Egyptian Journal of Animal Health

P-ISSN: 2735-4938 On Line-ISSN: 2735-4946
Journal homepage: https://ejah.journals.ekb.eg/

Control of sources of milk contamination with *Cronobacter sakazakii* using some sanitizers

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Received in 14/1/2025
Received in revised from 17/1/2025
Accepted in 9/2/2025

Keywords:

Cronobacter sakazakii, control
milk contamination
sanitizers

ABSTRACT

The current investigation directed to assess the effectiveness of commercial disinfectants (disinfectant I, sodium hypochlorite 400 ppm and disinfectant II, quaternary ammonium compound (QAC) 3 %) at inactivating field *C. sakazakii* strains previously isolated from diarrheic stool (A) and fresh cow milk (B) in watery suspension (planktonic cells) and that dried after being injected on stainless steel coupons. The results proved that, both disinfectants I and II had inhibitive effect on tested *C. sakazakii* strains in watery suspension and that inoculated and dried on stainless steel coupons with significant reductions ($P \leq 0.05$) in populations compared to the number of cells recovered from control and QAC was more potent than disinfectant I on tested *C. sakazakii* strains; the strain A was more resistant to these disinfectants than strain B and the longer the treatment time the more effectiveness of disinfectants. Therefore, it was concluded that; the routinely pre-rinsing ,cleaning and sanitization of equipment in dairy farms, milk collector centers and dairy processing plants with QAC 3 % disinfectant is the most crucial stage in managing the route of infection, spreading and contamination with *C. sakazakii* pathogens to avoid their public health hazards.

INTRODUCTION

Cronobacter sakazakii (*C. sakazakii*) is a Gram-negative, rod-shaped bacterium that is facultatively anaerobic, motile, and non-spore formers, possessing peritrichous flagella. It is a member of *Enterobacter* genus belonging to *Enterobacteriaceae* family **(Al-Aawadi and Weda, 2020).** This pathogen has been implicated in severe foodborne illnesses, demonstrating an ability to get past host defenses and avoid the host's immunological reaction. This feature allows it to inflict severe diseases like severe infant meningitis, necrotizing colitis, and septicemia capable of causing posterior fatality between 40 and 80 percent. What is more, it is related to a range of aspiration pneumonia, urinary tract infections, abscesses, wounds, diarrhea, and conjunctivitis in adults **(Shi et al. 2017; Kadlicekova et al. 2018; Henry and Fouladkhah, 2019; Abebe, 2020; Chauhan et al. 2022).**

C. sakazakii is considered A foodborne pathogen that is opportunistic has been isolated

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DOI: 10.21608/ejah.2025.442525

from diverse environmental sources. These include soil, water, various foods (such as fresh raw milk, powdered infant formula, milk powder, cheese products, dry cereals, and different vegetables), and food industry factory facilities **(Shaker et al. 2007; Berhilevych and Kasianchuk, 2017; Abebe, 2020; Csorba et al. 2022; Mohamed, Marwa et al. 2023).** The gastrointestinal tracts of humans, animals, insects, and rodents serve as natural harborage sites for the *Cronobacter* genus. Fecal-carried *C. sakazakii* can survive in soil and water for up to 120 days **(Molloy et al. 2008; Molloy et al. 2009).** Consequently, contamination of raw materials and finished products can occur via water, dust, and other environmental objects, particularly from stainless steel surfaces in dairy farms and processing plants, leading to contamination of milk and its products **(Bergilevich et al. 2013; Berhilevych and Kasianchuk, 2017; Lindsay et al. 2019; Chon et al. 2021; Mohamed, Marwa et al. 2023).**

Moreover, *C. sakazakii* exhibits numerous adaptations that contribute to its survival and persistence. These include its polysaccharide capsule, resistance to drying out, and the production of pigment "yellow carotenoid" that offers protection from radicals. Its ability to form **biofilms** allows the pathogen to adhere to stainless steel equipment surfaces and various packaging materials. Additionally, it tolerates a broad range of growth temperatures, ionic strength, and dryness. These adaptive mechanisms collectively protect the pathogen, leading to increased resistance to disinfectants and environmental stresses, thereby elevating the risk of contamination **(Singh et al. 2015; Brandão et al. 2017; Holý et al. 2019; Ling et al. 2020).**

