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MICROBIAL BIOFILMS IN RELATION TO MILK QUALITY

(With One Table and 4 Figures)

By

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الشريط الميكروبي الحيوي وعلاقته بجودة اللبن

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نظافة ماكينة الحليب من العمليات الهامة التي لها الدور الأكبر في إنتاج لبن خام عالي الجودة خالي من الميكروبات الضارة ولكن لصعوبة تركيب هذه الماكينات وملحقاتها فان تطهيرها يكون في منتهى الصعوبة وبالتالي هناك بعض بقايا اللبن المحلوب وبعض البكتيريا التي لها خاصية الالتصاق بسطح ماكينة الحليب من الداخل لتكون شريط حيوي بحيث يشكل مصدر لتلوث اللبن في الحلبة القادمة. أجريت هذه الدراسة في محلب مزرعة لإنتاج اللبن الجاموسي في محافظة الإسكندرية للتوصل للطريقة المثلي للتخلص من الشريط الميكروبي وذلك باستخدام بعض المطهرات مثل الايودفور ومركب رباعي كلوريد الأمونيوم. تم أخذ ٦٤ مسحة من كاس الحلمات، الأنابيب المطاطية والإستانلس، جرة اللبن وخزان اللبن (١٦ عينة من كل مكان) لمرتين بفارق زمني أسبوع بعد استخدام برنامج المزرعة للتطهير وتكرر أخذ هذه العينات بعد استخدام الايودفور ومركب رباعي كلوريد الأمونيوم كلاً على حدى وكذلك ٣٠ عينة من اللبن من خزان اللبن (١٠ عينات بعد كل معاملة). وقد أسفرت النتائج عن اختزال العد الكلي البكتيري باستخدام الايودفور ومركب رباعي كلوريد الأمونيوم بنسبة ٨٧,١٠&٩٤,٥٥؛ ١٦,١٦ & ٢٥,٢٨؛ ٩٦,٤٩& ٩٧,٣١؛ ٨٤,٩٥& ٩١,٥٤؛ ٨٦,٤٧ & ٩٣,٢٤% في المسحات من كاس الحلمات، الأنابيب المطاطية والإستانلس، جرة اللبن، خزان اللبن واللبن على التوالي. كانت نسبة الاختزال في الميكروبات القولونية هي ٢٩,٠٣&٥٣,٣٧ ؛ ٧٩,٩٠&٨٠,٩١ ؛ ٣١,٨٧&٦٢,٨٩ ؛ ٥٥,٦٥& ٧٨,٠٣ ؛ ٦٦,١٨& ٨٠,٣٧% على التوالي. وكانت نسبة الاختزال في الميكروب العنقودي الذهبي هي ٢٣,٠٨&٢٨,٢٢ ؛ ٦٧,٢٢&٦١,٦٢ ؛ ٩٣,٠٤&٨٦,٨٣ ؛ ٨٧,٣٠&٨٤,٢٤ ؛ ٩٠,٨٥&٨٩,٤٨% على التوالي. بينما كانت نسبة الاختزال في الميكروب المكور السبحي هي ٧٢,٤٥&٩٤,٠٥؛ ٧٤,٦٧&٩٢,١٣ ؛ ٣٧,٤٤&٩٠,٨١ ؛ ٨٠,٢٥&٩٥,٩٩ ؛ ٧٥,٤٦&٧٩,٣٧% على التوالي. ومن هذه النتائج اثبت مركب رباعي كلوريد الأمونيوم أنه أكثر كفاءة من الايودفور ولكن المركبان فشلا في التخلص النهائي من الشريط الحيوي في ماكينة الحليب.

