Effect of Certain Antiseptics on the Growth of *Escherichia coli* in Skim Milk

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ABSTRACT

The present study aims to evaluate the effect of certain antiseptics, such as polyhexamethylene biguanide (PHMB), polyhexamethylene guanide (PHMG), silver nitrate ($AgNO_3$), and glutamic acid on the behavior of *Escherichia coli* (ATCC 4229) in skim milk. Briefly, both PHMB and $AgNO_3$ showed significant killing effect against *E. coli*, with respect to the higher antibacterial efficacy of PHMB compared to $AgNO_3$. The high killing percentages and leakage of potassium content exhibited positive direction against *E. coli* with high antiseptic concentrations up to certain limits. Using PHMB combined with $AgNO_3$ and PHMG combined with $AgNO_3$ against the growth of *E. coli* revealed high killing percentage than using any of these antiseptics separately in skim milk. In the same way, when PHMB was combined with $AgNO_3$ or PHMG with $AgNO_3$ mixed with glutamic acid, the percentages of survival cells significantly decreased ($P \leq 0.05$) compared to the use of the antiseptics separately with glutamic acid.

Keywords: $AgNO_3$, Antiseptic, Escherichia coli, PHMB, and PHMG.

INTRODUCTION

Many detergents and antiseptics have been applied in Egyptian dairy farms and plants to achieve a high microbial quality of the equipment and the manufactured dairy products (Salwa *et al*., 2008). The antiseptics are used to kill the microorganisms in animate places, and antiseptics have multi-action mechanisms for the killing process (Fraise *et al*., 2012). Disinfection is considered a decisive step for desired hygiene aspects in different types of food production or processing plants. In addition, previous studies have shown that antiseptics such as PHMB and PHMG in solutions have bactericidal activity against both negative and positive- gram bacteria (Antonik *et al*., 2002, Amjad & Demadis, 2015). Antiseptics are biocides or substances that kill or inhibite the growth of bacteria, either intracellularly or extracellularly (McDonnell & Russell, 1999), and their justification was related to the disturbance functions of the microbial cell membranes. Furthermore, antimicrobials have a major role in controlling diseases and pathogen's spread. Additionally, their action in respect of reducing bacterial counts was clear (Brady *et al*., 2003, Ferrara *et al*., 2011). Bisbiguanides (i.e. chlorhexidine) include two cationic gatherings isolated by a hydrophobic crossing-over structure (hexamethylene), whilst the polymeric biguanides (i.e. PHMB) are polycationic linear polymers with a hydrophobic spine and numerous cationic groupings isolated by hexamethylene chains (Gilbert & Moore, 2005). In dermatology, it is known as polihexanide and is marketed under the brand names Lavasept, Serasept, Prontosan, and Omnicide. PHMB and PHMG are polymers that act as disinfectants, antiseptics, and biocides, and have thus been used in a variety of applications, including killing microbes (e.g. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, and *E. coli*) with a high treatment index, as well as industry, homes, and clinics (Oule *et al*., 2008, Müller *et al*., 2013). The European Chemical Agency categorized Polyhexamethylene guanidine as a category 2 carcinogen; however it is still permitted in small doses in cosmetics. Polyhexamethylene guanidine (PHMG) is a guanidine derivative used as a biocidal disinfectant, most commonly in the form of its salt. PHMG and its derivatives largely cause cell membrane damage by decreasing the function of cellular dehydrogenases. In respect of the killing effects of silver, it was reported that the killing rate sharply increased with low concentrations due to the greater part of the metal being chelated inside microbes, and then the killing effects were more smooth (Wakshlak *et al*., 2015).

The incidence of *E. coli* in raw milk, and *kariesh* cheese was detected in previous study (El Nahas *et al*., 2015). Furthermore, raw market milk had the highest prevalence of *E. coli* (52%), followed by Kareish cheese (48%) (Ibrahim *et al*., 2022). *E. coli* includes various strains which ordinarily occupy the intestines of both humans and animals. The majority of *E. coli* strains are not harmful, but some strains can cause illness in humans. It is noticeable that the animals and poultry are considered natural resources for *E. coli*; however, this microorganism grows excellently outside the body of the animal, and also in unclean food-handling equipment. Likewise, fecal contamination from humans or animals often considers a source of *E. coli*. The prevention measures for food infection from *E. coli* demand control measures at all production steps whether before or after production. Therefore, the main purpose of the present study was to evaluate the killing effects of several disinfectants, including PHMB, PHMG, $AgNO_3$, and glutamic acid severally or in mixtures as potential disinfectants against *E. coli* (ATCC 4229) in skim milk.