Despite the chance that *C. sakazakii* has long been detected, routine sanitization methods in the dairy sector may not be effective to sterilize because they are highly resistant to adverse stress conditions, like high acid, high temperature, and low water activity (**Ueda, 2017; Chen et al. 2019; Henry and Fouladkhah, 2019; Lin et al. 2023**). The mostly adopted sanitizing strategy in dairy farms and dairy industry were sodium hypochlorite, quaternary ammonium chloride (QAC), and hot water sanitizing because they had exceptional advantages (e.g., high effectiveness, easy application, and low cost) (**Kim et al. 2007; Beuchat et al. 2009; Chon et al. 2021; Panebianco et al. 2022; Tan et al. 2022 and Lin et al. 2023).** Nonetheless, research concerning the efficacy of the disinfectants against *C. sakazakii* is still underway across the world to date; there is a limited publication on the resistance of *C. sakazakii* to commercial disinfectants available in Egypt.

Therefore, the objective aim of this study was to investigate the impact of two common disinfectants approaches (sodium hypochlorite and quaternary ammonium compound (QAC), utilized in the dairy industry on *C. sakazakii* strains isolated from the clinical sample or from milk sample to clean, disinfect and sanitize the equipment used in dairy farms , milk collector centers and dairy processing plants.

MATERIALS AND METHODS

1.Preparations of *C. sakazakii* strains according to Chon *et al.* (2021)

Field *C. sakazakii* strains used in this study were previously isolated and identified phenotypically and genotypically by the authors from diarrheic stool (A) and fresh cow milk (B). Strains were inoculated individually into Tryptone soya broth (TSB) (High Media, India) and placed at 37 °C for 24 hours. Then a loopful of each incubated TSB was plated on Tryptone soya agar (TSA) (High Media, India) and placed at 25°C for (48-72 h.), four to five grown yellow produced colony from each *C. sakazakii* strain were cultured into tube of TSB and placed at 37°C for 24 hours. From this culture, serial dilutions in 0.1% peptone water up to 10^{-10} were overlaid on Tryptone soya agar (TSA) to determine the cell concentration. The cell count was adjusted to 10^{7}cfu/ml for *C. sakazakii* with tube dilution methods.

2. Preparation of disinfectants used

Two disinfectants were used at concentrations recommended by the manufacturers for detection of their efficacy in killing *C. sakazakii*. Disinfectant (I) sodium hypochlorite was adjusted to obtain 400 ppm concentration

by using distilled water, and disinfectant (II) Quaternary ammonium compound (QAC_s) was adjusted to obtain 3%concentration by distilled water.

3. Preparation of stainless steel coupons for spot inoculation and biofilm studies.

Stainless steel coupons (5 cm by 2 cm) were used after thoroughly washed by food grade soap and rinsed with sterile distilled water then sterilized by autoclaving.

4. Efficacy of disinfectants in killing planktonic *C. sakazakii*

Ten milliliters of each previously prepared *C. sakazakii* suspension was deposited in three sterile 25- by 150-mm test tubes; the first contain 10 ml of sterile distilled water (as control),the second contain10 ml of sodium hypochlorite 400 ppm and the third contain 10 ml of QAC 3%and mixed thoroughly. At zero time (within 10 seconds)and after treatment for 1, 5, and 10 minutes,1ml of each test tube serially diluted in 0.1% peptone water , then 0.1 ml of each test tube was surface plated on Tryptic soy agar and incubated at 37ºc for 48 hrs., then colonies were counted (**Chon et al. 2021**).

