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Effect of dissolved carbon dioxide (CO_2) on the growth of some pathogenic and spoilage microorganisms in milk and whey medium

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Abstract

The growth of pathogenic and spoilage strains in saturated milk or whey with carbon dioxide (CO_2) was studied. In saturated milk, the highest log reductions were 2.0 and 1.3 for *Staphylococcus aureus* and *Candida albicans*. After two weeks of cold storage, the log reductions were reached to 2.9, 2.48 and 2.0 for *Candida albicans, Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. Slightly inhibitory effect could be detected with some strains in saturated whey. The highest log reductions 0.2 was reported for *Pseudomonas aeruginosa* after cold storage of whey for two weeks. The log reduction of *Staphylococcus aureus* was 0.36 in milk with pH 5.5. In milk with pH 6.6, the log reductions were 2.0, 1.3 and 0.5 for *Staphylococcus aureus, Candida albicans* and *Bacillus cereus*, respectively. CO_2 addition was showed a good log reduction for *Candida albicans, Staphylococcus aureus* and *Pseudomonas aeruginosa* in milk with cold storage and pH 6.6. Therefore, the addition of carbon dioxide was more effective against pathogenic strains in saturated milk than in whey. In addition, the effect of CO_2 was independent according to strain type. Therefore, we can use dissolved carbon dioxide to reduce the microbial load and extend the shelf life of dairy products.

***Keywords*:** Carbon dioxide, microbial load, spoilage fungi, pathogenic bacteria, milk and whey**.**

Introduction

Nowadays, food safety authorities have recommended that the food industry reduce chemical additives and microbial overload in foods. In addition, the demand for natural antimicrobial agents is increasing due to consumer concern over health issues [1]. Dairy products are an excellent growth medium for pathogenic and spoilage microorganisms that produce extracellular enzymes that reduce the quality of milk, produce undesirable aromas and organic acids [2]. These microbial pathogens are causing many foodborne diseases because of the ingestion of contaminated foods**.** Thus, to reduce the microbial growth rates in raw milk and dairy products, several preservation methods have been developed for extending the shelf life of these products. These methods sometimes have undesired effects on the nutritional and organoleptic properties of foods [3]. Reducing the microbial load and controlling microbial growth improves the quality and safety of dairy products, increased storage time of raw milk and prolonged shelf life of pasteurized milk**,** bring economic benefits to the dairy industry by virtue of increased flexibility in milk utilization and distribution [4]. Moulds and yeasts are one of the main causes of microbiological contamination and destroy a large number of foods every year [5].

The global challenge of the 21st century is to use cold pasteurization of carbon dioxide CO_2 (dry ice) as antimicrobial agent in the food and dairy industries [6]. The use of carbon dioxide (CO_2) has several advantages in the inactivation of microorganisms in foods, such as low cost, ease of removal from the product after use, and is generally recognized as safe, non-toxic, inflammable and environmentally friendly [7, 8]. Dry ice is a solid form of carbon dioxide and historically, has been used to maintain the quality, value, extended shelf life of fruits, vegetables, grains, and meat products. Furthermore, carbon dioxide was used in modified atmosphere packaging systems for meat and poultry foods, as well as dairy products like ice cream, cheese, and milk, to extend shelf life [9, 10].

Furthermore, carbon dioxide aerosols and dry ice were used to reduce pathogenic bacteria biofilms

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on surfaces [11, 12]. Antimicrobial against attached cells because it is an ultra-pure product, free from toxicity, which can be easily removed from food products [13 14, 15]. The addition of CO_2 to raw milk and dairy products controls the growth of psychrotrophic bacteria at refrigeration temperatures [9]. Dry ice is a flavourless, colourless, odourless gas and has many functions in food preservation, such as penetrating the microbial cell wall and altering the permeability, producing carbonic acid, which reduces the pH of the cell, and interfering with several enzymatic and biochemical pathways inside the microbial cells [16].

Meanwhile, carbon dioxide extends the lag phase of spoilage microorganisms and has possibly both inhibitory and stimulatory effects on bacteria depending on factors such as the type of product and microorganism [17]. Many authors have shown that the use of CO_2 can achieve viral and bacterial inactivation [18]. Carbon dioxide can kill microorganisms by damaging the cell membrane, causing changes in cell metabolism, denaturation of DNA and lowering the pH of the cytoplasm [6, 19]. Numerous studies have explored the bactericidal effect of CO_2 on microbial growth of *Pseudomonas*, *Acenatobacter* and *Moraxella* [20]. Also, ingeniously created a micro bubble of CO_2 or dry ice for water disinfection and reported that these systems killed the faecal coliforms [21, 22].