MATERIALS AND METHODS

Raw materials and chemicals

Skim milk powder imported from France was purchased from local market (Ismailia, Egypt). In addition, PHMB, PHMG, and glutamic acid were imported from SOPURA, Belgium, and purchased from El Nasr-pharmaceutical Chemical Co., Cairo, Egypt. $AgNO_3$ was obtained from Algomhuria Co., Cairo, Egypt. *E. coli* (ATCC 4229) was obtained from SOPURA, Belgium. All chemicals used in the present work were of analytical grade.

Methods

Preparation of cell suspension

E. coli (ATCC 4229) was activated using a technique of three successive transfers at 37 °C. Then, *E. coli* cells were collected by centrifugation (Avanti J-26XP, Beckman Coulter, Inc. USA) at a speed of 8000 rpm for 10 min at 4 °C. After 10 h of incubation in a Luria-Bertani LB broth medium (Sezonov *et al*., 2007), the cells were washed twice with sterilized distilled water, and then suspended again in sterilized distilled water to give cell density of 1 mg cell dry weight per mL.

Killing effect of antiseptics

Skim milk samples were reconstituted using 8 g of skim milk powder in 100 mL of distilled water. The liquid milk was stored at 4 °C for 40 min. Milk samples (10 mL) were prepared with different antiseptics concentrations, and pasteurized for 15 sec at 72 ℃. Exactly 1 mL of *E. coli* suspension (1 mg cell dry weight per mL) was incubated with skim milk samples containing different antiseptics at 25 °C / 20 and 60 min. To measure the killing effect of antiseptics, the plate count technique was applied to determine *E. coli* through diluting of each sample into 10 folds of sterile saline, and colonized onto Lb agar. The culturable colonies of *E. coli* were counted as colony-forming units per mL (CFU mL^{-1}) after 48 h of incubation at 37 °C.

Determination of potassium leakage

Perkin-Elmer 290 B Atomic Absorption Spectrophotometer (Perkin-Elmer, Model: 290 B, USA) (Lambert & Hammond, 1973) was applied to determine the potassium content of *E. coli* cells.

Statistical analysis

SAS statistical software performed all statistical analysis of results (SAS, 1999) using the ANOVA procedure to analyze variance. The results were expressed as mean ± standard error, and the differences between means were tested for significance using Duncan's multiple ranges at ($P \leq 0.05$).

RESULTS AND DISCUSSION

Effect of inoculation time on the killing effect

The killing effect of $AgNO_3$ and PHMB on *E. coli* is shown in Figure (1). It was shown that the killing percentage via PHMB (1 ppm) against *E. coli* represented 26×10^6 cell/mL, which was significantly ($P \leq 0.05$) higher than that of $AgNO_3$ (50 ppb) through all the inoculation times (30 – 240 min). Moreover, the killing percentage of PHMB increased from 30 to 75 min, and then no changes were observed after 75 min. The killing percentage caused by $AgNO_3$ exhibited significant ($P \leq 0.05$) differences with all incubation times (30 – 240 min), but from 30 to 75 min, the increase of killing percentage was fast, and then the increasing level decreased after 75 min. It can be concluded that the killing percentages of both $AgNO_3$ and PHMB against *E. coli* were dependent on the time. A previous report indicated that silver enters into bacteria within half an hour of exposition, following the binding of silver with proteins, cytoplasm, and nucleic acids (Yamanaka *et al*., 2005, Jung *et al*., 2008).

