biochemical reactions and serological characterization of *Staph. auerus* bacteria are presented in Table 1. The quality control tests that were done included the Standard Plate Count (SPC), Coliform Count (CC) and *Staph. aureus* counts. The nutrient agar (Oxoid) was used for the determination of the total aerobic bacterial counts and the plates were incubated at 37 °C for 48 hrs, while the Violet red bile agar (Oxoid) was used for the determination of the coliform counts and the plates were incubated at 37 °C for 24hrs, typical pinkish to red colonies were counted and each bacterial analysis was made in duplicate for all microorganisms that affected the raw milk quality. The number of colonies in each bacterial dilution was multiplied by the reciprocal of the dilution factor and calculated as the colony forming units (cfu/ml) per milliliter of raw milk. Characterization of *Staph. aureus* isolates was carried out by using the colony morphology and cultural properties. Typical colonies of *Staph. aureus* were appeared on the selective differential chromagar-*Staph. aureus* as mauve in color. The *Staph. aureus* isolates had the ability to grow on the mannitol salt agar (MSA) and fermented the mannitol sugar and produced golden yellow colonies that surrounded by yellow zone around the colonies. All the isolates that grown on the (5% v/v) of sheep blood agar at 37 °C for 24 hrs gave β - hemolysis pattern. Further biochemical and serological tests identifications were done including the gram's reaction, coagulase test, catalase test, Dryspot *Staph. aureus* and Latex Mast Staph. The positive results for all positive *Staph aureus* isolates were indicated by agglutination of the Latex particles after about 20 seconds of serological reaction.

Table 1: The growth (cultural), biochemical and serological characteristics of the microorganisms that effected of the raw milk quality

microbial test	Cultural media	Cultural characteristics	Positive results		
			Biochemical characteristics	Serological characteristics	
				Dryspot *staph. aureus*	Latex mast staph
Staph. aureus	Chromogic agar	Mauve colonies	Gram positive	Agglutination	Agglutination
	Mannitol salt agar	Golden Yellow colonies	Catalase positive by oxygen bubbles		
	Blood agar	B-hemolysis pattern	Coagulase positive by agglutination		
Coliforms	Violet red bile agar VRB	Pinkish to dark red colonies	Gram negative		
Total Bacterial count	Nutrient agar	White to creamy colonies			

+ve reaction = Agglutination

3.2 Isolation of the microorganisms that affect the raw milk quality

In the current study the isolation percentages of the microorganisms that affect the raw milk quality are shown in Table. 2. Data revealed that there were significant differences in the percentages of microorganisms that affect the raw milk quality where the highest significant ($P \leq 0.05$) prevalence levels of contamination were with total aerobic bacteria and coliform in their raw milk samples where 30 (100%) out of 30 raw milk samples examined were found positive during the current study, while 18 (60%) out of 30 raw milk samples were found positive for the presence of *Staph. aureus* bacteria. Such high prevalence levels of contamination with these microorganisms pointed out the potential public health hazard The presence of these bacteria in the raw milk often emerge as a major public health concern, especially for those peoples who still drink raw milk. Storing fresh milk at high ambient temperature (30 °C) together with unhygienic practices in the all milking process, may also result in microbiologically inferior milk quality [14], This current study investigated the microbiological quality and safety of raw milk by studying the prevalence of the indicator microorganisms such as coliforms and *Staph. aureus*. Identification of *Staph. aureus* was based on their growth on the selective agar, colony morphology, Gram's reaction, rapid biochemical and serological tests [15].The high isolation percentage of *Staph. aureus* in the raw milk (60%) might be due to the contamination by handlers, since up to 65% of humans are nasal carriers of *Staph. aureus* bacteria and 5-20% of peoples are carrying the bacteria as part of their normal skin flora [16]. Coliforms are group of bacteria, which are inhabit the intestinal tracts of both human and animals, in this current study the high isolation percentage (100%) of Coliform bacteria might be due to contamination of the raw milk from different sources such as unclean milker "s hands, improperly cleaned and/or un- sanitized equipment's, faulty sterilization of raw milk utensils especially churns, milking machines, improper preparation of the dairy cow's manure, hair dropping in to the raw milk during different stages of milking, unclean water, dirty towels and udder not dried before milking process [17].