5. Efficacy of disinfectants in killing *C. sakazakii* spot inoculated and dried on stainless steel

Three sterile stainless steel coupons were placed on a wire screen elevated 7 cm above the work surface in a laminar-flow biosafety cabinet. 100µl of each previously prepared *C. sakazakii* suspension was deposited on each coupon. The inoculums were dried for 20 hrs. at 22±2 ºc in laminar-flow biosafety cabinet. The inoculated coupons were immersed in three sterile 25- by 150-mm test tubes; the first contain 25 ml of sterile distilled water (as control), the second 25 ml of sodium hypochlorite 400 ppm and the third contain 25 ml of QAC 3% and mixed thoroughly. After treatment for 0 min (within 10 s after immersing coupons in sterile water) and after treatment for 1, 5, and 10 min. in water or disinfectant solutions, three grams of sterile glass beads were added to each test tubes, then they were vortexes for one min. and1ml of each test tube serially diluted in 0.1% peptone water, then 0.1 ml of each test tube was surface plated on Tryptic soy agar and incubated at 37ºc for 48 hrs., then colonies were counted (**Kim et al. 2007**).

6. Statistical analysis

Three replicate experiments were performed for each *C. sakazakii* strainstudied (A and B), and all data were analyzed using Statistical Program. Duncan's multiple range tests was used to separate means using a level of significance of $p \leq 0.05$ using the computer software program **(SPSS, ver 20).**

RESULTS

1. Efficacy of disinfectants in killing planktonic *C. sakazakii*

The mean *C. sakazakii* count of diarrheic stool (A) and fresh cow milk (B) (Table, 1) suspended in water of control treated with disinfectants (control), did not change significantly $P<0.05$ within 10 min. Treatment of both strains suspended in water containing disinfectants for 10 min resulted in significant reductions ($P \leq 0.05$) in populations compared to the number of cells recovered from control. For disinfectant (I) sodium hypochlorite 400 ppm, the mean *C. sakazakii* strains (A) count was markedly declined ($P \leq 0.05$) from 7.71 ± 0.08 at zero time to7.60 ±0.01, 7.49±0.02 and 6.77 ± 0.09 log CFU/ml ±SD with reduction percentage of 22.08 %, 40.27 %,and 88.64% after 1st , 5th and 10th min of treatment, respectively. Meanwhile, the mean *C. sakazakii* strains (B) count was markedly declined ($P \leq 0.05$) from 7.74 ± 0.08 at zero time to 7.60 ± 0.01, 7.43 ± 0.02 and 6.54 ± 0.08 log CFU/ml ± SD with reduction percentage of 26.39 % ; 50.92 % and 93.56% after 1st, 5th and 10 th min of treatment, respectively. In case of using disinfectant (II) QAC 3 % the mean counts of strain (A) was decreased significantly ($P \leq 0.05$) from 7.71 ± 0.08 at zero time to 7.54 ±0.01 and 6.95 ± 0.10 CFU/ml ± SD with reduction percentage of 31.80% and 82.65 % after 1st and 5th min of treatment, respectively, but not detected at 10th min of treatment. Also, the mean *C. sakazakii* strains (B) count was decreased significantly ($P \leq 0.05$) from 7.74 ± 0.08 at zero time to 7.41 ± 0.03 and 6.50 ± 0.09 log CFU/ml ± SD with reduction percent-

age of 52.75% ; and 94.17% after 1st and 5 thmin of treatment, respectively, but not detected at 10th min of treatment(Fig.,1).