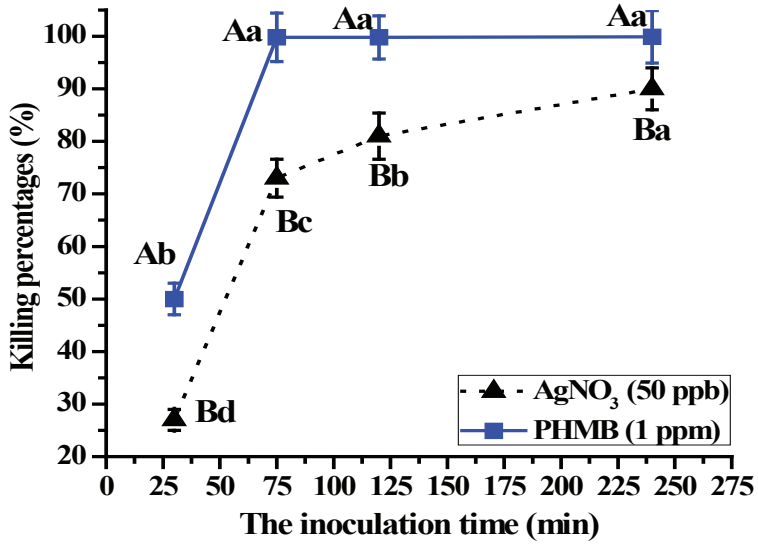

Fig. 1: Effect of inoculation time on the killing effect of *E. coli* via $AgNO_3$ and PHMB. Capital letters: with the different letters are significant ($P \leq 0.05$) with the different treatments; Small letters: with the different letters are significant ($P \leq 0.05$) with the same treatments

Effect of PHMB and $AgNO_3$ on the growth of *E. coli*

The killing percentages of the different antiseptics against *E. coli* are shown in Table (1). The differences between the killing percentages of *E. coli* which resulted from each PHMB and $AgNO_3$ were significant ($P \leq 0.05$). In addition, the increase of PHMB from 1 to 10 ppm resulted in the same trend of high killing percentages against *E. coli*, while the killing percentages of *E. coli* significantly ($P \leq 0.05$) increased with 10 to 500 ppb $AgNO_3$. Moreover, the concentrations of PHMB and 500 ppb $AgNO_3$ against *E. coli* resulted in high killing percentages. The killing effect of PHMB and $AgNO_3$ on *E. coli* might be due to the resultant changes in the cell. Previous findings imply that antiseptics enter susceptible bacterial cells via the outer membrane proteins 35-kDa OMP, and that subsequent resistance is connected with the absence of this protein (Winder *et al*., 2000), In addition, some biocides can exhibit specific interactions with the cell wall peptidoglycan (McMurry *et al*., 1998, Maillard & Pascoe, 2023), and phospholipids (Boeris *et al*., 2007). On the other hand, other reports stated that the killing mechanism by copper, silver, and zinc can result from their ability to bind with the substantial enzyme sulfhydryl (Huang *et al*., 2004, Huang *et al*., 2007). The previous study (Wakshlak *et al*., 2015) has indicated that silver concentrations (below 5 ppm) caused dead cells or bacterial inactivation, whereas a silver concentration of 10 ppm caused a bactericidal action. Therefore, the bacteriostatic and bactericidal effects depended on the compound concentrations (Wakshlak *et al*., 2015). As given in Table (2), the effect of cell density on killing percentages of antiseptics recorded high values, whereas the increase of cell density from 26 to 61 × 10^6 cfu / mL significantly ($P \leq 0.05$) decreased the killing percentages from 73% to 48%. The relation between the cell density of *E. coli* and the killing percentage via $AgNO_3$ was inverse.

Table 1: Effect of PHMB and $AgNO_3$ on the killing percentages of *E. coli* (61 × 10^6 cfu/ml)

Antiseptics concentrations		Killing percentages (%)
PHMB (ppm)	1	99.6±0.53[a]
	5	99.9±0.62[a]
	10	99.9±0.68[a]
$AgNO_3$ (ppb)	10	8.0±0.16[f]
	25	20.0±0.26[e]
	50	52.0±0.45[d]
	100	73.0±0.48[c]
	250	97.0±0.56[b]
	500	99.9±0.62[a]

Small letters: Average values with the different letters are statistically significant ($P \leq 0.05$) for the column.

The influence of PHMB, $AgNO_3$, and PHMG against *E. coli*

As shown in Table (3), the different types of antiseptics levels against *E. coli* displayed significant ($P \leq 0.05$) differences. After incubation at 35°C/30 min, the survival cell percentages of

Table 2: Effect of cell density on killing percentages of disinfectants

Cell density (cfu/ml)	Killing percentages (%) of $AgNO_3$ (50 ppb)
26×10^6	73±1.27[a]
61×10^6	48±0.86[b]

Referring to Table 1. cfu: colony forming unit.