Table 2: The isolation percentages of microorganisms that affected the raw milk samples quality that collected from distracts of Baghdad province

Quality control microorganisms	No. of examined samples	No of positive samples	Isolation percentage
Total Bacterial counts	30	30	100% A
Coliforms	30	30	100% A
Staph. aureus	30	18	60% B

X^2 =138.26

$P \leq 0.0001$

3.3 The viability of the microorganisms that effect the raw milk samples quality that subjected to the ozonation treatment at (0.5 ppm) for 10, 15 and 20 minutes after 24 hrs of storage at ambient temperature

The mean levels of the Total aerobic bacteria, Coliforms and *Staph. aureus* counts in the raw milk samples that subjected to the ozonation treatment at (0.5 ppm) for 10, 15 and 20 minutes after 24 hrs of storage at ambient temperature are shown in Table 3. Total aerobic bacteria, coliforms and *staph. aureus* counts in raw milk samples that subjected to ozonation treatment for 10, 15 and 20 minutes after 24 hrs of storage at ambient temperature had significantly ($P \leq 0.05$) influenced by the exposure times where exposure the raw milk samples to the ozonation treatment at (0.5 ppm) for 10 minutes caused a significant ($P \leq 0.05$) reduction in the total aerobic bacteria coliforms and *staph aureus* counts, the mean log values of starting initial counts of 8.83 ± 0.06 log cfu/ml, 7.79±0.13 log cfu/ml and 5.52±0.05 log cfu/ml respectively, to 7.46±0.07 log cfu/ml, 6.58±0.12 log cfu/ml and 5.28±0.01 log cfu/ml respectively, while after 15 minutes of exposure to the ozonation caused a significant ($P \leq 0.05$) reduction in the total aerobic bacteria, coliforms and *Staph aureus* counts to 4.36±0.08 log cfu/ml, 4.33±0.11 log cfu/ ml and 4.03±0.04 respectively Exposing of raw milk to the ozonation treatment to the 20 minutes had a greatly significant ($P \leq 0.05$) reduction in the total aerobic bacteria, coliforms and *staph aureus* counts to 3.82±0.03 log cfu/ml, 3.15 ± 0.17 log cfu/ml and 2.14 ± 0.04 log cfu/ml respectively (Table 3.). The Temperature of storage is important since the temperature influences the microbial growth, as well as highlighting the importance of a proper cool chain to maintain the raw milk quality. The milk and dairy products should be kept under refrigeration at all circumstances and the practice and display at room temperature should be discouraged. The refrigeration storage temperature can cause a dramatic reduction in the bacterial population, as the refrigeration at temperatures 4ºC is one of the best ways for preventing growth of many kinds of bacteria such as *Staphylococcus aureus* and consequently the formation of staphylococcal toxin. [18] Raw milk is known to possess several natural anti-microbial agents, but without

refrigeration facilities the bacteria can grow and multiply, and the bacterial number count could be doubled in less than three hours in un chilled raw milk, where the initial numbers of microbial growth will depend on the temperature at which the milk is held immediately after milking [17]

Table 3: Differences in the mean levels (log_{10}cfu/ml) of the microorganisms that effect the raw milk samples quality that subjected to the ozonation treatment at (0.5 ppm) for 10, 15 at ambient temperature and 20 minutes after 24 hrs of storage

Parameters	Exposure time /Minutes			
	0	10	15	20
	Means± SE			
Total aerobic bacteria	8.83±0.06 Aa	7.46±0.07 Ba	4.36±0.08 Ca	3.82±0.03 Da
Coliforms	7.79±0.13 Ab	6.58±0.12 Bb	4.33±0.11 Ca	3.15±0.17 Db
Staph. aureus	5.52±0.05 Ac	5.28±0.01 Ac	4.03±0.04 Bb	2.14±0.04 Cc
LSD	0.2677			

*Different capital letters in the row denote significant ($P \leq 0.05$) differences in the microbial counts between different exposure times
*Small letters in a column denote significant ($P \leq 0.05$) differences between the bacterial species
*SE=Standard error