Faecal coliforms, such as *E. coli* and bacteriophageT4, bacteriophages MS2 and Qβ can be effectively inactivated by dissolved CO_2 at 0.3–0.6 MPa within 20–30 min [18]. The other advantage of the use of CO_2 is to retard the oxidative rancidity in milk and milk products [23]. The direct addition of dissolved carbon dioxide to dairy and other products has been commercially successful and economically feasible and extended shelf life from 200% to 400% [24]. Therefore, the main goal of this study was planned to study the antimicrobial effects of dissolved carbon dioxide in milk and whey medium against some pathogenic and spoilage microorganisms that cause defects in food and dairy products.

2. Materials and Methods

2.1. Materials

The dry ice was purchased from DIFFCO2 Company in a closed foam box to prevent it from sublimating during transport. All the microbial media used in this study was obtained from Oxide.

2.1.1. Microbial sources

The tested pathogenic strains used in the present were originated from: *Bacillus cereus* B-3711 and *Aspergillus flavus* B-3357 were provided by the Northern Regional Research Lab., (NRRL) Illinois, USA. *Salmonella typhimurium* 14028 was obtained from dairy microbiological Lab., National Research Centre. *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* 0157:H7 ATCC 8739 and *Candida albicans* ATTC 10231 were provided by Northern Regional Research Laboratory, Illinois, USA.

2. 2. Methods

2.2.1. Growth conditions

Five pathogenic strains such as *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Staphylococcus aureus* were firstly activated overnight twice in nutrient broth at 37° C for 24 h to achieve the intended cell concentration in the resulting suspensions was generally about 10^8 cfu/ml. In addition, three yeast and fungal strains were used in the experiments, *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* were activated on potato dextrose agar slants at 25°C for 3-5 days. Then, spores were harvested by adding 10 ml of sterile distilled water to get the final concentration to 10^{12} spores/ml [25].

2.2.2. The antagonistic effect of dry ice (co_2) against pathogenic strains activity

To determine the antimicrobial effect of carbon dioxide (co_2) in milk or whey, the milk or whey was prepared into one-liter conical flasks (2000 ml) and sterilized at 120°C for 15 min. The experiment was divided into two portions (treated and non-treated by dry ice). Dry ice was dissolved into the conical flasks at a rate of 1.5 % (by weight) until saturation and the temperature dropped to 5°C.

The pressure rose gradually with the extension of the sublimation of the dry ice in the closed flasks, and eventually reached a stable point when the dry ice was completely exhausted into the milk or whey. After saturated milk or whey with carbon dioxide, it was distributed into sterilized bottles (50 ml). On the other hand, the control bottles without carbon dioxide were distributed into sterilized bottles (50 ml), and then all treated and non-treated bottles were inoculated with separately examined pathogenic strains (1 flask treatment and the other kept as a control). All inoculated bottles were divided into two groups, the first group was incubated aerobically at 37°C for 24 h for bacterial strains and at 25°C for 5 for fungi. The second group was stored in the refrigerator at 7°C for 2 weeks (Fig. 1 A&B).

2.2.3. Enumeration of pathogenic bacteria

All pathogenic strains under study were enumerated by using standard plate count agar medium (Oxoid) according to [26]. The plates were incubated at 32 ± 2° C for 48 h.

2.2.4. Enumeration of fungi and yeast

Fungi and yeast counts in all samples were determined using acidified potato dextrose agar medium (Oxoid) according to the method described by [27]. The plates were incubated at 25° C for 3-5 days.