SUMMARY

The attachment of bacteria with subsequent development of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage or transmission of diseases. The present study was carried out in a buffalo dairy farm in Alexandria governorate for studying the biofilm of milking machine. A total of 192 swabs were taken from teatcups, milk jars, milk pipelines (rubber and stainless steel) and bulk tank milk (48 of each) as well as 30 bulk tank milk samples after using farm program, and after application of Iodophores and QACs. By application of Iodophores and QACs, Total bacterial counts reduced by 29.03 & 53.37; 79.90 & 80.91; 31.87 & 62.89; 55.65 & 78.03 and 66.18 & 80.37 %, respectively, While Coliforms were suppressed by 29.03 & 53.37; 79.90 & 80.91; 31.87 & 62.89; 55.65 & 78.03 and 66.18 & 80.37 %, respectively. *S.aureus* forming biofilm in teatcups, milk pipelines, milk jars and tank milk was reduced by 28.22 & 23.08; 67.62 & 61.62; 93.04 & 86.83 and 87.30 & 84.24 %., respectively. The reduction percentages for Enterococci counts of teatcups, milk pipelines, milk jars and tank milk and bulk tank milk after using Iodophores and QACs were 72.45 & 94.05; 74.67 & 92.13; 37.44 & 90.81; 80.25 & 95.99 and 75.46 & 79.37%, respectively. It could be concluded that QACs is more efficient on TBC, Coliforms and Enterococci counts, while Iodophores is more efficient on *S.aureus*. Both Iodophores and QACs are failed to overcome the microbial biofilm of milking machine.

Key words: *Biofilm, microbial attachment, milking machine.*

INTRODUCTION

Dairy animals are normally milked twice daily, and the milking machine has to be cleaned after each milking. Because of the complexity of milking machines and some of their components, cleaning and, in particular, disinfection may not be fully effective. So, milk residues and associated bacteria are not completely removed from the equipment and tend to accumulate daily. Except in very cold weather bacteria multiply in the equipment between milkings and their numbers may increase more rapidly than visible residues. Unfortunately, bacterial contamination cannot be determined simply by inspection (Bramley and Mckinnon, 1990).

The attachment of bacteria with subsequent development of biofilms in food processing environments is a potential source of

contamination that may lead to food spoilage or transmission of diseases. The surfaces of equipment used for dairy processing are recognized as major sources of microbial contamination even with acceptable Clean-In-Place (CIP) system, bacteria can remain on equipment surfaces. These organisms may survive for prolonged periods, depending on the amount and residual soil, temperature, and relative humidity (Zottola, 1994 and Bremer *et al.*, 2006). Areas that are prone to biofilm development include dead ends, joints, valves and gaskets.

Several possible mechanisms by which microbial cells attach and form biofilm on equipment surfaces have been suggested. It was suggested that the attachment occurs in two stages, in the first stage, which is reversible, a cell is weakly held to the surface by a weak force (electrostatic force). In the second stage, a cell produces complex polysaccharide molecules to attach its outer surface to the equipment surface and the process is irreversible (Notermans *et al.*, 1991 and Lehner *et al.*, 2005).

Microbial attachment and biofilm formation to solid surface provide some protection of the cells against physical removal of the cells by washing and cleaning. These cells seem to have greater resistance against sanitizers and heat. Thus spoilage and pathogenic microorganisms attached to surfaces can not be easily removed by washing, and later they can multiply and reduce the stability of dairy products (Wong, 1998 and Ray, 2001).

The level of attachment of microorganisms to food processing equipment surfaces is found to be directly related to contact time. As the contact time is prolonged, more cells attach to the surface, the size of the microcolony increases, and attachment between cells increases (Mattila and Wirtanen, 1992).

Temperature, relative humidity, soil and surface affect the behavior of surface associated microorganisms. In addition, the nature of the attachment affects the efficacy of sanitizers. Similarly, the nature of the soil affects cleaning of the milking system (Wong, 1998).

Biofilm formation by the cells occurs faster at optimum temperature and pH of growth. Several species of *Pseudomonas* were found to attach to stainless steel surfaces within 30 minutes at 25°C to 2hrs at 4°C as well as *E.Coli* (Kives *et al.*, 2005). Studies showed that when microorganisms such as *Pseudomonas fragi* and *Listeria monocytogenes*, are grown together, they form a more complex biofilm than when either is grown separately (Wong, 1998).

Quaternary ammonium compounds (QACs) are cationic detergents and effective against vegetative bacteria and some fungi but not against spores. They are inactivated by protein, by a variety of natural and plastic materials and by anionic detergents and soap. They mentioned that Iodophores like chlorine compounds, as they are effective against vegetative bacteria, spores, fungi, and both lipid containing and non-lipid containing viruses. They are rapidly inactivated by proteins and to a certain extent by natural and plastic substances and are not compatible with anionic detergents. They are active as long as they remain brown or yellow. Iodophores are relatively harmless to skin but some eye irritation may be experienced (Collins *et al.*, 1995)

The concept and importance of microbial attachment and biofilm formation in solid food equipment and food environments is now recognized. The limited studies have shown that under suitable conditions many of microorganisms important in food can form biofilm. So, the present work was carried out to study the effect of microbial biofilms in milking machine on milk safety and quality and its control by application of different types of chemical sanitizers.