3.4 The viability of the microorganisms that effected of the raw milk quality subjected to the ozonation treatment at (0.5 ppm) for 10, 15 and 20 minutes after 24 hrs of storage at refrigeration temperature

The mean levels of the Total aerobic bacteria, Coliforms and *Staph. aureus* counts in the raw milk samples that subjected to the ozonation treatment at (0.5 ppm) for 10, 15 and 20 minutes after 24 hrs of storage at refrigeration temperature (4 °C) are shown in (Table 4). Total aerobic bacteria, coliforms and *Staph. aureus* counts in raw milk samples that subjected to ozonation treatment for 10, 15 and 20 minutes after 24 hrs of storage at refrigeration temperature had significanty ($P \leq 0.05$) influenced by the exposure time where exposure the raw milk samples to the ozonation treatment at (0.5 ppm) for 10 minutes caused a significant ($p \leq 0.05$) reduction in the total aerobic bacteria, coliforms and *Staph. aureus* counts from the mean log values of starting initial counts 8.83 ± 0.06 log cfu/ml, 7.79 ± 0.13 log cfu/ml and 5.52±0.05 log cfu/ml respectively, to 6.52 ± 0.11 log cfu/ml, 6.45 ± 0.04 log cfu/ml and 5.19 ± 0.06 log cfu/ml respectively, while after 15 minutes of exposure to the ozonation caused a significant ($P \leq 0.05$) reduction in the total aerobic bacteria, coliforms and *Staph aureus* counts to 4.70±0.09 log cfu/ml, 4.28±0.07 log cfu/ ml and 3.01±0.02 respectively Exposing of raw milk to the ozonation treatment for 20 minutes had a greatly significant ($P \leq 0.05$) reduction in the total aerobic bacteria, coliforms and *staph aureus* counts to 2.94±0.07 log cfu/ml, 3.47±0.04 log cfu/ml and 1.81 ± 0.10 log cfu/ml respectively.

Table 4: Differences in the mean levels (log_{10}cfu/ml) of the microorganisms that effect the raw milk samples quality that subjected to the ozonation treatment at (0.5 ppm) for 10, 15 and 20 minutes after 24hrs of refrigeration storage at 4 °C.

Parameters	Exposure time/Minutes			
	0	10	15	20
	Means± SE			
Total aerobic bacteria	8.83±0.06 Aa	6.52±0.11 Ba	4.70±0.09 Ca	2.94±0.07 Db
Coliforms	7.79±0.13 Ab	6.45±0.04 Ba	4.28±0.07 Cb	3.47±0.04 Da
Staph. aureus	5.52±0.05 Ac	5.19±0.06 Bb	3.01±0.02 Cc	1.81±0.10 Dc
LSD	0.2462			

*Different capital letters in the row denote significant ($P \leq 0.05$) differences in the microbial counts between different exposure times
*Small letters in a column denote significant ($P \leq 0.05$) differences between the bacterial species
*SE=Standard error

Ozone is used in combination with low refrigeration temperatures (as stress profile) to decrease the amount of antimicrobial agent that can be used to increase the microbial inactivation for the microorganisms that may affecte the raw milk quality [19, 20] Many studies reported that moulds were more resistant to ozonation treatment than yeasts and yeasts were more resistant than bacteria, while gram-negative bacteria were more sensitive than gram positives bacteria and ozone with less efficacy against both fungal and bacterial spores than vegetative bacterial cells [21, 22], Bacteria cannot grow at the refrigeration temperatures and remain at low numbers, indicating that the temperature plays an important role for controlling the prevalence and proliferation of specific microorganisms in the raw milk [23]

4. Conclusions

The gaseous ozone bubbling in the raw milk that could be used as a preservation method to reduce the microbial contamination was depending on the concentration of ozone, exposure time and storage temperature. Also the microorganisms that affect the raw milk quality which used in this study showed different sensitivity to the ozone treatment at 0.5 ppm for 10, 15 and 20 minutes at different storage temperature but the bacterial reductions was possibly not enough for improving both the quality and the shelf life of raw milk.

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