2. Efficacy of disinfectants in killing *C. sakazakii* spot inoculated and dried on stainless steel surface

The efficacy of disinfectants I and II on ***C. sakazakii*** strains A, B inoculated and dried on stainless steel coupons (Table, 2) revealed that, the mean *C. sakazakii* strains A and B counts extracted from the surface of stainless steel coupons treated with disinfectant (I) sodium hypochlorite 400 ppm were reduced significantly ($P \leq 0.05$) from 7.62 ± 0.05 at zero time to 7.46± 0.05; 7.32 ± 0.01;6.61 ± 0.011 og CFU/coupon ± SD with reduction percentage of 31.20%; 49.54%; 90.14% for strain A after 1st , 5th and 10 th min of treatment, respectively and from 7.37 ± 0.01 at zero time to7.44 ± 0.06; 7.22 ± 0.02; 6.33 ± 0.03 log CFU/coupon ± SD with reduction percentage of 40.89% ; 64.30% and 95.41% for strain B after 1st ,5th and 10th min of treatment, respectively. For disinfectant (II) QAC 3 %were decreased significantly ($P \leq 0.05$) from7.62 ± 0.05 at zero time to 7.38 ± 0.03 ;6.64± 0.09 log CFU/ coupon ±SD with reduction percentage of 42.22% ; 89.49% for strain A after 1stand 5th-min of treatment, respectively but not detected at 10th min of treatment, and from 7.37 ± 0.01 at zero time to 7.28 ± 0.01; 6.42 ± 0.07 log CFU/coupon ±SD with reduction percentage of 59.11% ; 94.30% for strain B after 1st and 5th min of treatment, respectively but not detected at 10 th min of treatment(Fig., 2).

Table 1. Efficacy of disinfectants in controlling planktonic *C. sakazakii* (log CFU/ml ±SD)

Disinfectant	Strain	Zero time	1 min.	R1%	5 min.	R5%	10 min	R10%
Control	A	7.71 ± 0.08^{aD}	7.72 ± 0.06^{aC}	-	7.74 ± 0.08^{aB}	-	7.75 ± 0.08^{aA}	-
	B	7.74 ± 0.08^{aC}	7.75 ± 0.08^{aB}	-	7.75 ± 0.08^{aA}	-	7.76 ± 0.01^{aA}	-
T1	A	7.71 ± 0.08^{aA}	7.60 ± 0.01^{bB}	22.08	7.49 ± 0.02^{bC}	40.27	6.77 ± 0.09^{b}	88.64
	B	7.74 ± 0.08^{aA}	7.60 ± 0.01^{bB}	26.39	7.43 ± 0.02^{bC}	50.92	6.54 ± 0.08^{b}	93.56
T2	A	7.71 ± 0.08^{aA}	7.54 ± 0.01^{cB}	31.80	6.95 ± 0.10^{cC}	82.65	ND	>99.99
	B	7.74 ± 0.08^{aA}	7.41 ± 0.03^{cB}	52.75	6.50 ± 0.09^{cC}	94.17	ND	>99.99

abc Different superscript letters within the same **column**, the same time of treatment, and the same strain means significant difference ($P \leq 0.05$).
ABCD Different superscript letters within the same **row**, the same time of treatment, and the same strain means significant difference ($P \leq 0.05$)