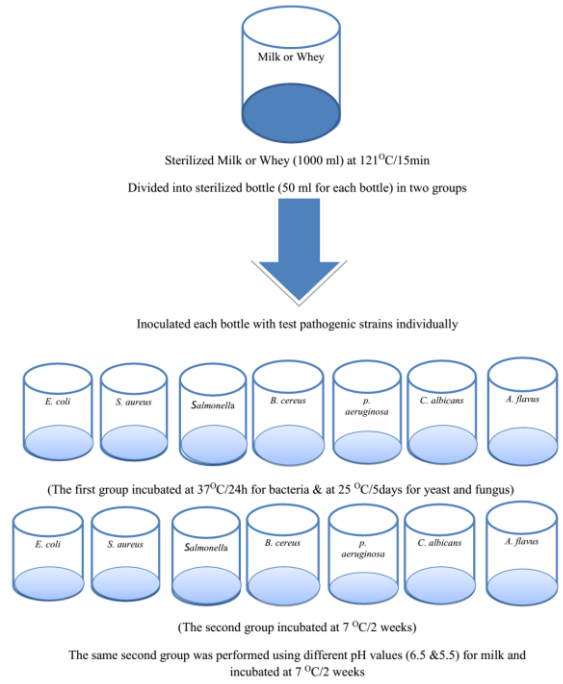

Fig. 1A Schematic Diagram for control samples

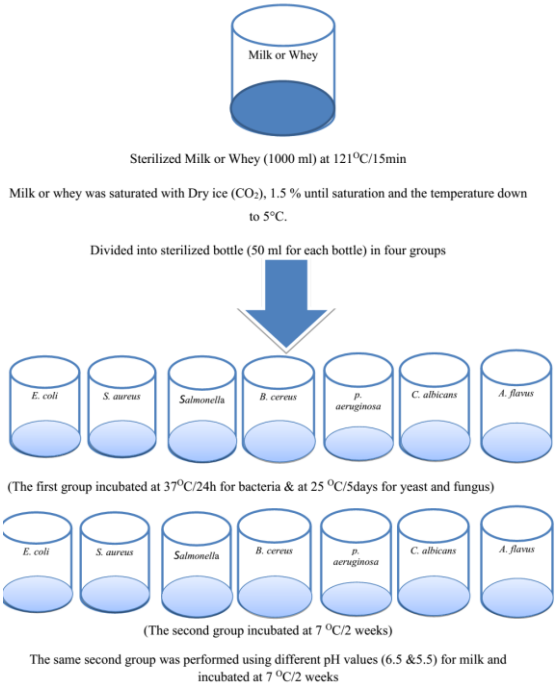

Fig. 1B Schematic Diagram for treated samples

3. Results and discussion

3.1. The effect of dissolved carbon dioxide on growth of microbial strains after 24h of incubation in milk

The effects of dissolved carbon dioxide in the growth of different microorganisms in sterilized milk after 24 h are presented in Table 1. From the obtained results, it was noticed that the effects of carbon dioxide on the tested microbes were varied according to the type of strain. The log reductions were 2.0, 1.3, 0.5 and 0.2 with *Staphylococcus aureus*, *Candida albicans*, *Bacillus cereus* and *Aspergillus flavus* respectively. On the other hand, the addition of CO_2 to the milk didn't have effect on the *Salmonella typhimurium* followed by *Pseudomonas aeruginosa* then *Escherichia coli.* Where, the count of these microbes has remained in the same log counts as control after incubation. The results were in agreement with those reported by [28]. They mentioned that, the addition of CO_2 was suppressed the growth of bacteria in both raw and pasteurized milk at refrigeration temperatures. In addition, from incubation at 24h only, our data noticed that the strains *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli* were little affected by the CO2 concentration used in this experiment

3.2. The effect of dissolved carbon dioxide on growth of microbial strains after two weeks of incubation in cold storage using milk

The effects of dissolved carbon dioxide on the growth of different microorganisms in sterilized milk during refrigeration storage at 7° C are shown in Table 2. After one week of cooling storage, *Escherichia coli* and *Aspergillus flavus* were not affected by carbon dioxide addition and log reduction was zero (not detected according to the microbial log counts after incubation) but the count of *Bacillus cereus* was recorded 3.9 and 4.00 CFU/ml in control and treatment, respectively. However, the CO_2 addition with cold storage did not have an influence on the viability of *Escherichia coli*, *Bacillus cereus* and *Aspergillus flavus*. Conversely, with extending the cooling storage to 2 weeks, all tested strains were affected except *Bacillus cereus*. The log reductions were increased and reached 2.9, 2.48 and 2.0 with the strains *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. This means the cooling temperature enhanced the ability of $CO_2$for inhibiting the growth of tested strains. Our obtained results are in the harmony with findings by [29] they found that, the use of CO_2 concentration significantly inhibited the different bacterial populations of *Pseudomonas fluorescens*, *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Enterococcus faecalis* in raw milk.

Table 1 Effect of CO_2 (dry ice) on tested pathogenic strains in milk medium after 24h incubation at 25 and 37° C.