MATERIALS and METHODS

The present study was carried out in a buffalo dairy farm in Alexandria governorate for studying the biofilm of milking machine. A total of 192 swabs from teatcups, milk jars, milk pipelines (rubber and stainless steel) and bulk tank milk (48 of each) as well as 30 bulk tank milk samples after using farm program for sanitation, and after application of Iodophores and QACs separately, respectively.

1. Collection of samples:

1.1. Swabs:

Cotton swabs were prepared from non-absorbent cotton with a length of 2 cm and thickness of 0.5 – 1 cm on stiff stainless steel wire. The swabs were kept in their tubes each containing 10 ml of quarter strength Ringer's solution then autoclaved (Harrigan and McCance, 1976).

1.1.1. Teatcups: Swabs were taken from the four teatcups and then pooled as one sample.

1.1.2. Pipelines: After removal of the rubber from the milk jar, swabs were taken from milk pipelines both rubber and stainless steel and the junctions between them. The samples were pooled as one sample.

1.1.3. Milk jars: Samples were taken from the upper and lower poles of the jar then one pooled sample was made.

1.1.4. Bulk tank milk: The samples were taken from different sites of the tank using a prepared sterile cotton swab, and then pooled to one sample.

1.2. Milk samples:

1.2.1. Bulk tank milk samples: Samples of milk from the bulk milk tank were collected in sterile milk sample bottles (250 ml).

All samples either swabs or fluid milk were collected twice after using farm program for sanitation with one week interval.

2. Application of Sanitizers:

2.1. Iodophores: The commercial preparation used was Iodophores 1% (1liter/200liter H_2O at 37°C in closed system for 15 minutes).

2.2. Quaternary ammonium chloride: The commercial preparation used was Quaternary active sterilizer (2ml/liter H_2O at 70°C in closed system for 15 minutes).

The number of collected samples was repeated after application of Iodophores and QACs, separately.

3. Preparation of 10-fold serial dilution (APHA, 1992):

One ml of swabbing solutions and milk samples were added to 9 ml sterile saline solution and thoroughly mixed to make a dilution of 1:10 from which decimal dilutions were prepared.

4. Microbiological evaluation:

4.1. Total bacterial count (TBC) according to APHA (1992).

4.2. Coliforms count (MPN/g) according to Harrigan (1998).

4.3. Staphylococcus aureus count according to I.C.M.S.F. (1986).

4.4. Enterococci count according to Efthymiou *et al.* (1974).

RESULTS

Results of bacteriological examination of teatcups, milk jars, milk pipelines bulk tank milk as well as bulk tank milk samples after using farm program, and after application of Iodophores and QACs are presented in Table 1 and Figures 1-4

Table 1: counts using farm program for sanitation and reduction percent after application of Iodophores and QACs