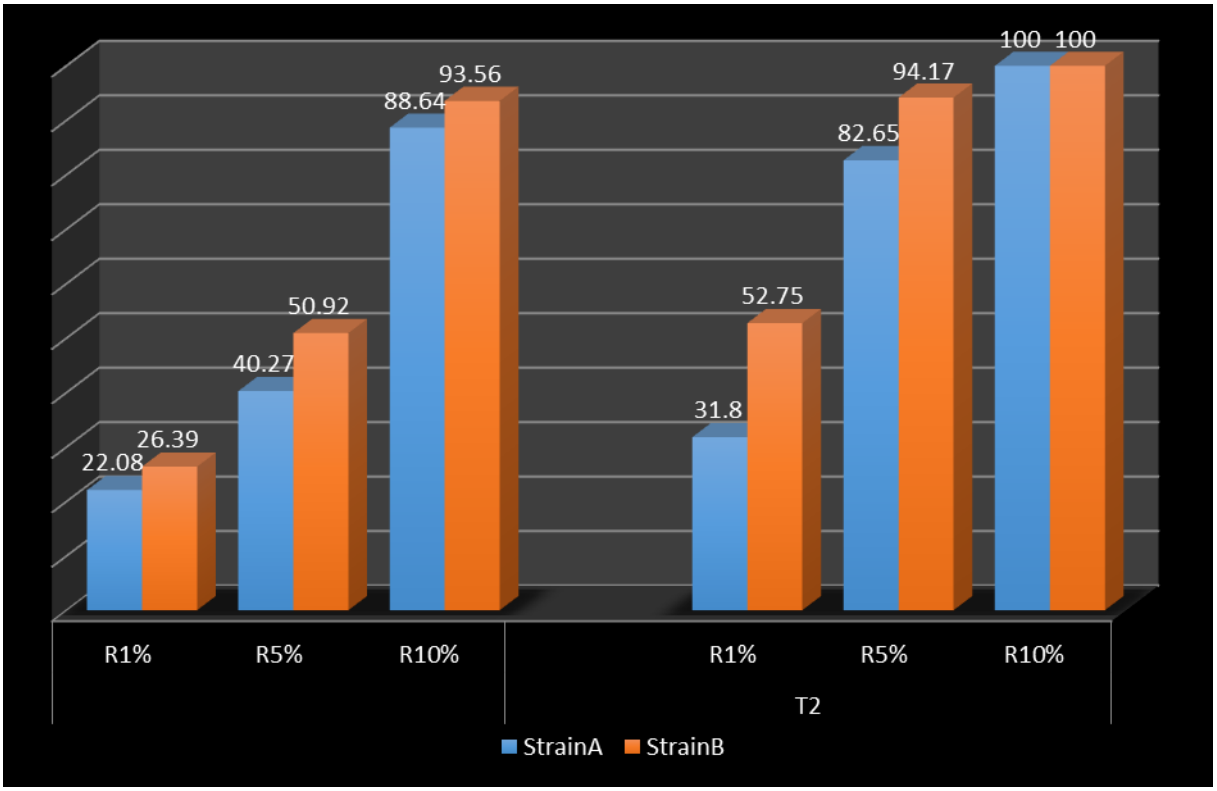

Fig. (1):Reduction percentage of planktonic *C. sakazakii* strains after treatment with disinfectant (I) sodium hypochlorite 400 ppm(T1) and disinfectant (II) QAC 3 % (T2)

Table 2. Efficacy of disinfectants in killing *C. sakazakii* spot inoculated and dried on stainless steel (log CFU/coupon ± SD)

Disinfect-ant	Strain	Zero time	1 min.	R1%	5 min.	R5%	10 min	R10%
Control	A	7.62 ± 0.05^{aA}	7.35 ± 0.01^{aC}	-	7.37 ± 0.01^{aC}	-	7.42 ± 0.01^{aB}	-
	B	7.37 ± 0.01^{aB}	7.40 ± 0.05^{aB}	-	7.44 ± 0.07^{aB}	-	7.53 ± 0.09^{aA}	-
T1	A	7.62 ± 0.05^{aA}	7.46 ± 0.05^{bB}	31.20	7.32 ± 0.01^{bC}	49.54	6.61 ± 0.01^{bD}	90.14
	B	7.37 ± 0.01^{aB}	7.44 ± 0.06^{aA}	40.89	7.22 ± 0.02^{bC}	64.30	6.33 ± 0.03^{bD}	95.41
T2	A	7.62 ± 0.05^{aA}	7.38 ± 0.03^{cB}	42.22	6.64 ± 0.09^{cC}	89.49	ND	>99.9
	B	7.37 ± 0.01^{aA}	7.28 ± 0.01^{bB}	59.11	6.42 ± 0.07^{cC}	94.30	ND	>99.9

abc Different superscript letters within the same column, the same time of treatment, and the same strain means significant difference ($P \leq 0.05$)

^{ABCD} Different superscript letters within the same **row**, the same time of treatment, and the same strain means significant difference ($P \leq 0.05$)