Tested Strains	Log count CFU/ml		
	control	Treated	Log reduction

Escherichia coli	7.6	7.62	-0.02
Staphylococcus aureus	8.9	6.9	2.0
Salmonella typhimurium	7.1	8.3	-0.8
Bacillus cereus	7.1	6.6	0.5
Pseudomonas aeruginosa	7.4	7.3	-0.1
Candida albicans	6.9	5.6	1.3
Aspergillus flavus	8.6	8.4	0.2

Log reduction = Log counts of control - Log counts treated with Co_2
Control without CO_2; Treatment with Co_2; Saturation ~ 1.5%

Table 2 Effect of CO_2 (dry ice) on tested pathogenic strains in milk during refrigeration storage at 7° C.

Tested Strains	Time of storage					
	1 week			2 weeks		
	Log CFU/ml					
	control	Treated	Log reduction	control	Treated	Log reduction
Escherichia coli	6.5	6.5	0.0	5.48	5.38	0.1
Staphylococcus aureus	4.6	2.6	2.0	2.48	00	2.48
Salmonella typhimurium	5.08	5.0	0.08	5.47	5.3	0.17
Bacillus cereus	3.9	4	-0.1	4.47	4.5	-0.03
Pseudomonas aeruginosa	6.38	5.17	1.21	6.47	5.46	2.0
Candida albicans	5.6	4.6	1.0	2.9	0.0	**2.9**
Aspergillus flavus	0.0	0.0	0.0	8.9	7.8	**1.1**

Log reduction = Log counts of control - Log counts treated with Co_2
Control without CO_2; Treatment with Co_2; Saturation ~ 1.5%.

3.3. The effect of dissolved carbon dioxide on growth of microbial strains after 24h of incubation in whey

The effects of dissolved carbon dioxide in the growth of different microorganisms in sterilized whey after 24 h at 37° C were presented in Table 3**.** The log reduction of the tested strains was 0.4, 0.1, 0.3, 0.1, and 0.1 with strains like *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Candida albicans*, respectively. The data **i**ndicated a small effect rate of using CO_2 with whey to suppress the pathogen counts after incubation. The data was indicated small effect rate of using CO_2 with whey to suppressed the pathogens counts after incubation. Only *Aspergillus flavus* was little improved and reached 8.3and 8.68 log CFU/ml for control and treated samples, respectively. [20] discovered that the use of CO_2 led to inhibition of activity against Gram-negative and Gram-positive bacteria.

3.4. The effect of dissolved carbon dioxide on growth of microbial strains after two weeks of incubation in cold storage using whey

The antimicrobial effects of dissolved carbon dioxide against different microorganisms in whey during refrigeration storage at 7° C are showed in Table 4. After one week of cooling storage, the results were found that the only affected strains were *Staphylococcus aureus* and *Salmonella typhimurium* with little log reduction of 0.1 and 0.24, respectively. However, the others tested strains didn`t affect, where the counts of these strains (*Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus flavus*) were found in the same log cycles or little improved than control. In addition, the microbial counts under dissolved carbon dioxide effect in whey after 2 weeks were nearly the same and both *Bacillus cereus* and *Pseudomonas aeruginosa* were the only affected strains with prolonging the cooled storage and the log reduction was 0.14 and 0.2, respectively. The carbon dioxide effect was higher against tested microorganisms in milk than whey. The inhibition effect caused by the addition of CO_2 appears to be the acidification ability of the medium and has a direct effect on the cell metabolism of microorganisms and acidification [30].

3.5. The effect of dissolved carbon dioxide on growth of microbial strains after incubation in cold storage using different milk pH values

The effect of dissolved carbon dioxide against different strains in different milk pH values after one week of refrigeration storage was illustrated in Table 5. The results showed that, the log reduction in milk media with pH 5.5 were 0.36, 0.1 and 0.03 with the tested strains of *Staphylococcus aureus*, *Salmonella typhimurium* and *Bacillus cereus*, respectively. In addition, in milk media with pH 6.6 the log reduction were 2.0, 1.3, 0.5 and 0.2 with strains *Staphylococcus aureus*, *Bacillus cereus* B-311, *Candida albicans* and *Aspergillus flavus* respectively. So, the data found that pH 6.6 was more effective than pH 5.5 at

reducing the pathogen counts during cold storage for one week.

Also, the results in Table 5 indicated that, the effects of dissolved carbon dioxide against microorganisms tested in different milk pH values after two weeks of refrigeration storage time. The highest log reduction was recorded in milk with pH 5.5 against *Staphylococcus aureus* (3.0). Also, the counts of treated strains in milk with pH 5.5 were near to the microbial counts to control. Nevertheless, in the milk with pH 6.6, the more log reduction was 2.9 against *Candida albicans*, followed by *Staphylococcus aureus* (2.48), *Pseudomonas aeruginosa (*2.00), against *Aspergillus flavus* (1.10), *Salmonella typhimurium* (0.17) and *Escherichia coli* (0.1). The data showed that, the log reduction against pathogenic strains was higher at pH 6.6 after 2 weeks of refrigeration storage at 7º C.