	TBC	R%	Coliforms	R%	S.aureus	R%	Enterococci	R%
Teat cup swabs:								
• Farm program	$2.82x10^5 \pm 10x10^2$		$2.44x10^4 \pm 1.4x\ 10^2$		$4.37x10^2 \pm 1.25x\ 10^2$		$4.56x10^3 \pm 1.5x\ 10$	
• Iodophores	$3.63x10^4 \pm 1.5x\ 10^2$	87.10	$1.73x10^4 \pm 1.5x\ 10^2$	29.03	$3.14x10^2 \pm 0.25\ x\ 10$	28.22	$1.23x10^3 \pm 2.0x\ 10$	72.45
• QACs	$1.53x10^4 \pm 1.5x10^2$	94.55	$1.14x10^4 \pm 1.0x\ 10^2$	53.37	$3.36x10^2 \pm 1.55x\ 10$	23.08	$2.65x10^2 \pm 0.45x\ 10$	94.05
Pipelines swabs:								
• Farm program	$4.33x10^4 \pm 2.0x10^2$		$1.09x10^4 \pm 3.0x\ 10^2$		$1.35x10^3 \pm 1.5x\ 10^2$		$5.43x10^3 \pm 1.0x\ 10$	
• Iodophores	$3.63x10^4 \pm 1.0x\ 10^2$	16.16	$2.19x10^3 \pm 1.0x\ 10$	79.90	$4.42x10^2 \pm 0.75x\ 10$	67.22	$1.37x10^3 \pm 2.5x\ 10$	74.67
• QACs	$3.23x10^4 \pm 2.5x\ 10^2$	25.28	$2.08x10^3 \pm 2.0x\ 10$	80.91	$5.18x10^2 \pm 0.3x\ 10$	61.62	$4.27x10^2 \pm 0.5x\ 10$	92.13
Milk jar swabs:								
• Farm program	$3.95x10^5 \pm 5.0x\ 10^3$		$6.4x10^4 \pm 10.0x\ 10^2$		$1.56x10^3 \pm 0.25x\ 10$		$2.77x10^3 \pm 2.25x\ 10$	
• Iodophores	$1.38x10^4 \pm 1.5x\ 10^2$	96.49	$4.36x10^4 \pm 1.0x\ 10^2$	31.87	$1.09x10^2 \pm 0.1x\ 10$	93.04	$1.73x10^3 \pm 1.7x\ 10$	37.44
• QACs	$1.06x10^4 \pm 4.0x\ 10^2$	97.31	$2.37x10^4 \pm 2.5x\ 10^2$	62.89	$2.11x10^2 \pm 0.1x\ 10$	86.83	$2.55x10^2 \pm 0.5x\ 10$	90.81
Tank milk swabs:								
• Farm program	$1.65x10^5 \pm 2.5x\ 10^3$		$2.87x10^4 \pm 2.5x\ 10^2$		$1.87x10^3 \pm 2.5x\ 10$		$2.89x10^3 \pm 4.50x\ 10$	
• Iodophores	$2.49x10^4 \pm 5.0x\ 10^2$	84.95	$1.27x10^4 \pm 2.6x\ 10^2$	55.65	$2.38x10^2 \pm 0.2x\ 10$	87.30	$5.71x10^2 \pm 0.15x\ 10$	80.25
• QACs	$1.4x10^4 \pm 1.0x\ 10^2$	91.54	$6.31x10^3 \pm 0.5x\ 10$	78.03	$2.95x10^2 \pm 1.05x\ 10$	84.24	$1.16x10^2 \pm 0.4x\ 10$	95.99
Bulk tank milk samples								
• Farm program	$4.25x10^5 \pm 2.5x\ 10^3$		$6.72x10^4 \pm 0.75x\ 10^2$		$4.2x10^3 \pm 5.0x\ 10$		$7.83x10^3 \pm 1.5x\ 10$	
• Iodophores	$5.75x10^4 \pm 4.5x\ 10^2$	86.47	$2.27x10^4 \pm 2.5x\ 10^2$	66.18	$3.48x10^2 \pm 0.3x\ 10$	90.85	$4.49x10^2 \pm 0.55x\ 10$	75.46
• QACs	$2.87x10^4 \pm 2.5x\ 10^2$	93.24	$1.32x10^4 \pm 1.25x\ 10^2$	80.37	$4.41x10^2 \pm 0.85x\ 10$	89.48	$3.78x10^2 \pm 0.35x\ 10$	79.37