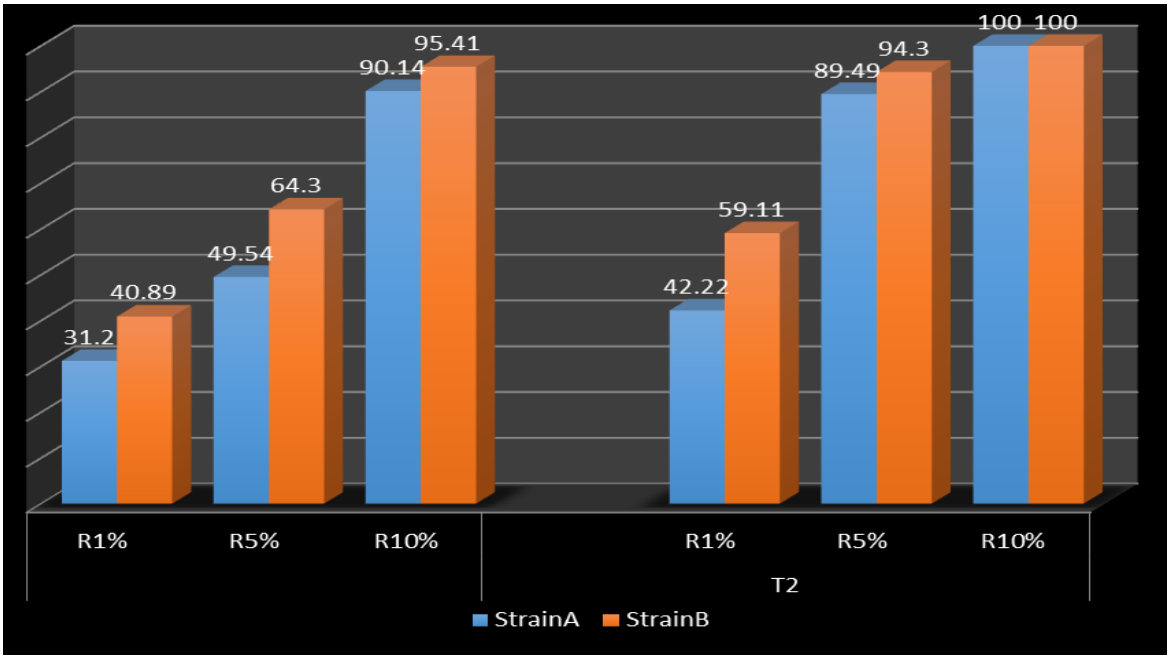

Fig. (2): Reduction percentage of *C. sakazakii* strains spot inoculated and dried on stainless steel after treatment with disinfectant (I) sodium hypochlorite 400 ppm(T1) and disinfectant (II) QAC 3 % (T2)

DISCUSSION

Cronobacter sakazakii has been recognized as a new multi-drug resistant (MDR) foodborne opportunistic pathogen **(Ling et al. 2020)** that has been very difficult control in dairy farms, milk collecting centers and milk product industries due to its ability to grow under high temperature or refrigeration conditions **(Kim et al. 2006; Beuchat et al. 2009 and Selim et al. 2020).** So, the present study tried to evaluate the efficacy of two common disinfectants (sodium hypochlorite and quaternary ammonium compounds) on *C. sakazakii* strains isolated from the fecal and milk samples to clean, disinfect and sanitize the equipment used in dairy farms, milk collector centers and dairy processing plants.

The obtained results in this study (Table 1 and Fig. 2) cleared that, both sodium hypochlorite 400 ppm and QAC 3 % disinfectants had inhibitive effect on tested planktonic *C. sakazakii* strains of diarrheic stool (A) and fresh cow milk (B) with significant reductions ($P \leq 0.05$) in their counts with reduction percentage of 22.08 %,26.39 % ; 40.27 %, 50.92 % ; 88.64% and 93.56% after 1st ,5th and 10th min of treatment with sodium hypochlorite, respectively, on the other hand 31.80 %, 52.75% ; 82.65 %, 94.17% after 1st and 5th min of treatment with QAC 3 % , respectively, but not detected (< 1 log cfu/ml) at 10th min of treatment. These results came in accordance with **Kim et al. (2007); Ha and Ha (2011); Chon et al. (2021)**and **Lin et al. (2023).**