Table 3. Killing effect of PHMB, $AgNO_3$, and PHMG on *E. coli* (60×10^6 cfu/ml)

Antiseptics		Survival cell (%)
PHMB (ppm)	1	0.40±0.04[d]
	5	0.01±0.01[d]
$AgNO_3$ (ppb)	10	92.0±2.05[a]
	50	49.2±0.87[b]
	100	28.0±0.34[c]
	500	0.01±0.01[d]
PHMB (ppm) + $AgNO_3$ (ppb)	1+10	0.04±0.01[d]
	1+50	0.01±0.01[d]
	1+100	0.01±0.01[d]
PHMG (ppm)	1	0.30±0.02[d]
PHMG (ppm) + $AgNO_3$ (ppb)	1+50	0.01±0.01[d]

Referring to Table 1.

E. coli significantly ($P \leq 0.05$) decreased from 92 to 0.01% with the increase of $AgNO_3$ concentration (from 10 to 500 ppb). In addition, the killing percentage was associated with concentration of $AgNO_3$. Despite the antiseptics of PHMB, (PHMB+$AgNO_3$), PHMG, and (PHMG+$AgNO_3$) exhibited no significant ($P \geq 0.05$) activity on the survival cell but recorded low survival cell percentages, also the effectiveness of previous antiseptics against *E. coli* compared to $AgNO_3$ were higher. Remarkably, the use of $AgNO_3$ with PHMB and PHMG supported the killing action, thus the synergetic effect between PHMB, PHMG, and $AgNO_3$ against the growth of *E. coli* can be attributed to the cell permeability and enzymatic activity.

Potassium leakage of *E. coli*

The data presented in Table (4) show the potassium leakage of *E. coli*. It is noticeable that the differences in potassium leakages of *E. coli* were significant ($P \leq 0.05$) with the increase of each $AgNO_3$ or PHMB concentration, while PHMB and $AgNO_3$ together caused insignificant ($P \geq 0.05$) changes. The leakage is considered one of the first indices for the loss of cell membrane permeability (El-Zayat & El-Bagoury, 1983). Both $AgNO_3$ or PHMB caused the potassium leakage and the amount of leakage was concentration dependent up to certain limits (25 ppb of $AgNO_3$ or 5 ppm of PHMB), and then decreased due to the blockage of leakage sites (El-Zayat, 1988), which related to the precipitation of the leaked ingredients with the cytoplasmic contents, or the damaged membrane blocked by the precipitated contents (El-Zayat, 1985). Furthermore, the increase in antiseptics could induce an increase in leakage and cell membrane permeability (El-Zayat & Omran, 1983). The present study was in agreement with the results reported previously (Lambert & Hammond, 1973) in respect of potassium leakage due to membrane damage and loss of semi permeability. The PHMG could cause lysis limited for the spheroplast or the permeability of intact bacteria (Barros *et al*., 2022, Johnston *et al*., 2003). Potassium is the most common monovalent intracellular cation in *E. coli* and other bacterial and eukaryotic cells. Potassium plays four major roles in *E. coli*: it is an osmotic solute, an activator of intracellular enzymes, a regulator of intracellular pH, and a second messenger to stimulate the accumulation of compatible solutes. In bacteria, cytoplasmic pools of K^+ are closely regulated by a variety of transport mechanisms that differ in terms of kinetics, energy coupling, and regulation.

Table 4. Effect of PHMB and $AgNO_3$ on the leakage of potassium (as the percentage of cell dry weight of *E. coli*)

Antiseptics		K (ppm)
$AgNO_3$ (ppb)	0	0.55±0.01[d]
	10	0.60±0.02[c]
	25	0.65±0.03[ab]
	50	0.58±0.01[cd]
	100	0.61±0.03[bc]
	250	0.55±0.02[d]
PHMB (ppm)	0	0.55±0.03[d]
	1	0.55±0.01[d]
	5	0.68±0.03[a]
	10	0.62±0.04[bc]
	20	0.55±0.01[d]
	50	0.60±0.02[c]
PHMB (ppm) + $AgNO_3$ (ppb)	0	0.55±0.04[d]
	1+10	0.55±0.03[d]
	1+25	0.55±0.03[d]
	1+50	0.54±0.01[d]
	1+100	0.54±0.04[d]
	1+250	0.54±0.02[d]

Referring to Table 1.

The influence of glutamic acid on the activity of the disinfectant

The effects of glutamic acid combined with different types of antiseptics on the survival of *E. coli* in skim milk are presented in Figure (2). The synergistic effect of glutamic acid with PHMB (1 ppm), PHMG (1 ppm), and $AgNO_3$ (50 ppb) represented 0.52%, 0.31%, and 50.82% survival cells, respectively. On the other hand, the synergistic effect of glutamic acid with the antiseptics (PHMB + $AgNO_3$ or PHMG + $AgNO_3$) appeared in fewer survival cells, therefore the increase of synergistic effect was in line with the increase of antiseptics types besides glutamic acid. Another study has reported that a complex of silver and glutamic acid exhibited a predominantly antimicrobial activity with Gram-negative test microorganisms, whereas a complex of silver and arginine resulted in higher antimicrobial activity (Legler *et al*., 2001).