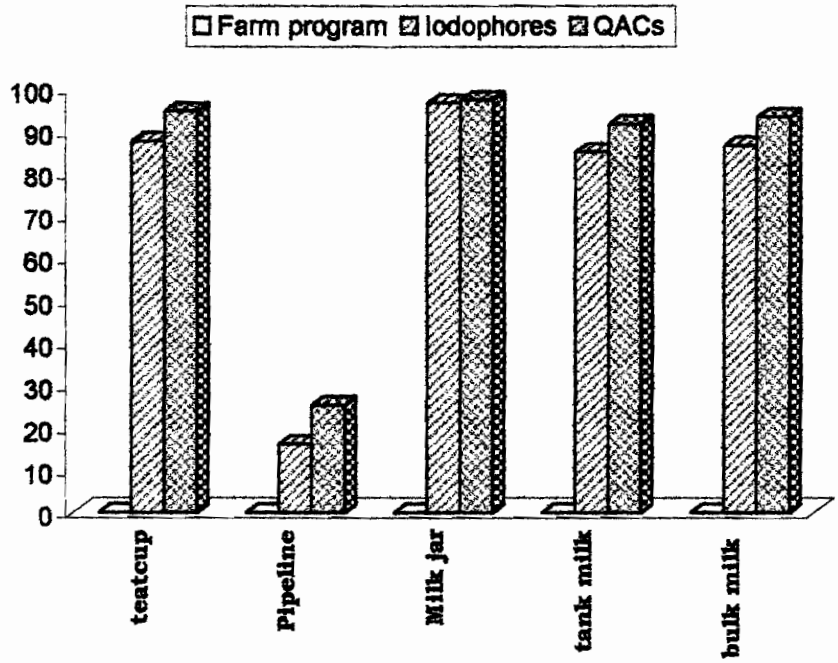

Fig. 1: Reduction percent of TBC by application of iodophores and QACs.

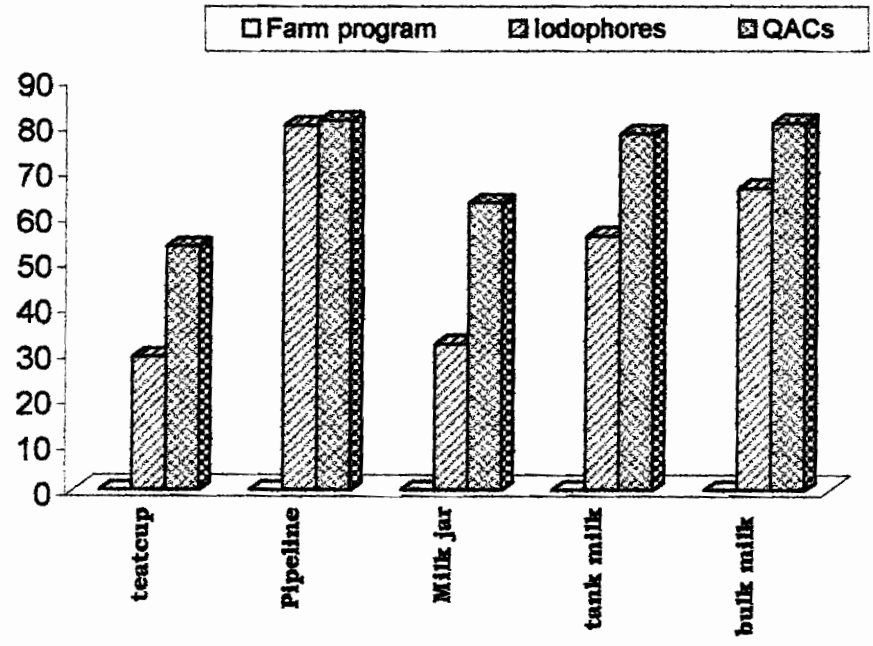

Fig. 2: Reduction percent of Coliforms by application of iodophores and QACs.

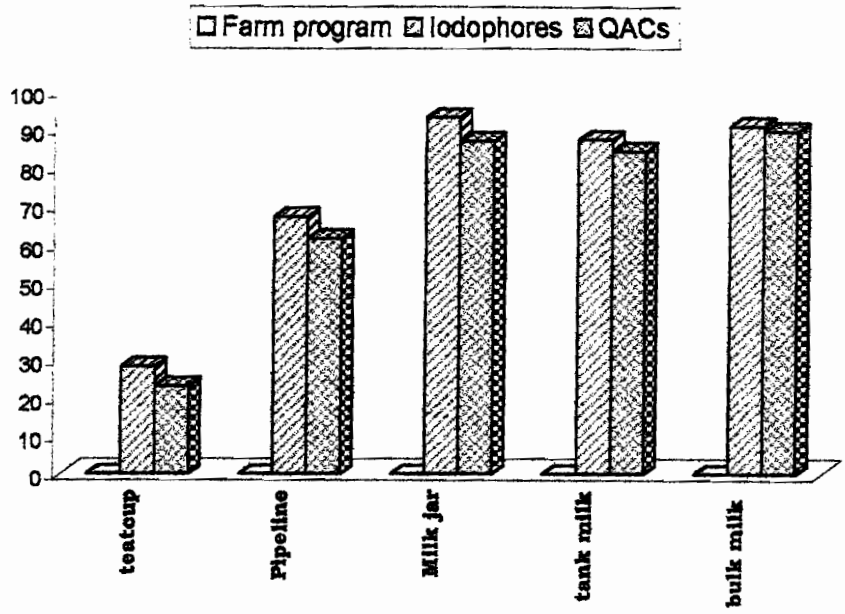

Fig. 3: Reduction percent of S. aureus by application of iodophores and QACs.