Table 3 Effect of CO_2 (dry ice) on tested pathogenic strains in whey after incubation at 25 and 37º C.

Tested Strains	Log count CFU/ml		Log reduction
	control	Treated	
Escherichia coli	9.3	8.9	0.4
Staphylococcus aureus	9.3	9.2	0.1
Salmonella typhimurium	8.9	8.8	0.1
Bacillus cereus	9.2	8.9	0.3
Pseudomonas aeruginosa	9.3	9.2	0.1
Candida albicans	8.8	8.7	0.1
Aspergillus flavus	8.3	8.68	-0.38

Log reduction = Log counts of control - Log counts treated with Co_2

Control without CO_2; Treatment with Co_2; Saturation ~ 1.5%

Table 4 Effect of CO_2 (dry ice) on tested pathogenic strains during refrigeration storage at 7º C in whey.

Tested Strains	Time of storage					
	1 week			2 weeks		
	Log CFU/ml					
	Control	Treated	Log reduction	Control	Treated	Log reduction
Escherichia coli	8.9	9.0	-0.1	8.89	9.03	-0.17
Staphylococcus aureus	9.1	9.0	0.1	8.36	8.78	-0.42
Salmonella typhimurium	9.24	9.0	0.24	8.4	8.4	00
Bacillus cereus	9.0	9.2	-0.2	8.41	8.27	0.14
Pseudomonas aeruginosa	8.9	9.2	-0.3	8.7	8.5	0.2
Candida albicans	8.2	8.3	-0.1	8.3	8.7	-0.4
Aspergillus flavus	8.47	8.47	00	8.52	8.84	-0.32

Log reduction = Log counts of control - Log counts treated with Co_2

Control without CO_2; Treatment with Co_2; Saturation ~ 1.5%

Table 5 Effect of CO_2 (dry ice) on tested pathogenic strains during refrigeration storage at 7º C and different milk pH values during storage.

	Log CFU /ml after 1week					
	pH 5.5			pH 6.6		
Tested Strains	Control	Treated	Log reduction	Control	Treated	Log reduction
Escherichia coli	6.29	6.4	-0.11	7.6	7.62	0.02
Staphylococcus aureus	5.43	4.7	0.36	8.9	6.9	2.0
Salmonella typhimurium	5	5.1	0.1	7.1	8.3	-0.8
Bacillus cereus	6.32	6.29	0.03	7.1	6.6	0.5
Pseudomonas aeruginosa	3.84	3.96	-0.12	7.4	7.3	0.1
Candida albicans	3.69	3.84	-0.15	6.9	5.6	1.3
Aspergillus flavus	8.06	8.54	-0.48	8.6	8.4	0.2
Log CFU /ml after 2 week						
	Control	Treated	Log reduction	Control	Treated	Log reduction
Escherichia coli	5.2	5.52	-0.32	5.48	5.38	0.1
Staphylococcus aureus	3.0	0	3.0	2.48	00	2.48
Salmonella typhimurium	6.04	6.04	0	5.47	5.3	0.17

Bacillus cereus	5.48	5.34	0.14	4.47	4.5	-0.03
Pseudomonas aeruginosa	3.6	3.95	-0.35	6.47	5.46	2.0
Candida albicans	00	00	00	2.9	00	2.9
Aspergillus flavus	8.9	8.8	0.1	8.9	7.8	1.1

Log reduction = Log counts of control - Log counts of treated with Co_2
Control without CO_2; Treatment with Co_2; Saturation ~ 1.5%

Finally, carbon dioxide extends the lag phase of spoilage microorganisms and has possibly both inhibitory and stimulatory effects on bacteria depending on factors such as the type of product and microorganism [17]. In addition, [11] and [12] found that carbon dioxide aerosols and dry ice could be used to reduce biofilms of pathogenic bacteria in surfaces.

Conclusion

The effects of dissolved carbon dioxide (Dry ice) on the growth of some pathogenic bacteria and spoilage fungi were strain independent. In addition, the effect of dissolved carbon dioxide was increased on milk than whey when inoculated with tested pathogenic strains. So, the use of dissolved carbon dioxide may be effective for maintaining milk from contamination during handling.

Conflict of Interest

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

There is no Informed consent.

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