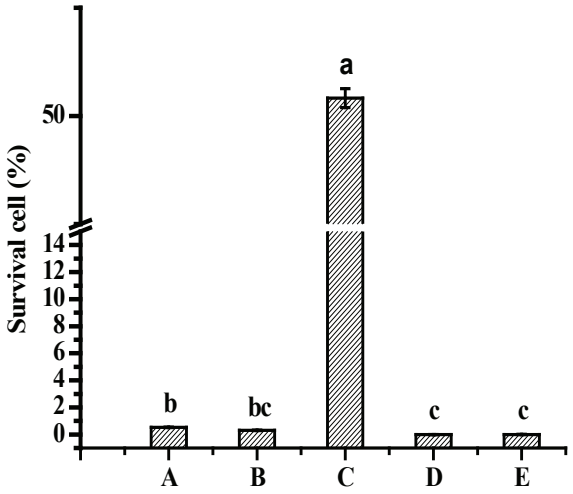

Fig. 2: The effect of glutamic acid with PHMB, PHMG, and $AgNO_3$ on the antiseptics activity of *E. coli*. A: PHMB (1 ppm)+GA (1 ppm); B: PHMG (1 ppm)+GA (1 ppm); C: $AgNO_3$ (50 ppb)+GA (1 ppm); D: PHMB (1 ppm)+$AgNO_3$ (3 ppb)+GA (1 ppm) and E: PHMG (1 ppm)+$AgNO_3$ (3 ppb)+GA (1 ppm). Referring to Fig. 1.

CONCLUSIONS

The use of PHMB, PHMG, and $AgNO_3$ as antiseptics in skim milk revealed the efficiency against the growth of *E. coli* through the results of inoculation times, killing percentages, and survival cells. The leakage of potassium content increased with the increase of antiseptics concentrations until certain limits. Moreover, the synergistic effect via glutamic acid with PHMB, PHMG, and $AgNO_3$ was noticeable against *E. coli*. Eventually, *E. coli* growth rates are significantly reduced when PHMB or PHMG are coupled with $AgNO_3$ and glutamic acid.

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تأثير بعض المطهرات على نمو بكتريا الإيشيريشيا كولاي في اللبن الفرز

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تهدف الدراسة الحالية إلى تقييم تأثير بعض المطهرات المتواحدة بعينات اللبن الفرز مثل البولي هكساميثيلين بايجوانيد PHMB، البولي هكساميثيلين جوانيد٣ PHMG، ونترات الفضة AgNo3 على معدلات نمو الإيشيريشيا كولاي. أظهرت نتائج الدراسة أن كل من PHMB وAgNo3 لهما تأثيرات مميتة في معدلات الخلايا الحية لبكتريا الإيشيريشيا كولاي، واتضح ذلك عند وجود مادة PHMB، في وسط نمو الإيشيريشيا كولاي بحيث أظهرت تأثيرًا قاتلًا أعلى بالمقارنة بالـ AgNo3. أظهرت النتائج أيضًا أن أعلى نسب لقتل الإيشيريشيا كولاي وترشيح محتوى البوتاسيوم كانت متزايدة كلما زادت تركيزات المواد المطهرة سالفة الذكر حتى حدود معينة من المواد المطهرة ومن اللافت للنظر إن إضافة مادة AgNo3 بالتبادل مع كل من الـ PHMB و PHMG أظهرت أعلى تأثير قاتل على الإيشيريشيا كولاي بالمقارنة من معاملة هذه المواد المطهرة على حدة. علاوة على ذلك، وجد أن النسبة المتبقية من الخلايا الحية لبكتريا الإيشيريشيا كولاي انخفضت بشكل ملحوظ كلما تنوعت المواد المطهرة المستخدمة مع حمض الجلوتاميك بالمقارنة باستخدام هذه المواد المطهرة بمفردها. لذا، فاستخدام PHMB وPHMG في كبح نمو البكتريا أثبت فعالية أكثر في وجود مطهرات أخرى سواء AgNO٣ أو حمض الجلوتاميك.