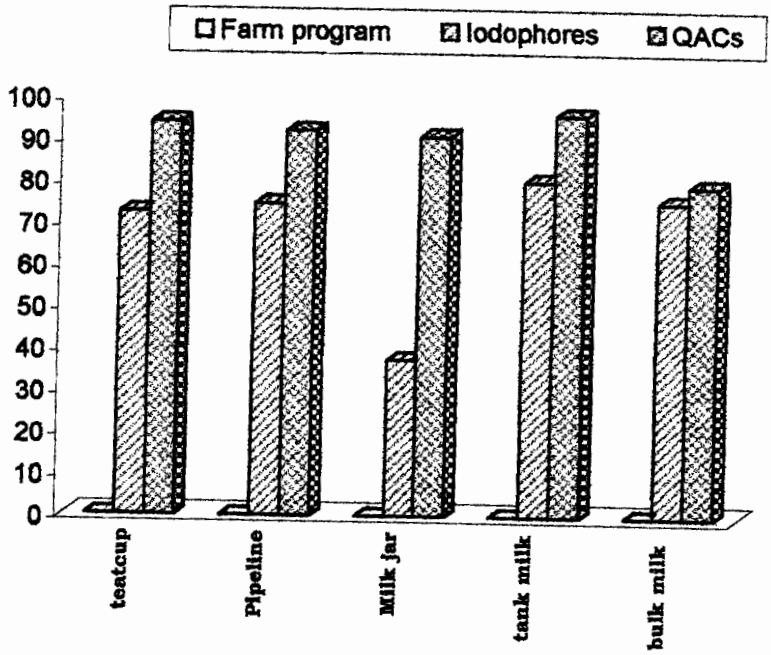

Fig. 4: Reduction percent of Enterococci by application of iodophores and QACs

DISCUSSION

In most countries, bacterial content is one of the factors considered in determining the level of payment for milk. Cleaning and disinfection are complementary processes; neither process alone will achieve the desired end result. Milk with low bacterial and somatic cell counts cannot be produced unless milking equipment are effectively cleaned and disinfected between milkings as well as the cows are kept healthy (Hayes, 1992).

1. Total bacterial count (TBC):

Total bacterial count is an index of the effectiveness of sanitary procedures of milk machine and ignorance of the residents toward the fundamental aspects of good and safe house keeping of dairy animal as well as possible presence of subclinical mastitis (Leues *et al.,* 2003). Data recorded in Table (1) and Fig (1) showed that the mean values of TBC in teatcups, milk pipelines, milk jar and tank milk swabs and bulk tank milk samples using farm program for sanitation were $2.82x10^5 \pm 10x10^2$, $4.33x10^4 \pm 2.0 x10^2$, $3.95 x 10^5 \pm 5.0 x 10^3$, $1.65x10^5 \pm 2.5x 10^3$, $4.25x10^5 \pm 2.5x 10^3$, respectively. By application of Iodophores and QACs, these counts reduced by 87.10& 94.55; 16.16 & 25.28; 96.49 & 97.31; 84.95 & 91.54 and 86.47 & 93.24%, respectively. This means that Iodophores and QACs were efficient on TBC in comparison with the farm program. QACs are more efficient on TBC than Iodophores.

Some machines are heavily contaminated, probably because of faults in design, incorrect layout of components, incorrect adjustments leading to an unbalanced flow of solutions or the use of solutions that are not hot enough. Robinson, (2002) found that the numbers of microorganisms recovered by rinsing these machines ranged from $< 5.0X10^5$ to $>1x10^9$ cfu/m^{-2}

2. Coliforms (MPN/g):

Coliforms count provides an indication of both the effectiveness of cow preparation procedures during milking and the cleanliness of the cow environment as a major source of Coliforms in bulk tank milk is transportation of soil from the teats and udders into the milking machine. Coliforms can also incubate on residual films of milking equipment, however Coliforms counts less than 10/ml indicate excellence in both pre-milking hygiene and equipment sanitation (Reinemann, 2002).