Concerning to the efficacy of disinfectants I and II on *C. sakazakii* strains A, B inoculated and dried on stainless steel coupons, as once equipment used in dairy farms, milk collector centers and dairy plants become contaminated with*C. sakazakii*, it is very difficult to eliminate because of its adhesion to surfaces and strong resistance to desiccation; certain capsulated *C. sakazakii* strains have been brought back to life after 2.5-years in a dry environment (**Barron and Forsythe, 2007**; **Berhilevych and Kasianchuk, 2017; Henry and Fouladkhah, 2019**). Consequently, this study simulated the adhesion of *C. sakazakii* strains to the surface of stainless steel, resulting in markedly decrease ($P \leq 0.05$) of mean *C. sakazakii* strains A and B counts recovered from the surface of stainless steel coupons treated with disinfectant (I) sodium hypochlorite 400 ppm from 7.62 ± 0.05, 7.37 ± 0.01 at zero time to 6.61 ± 0.01, 6.33 ± 0.03 log CFU/coupon with reduction percentage

of90.14% and 95.41% for strain A and B, after10 min of treatment, respectively. Meanwhile, for disinfectant (II) QAC 3 %were decreased significantly ($P \leq 0.05$) to 6.64 ± 0.09, 6.42 ± 0.07 log CFU/coupon with reduction percentage of 89.49% and 94.30% for strain A and B, after five min of treatment, respectively but not detected at 10 min of treatments and the results in Table (2) were also appeared that, the QAC 3 % was more effective than sodium hypochlorite 400 ppm on tested *C. sakazakii* strains .Strain A was more resistant to these disinfectants than strain B. These findings are in synchronization with those informed by **Iversen and Forsythe (2003); Kim et al. (2007); Beuchat et al. (2009) ; Bayoumi et al. (2012); Torlak et al. (2015); Ling et al. (2020).**

Moreover, the results in Tables (1&2) revealed that, strain A from diarrheic stool was more resistant to these disinfectants than strain B from milk and the longer the treatment time the more effectiveness of disinfectants, these results came in harmony with **Mosteller and Bishop (1993); Kim et al. (2007); Ha and Ha (2011); Chon et al. (2021)** who reported that, these may be due to QAC is hydrophilic , negatively charged ,easily adsorbed to *C. sakazakii* surface and then could penetrate the cell wall resulting in destroy the cytoplasmic membrane.

As *C. sakazakii* can adhere to stainless steel coupons resemble to milk collecting containers and secrete massive quantity of extracellular polymeric substances, (EPSs), aggregate together to form a thickened biofilm (**Lehner et al. 2005; Ling et al. 2018; Ling et al. 2020).** Therefore, pre-washing prior to sanitization of equipment is a very valuable process in getting rid of the attachment of microorganisms to stainless steel equipment and is capable of physically sweeping potential organic components (e.g. milk or whey deposits) off equipment surfaces, in dairy farms, milk collecting centers and milk product industries, which could reduce or eliminate the effectiveness of disinfectants and sanitizers leading to cross-contamination (**Bremer et al. 2006; Thomas and Sathian, 2014; Panebianco et al. 2022** and **Lin et al. 2023**).

CONCLUSION

Finally, the present study estimated that; both sodium hypochlorite 400 ppm and quaternary ammonium compound (QAC) 3 % disinfectants had inhibitive effect on tested *C. sakazakii* strains in watery suspension (planktonic cells) and that inoculated and dried on stainless steel coupons; the QAC was more effective than sodium hypochlorite on tested *C. sakazakii* strains, the strain A from diarrheic stool was more resistant to these disinfectants than strain B from milk and the longer the treatment time the more effectiveness of disinfectants. Therefore, it was concluded that the routinely pre-rinsing ,cleaning and sanitization of equipment in dairy farms, milk collector centers and dairy processing plants with QAC 3 % disinfectant is The most crucial phase in managing the route of infection, spreading and contamination with *C. sakazakii* pathogens to avoid their public health hazards.

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