Table (1) and Fig (2) revealed that the mean values of Coliforms in teatcups, milk pipelines, milk jar and tank milk swabs and bulk tank milk samples in farm program were $2.44x10^4 \pm 1.4x 10^2$, $1.09x10^4 \pm 3.0x$

$10^{2,}$ 6.4x10^4 ±10.0x $10^{2,}$ 2.87x10^4 ±2.5x $10^{2,}$ and 6.72x104 ±0.75x 10^2, respectively. By application of Iodophores and QACs, these counts reduced by 29.03 & 53.37; 79.90 & 80.91; 31.87 & 62.89; 55.65 & 78.03 and 66.18 & 80.37 %, respectively. From the above results, it is clear that QACs are more efficient on Coliforms than Iodophores but both of them could not be able to eliminate their presence and subsequently their dangers.

3. *Staphylococcus aureus* count:

Data summarized in Table (1) and Fig (3) showed the effect of application of Iodophores and QACs as chemical sanitizers in reducing S.aureus forming biofilm in teatcups, milk pipelines, milk jar and tank milk by 28.22 & 23.08; 67.62 & 61.62; 93.04 & 86.83 and 87.30 & 84.24 %., respectively. From the previous results, it could be observed that Iodophores is more efficient than QACs in controlling S.aureus. This result is in accordance with that obtained by Babakhanyan and Asatryan (1990) who found that a 0.1% solution QACs has bactericidal effect on S.*aureus* when they were exposed for 10 min at 20 °C. They concluded that QACs are recommended for use in combination with surface active agents for cleaning/disinfection of the dairy equipment.

4. Enterococci count:

Table (1) and Fig (4) showed that the reduction percentages for Enterococci counts of teatcups, milk pipelines, milk jar and tank milk and bulk tank milk after using Iodophores and QACs were 72.45 & 94.05; 74.67 & 92.13; 37.44 & 90.81; 80.25 & 95.99 and 75.46 & 79.37%, respectively. It could be concluded that both Iodophores and QACs were efficient in suppression of Enterococci counts. These results were agreed with that obtained by Katie and Stojanovic (1990) who used Iodophores sanitation of milking machine in a concentration of 20 ppm available iodine. They reported that Iodophores had a good bactericidal effect against *Str.agalactiae, Str. dysgalactiae,* and Str. *uberis.*

It could be concluded that QACs are more efficient on TBC, Coliforms and Enterococci counts, while Iodophores are more efficient on *S.aureus.* QACs are more efficient preferable than Iodophores as after disinfection surfaces treated with QACs retain a bacteriostatic film due to the absorption of the disinfectant on the surface; this film prevents the subsequent growth of residual bacteria (Hayes, 1992).

Several reasons can account for the reduced sensitivity of bacteria within a biofilm. There may be reduced access of a disinfectant to the cells within the biofilm, chemical interaction between the disinfectant and the biofilm itself, modulation of the environment,

production of degradative enzymes and neutralizing chemicals or genetic exchange between cells in a biofilm (Augustin *et al.*, 2004).

Bacteria growing as surface associated biofilms are much harder to treat with a disinfectant, thus, potentially leading to the build up of dangerous infections. The growth patterns, coverage and adherence of the biofilms depend critically on the substrate roughness, composition and types of microorganisms (Schwartz *et al,* 2003).

Overall, it is important to understand the interactions between bacteria and surfaces in specific food processing and food services environment and the impacts of surface-associated bacteria on cleaning and sanitizing to provide more effective measures for prevention of biofilm formation and for biofilm removal (Wong, 1998).

Some milk producers fair to use chemical sanitizers especially on production of milk intended for manufacture of cultured dairy products as they thought that any residue of sanitizer will interfere with the action of added starters and subsequently great economic losses. So, some modifications may be necessary to overcome the problem, especially in food contact and processing equipment surfaces. Biofilms are difficult to remove if they are left to grow and should be removed when they are still young. In a dairy processing operation, cleaning and sanitation need to be done every few hours. A treatment with some suitable hydrolyzing enzymes may be initially administrated. EDTA, along with a quaternary ammonium compound treatment following an enzyme treatment can be helpful (Scott, 2000).

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