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Application of lactoferrin as a trial to control *E. coli* O1 and O26 in pasteurized milk

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A B S T R A C T

Lactoferrin has amajor effects on enteropathogenes as it inhibits growth. so, This work adopted to study the antibacterial activity of lactoferrin on growth of *E. coli non O157* in broth and pasteurized milk. Two strains of *E. coli (* O1 carry *stx2* and *hly* gene and O26 carry *hly* gene) were used. Different concentrations of lactoferrin (zero, 0.5, 1.0, 5.0, 10 and 20 mg/ml) were used in Lauria broth. Based on our results, LF showed various inhibition activity on *E. coli non O157* growth in Lauria broth. Significant decrease observed on growth of *E. coli* O1. Higher concentrations of lactoferrin caused a significant decrease in *E. coli* O26 growth. Regarding its effect in pasteurized milk, a significant decrease in the count of *E. coli* O1and *E. coli* O26 at the concentrations of 10 and 20 mg/ml lactoferrin was noticed. So, lactoferrin could become a promising method to decrease growth of *E. coli non O157* in pasteurized milk consequently decrease *E.coli non O157* associated illness in humans.

Keywords: *Lactoferrin, Lauria broth ,Pasteurized milk, E. coli O1, E. coli O26.*

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1. INTRODUCTION

Cattle and other ruminants are the most important reservoir of zoonotic Shiga toxin–producing E. coli (STEC), which transmitted to human through the ingestion of foods or water contaminated with animal feces, or through direct contact with the infected animals or their environment. The main sources of STEC infection of cattle on-farm are the drinking water, the feed, and the immediate environment of the animal (Fairbrother and Nadeau, 2006).

Escherichia coli strains belonging to serogroups O1 are frequently associated with human infections ,especially extra-intestinal infections such as bloodstream infections or urinary tract infections (Delannoy et al .,2017). On the other hand, Entero-hemorrhagic Escherichia coli (EHEC) O26 has emerged as a significant cause of hemolytic uremic syndrome(HUS) (Allerberger et al., 2003).

Naturally occurring antimicrobials are widely distributed in environment. Numerous antimicrobial agents exist in animal and plants where they evolved in host defense mechanisms. These compounds may exhibit antimicrobial activity in food as natural ingredients or may be used as additives to other food. These antimicrobials have been limited to five different classes of natural system . They are phyto-antimicrobials (flavonoids), acid-antimicrobials (lactic acid, acetic and citric acid), Bacto-

antimicrobials (probiotics), Ovo-antimicrobials (lysozyme) and Lacto-antimicrobials (Lactoferrin "LF") (Naidu, 2000).

Lactoferrin (LF), a member of the transferrin protein family, is an iron-binding glycoprotein that is found in many exocrine secretions, including milk, tears, saliva, and serum (Rybarczyk et al., 2017). Lactoferrin (LF), is primarily extracted from bovine milk. Bovine colostrum contains 1.5 mg/ml lactoferrin and the lactoferrin concentration in milk ranges from 0.02 mg/ml to 0.20 mg/ml (Shimazaki et al., 2000 and Ochoa and Cleary, 2009). Lactoferrin has been used in a wide variety of products since it was first added to infant formula in 1986 (Tomita et al., 2002). Now it was added into many commercial products such as cosmetics, nutritional supplements, and toothpaste (Wang et al., 2019).

The antimicrobial activity of lactoferrin is mainly explained by two mechanisms; the first one is the absorption of the iron from the infection sites which is the food source of the microorganisms. This creates a bacteriostatic effect. The second one is the direct interaction of lactoferrin with the infection agent as Lactoferrin has high levels of amylase, DNase, RNase and ATPase activity. Therefore, LF can damage the nucleic acids of bacteria through hydrolysis and can inhibit the organism (González-Chávez et al., 2009). Furthermore, Lactoferrin has major effects on enteric pathogens: it inhibits growth and it impairs function of surface expressed virulence factors thereby decreasing their ability to adhere or to invade mammalian cells (Ochoa and Cleary, 2009).

This work adopted to study the antibacterial activity of lactoferrin on growth of E. coli non O157 in broth and pasteurized milk.

2. MATERIAL AND METHODS

2.1.Lactoferrin (LF) preparation:

Lactoferrin was purchased from Jarrow FORMULAS, Superior Nutrition and Formulation, Los Angeles, CA 90035-4317. The LF was disolved in sterile distilled water and stored at − 20° C until needed.

2.2 Tested strains:

Two strains of E. coli (O1 carry stx2 and hly gene and O26 carry hly gene) were used. These strains were previously isolated from Kariesh Cheese and plain yogurt samples and differentiated serologically and by using PCR technique in Biotechnology unit in Animal Health Research Institute, Doki, Egypt.

2.3. Culture preparation:

The strains were inoculated into trypticase soy broth at 37 °C for 24 h and then tenfold serial dilution was done and plated on EMB agar for enumeration. The concentration of each strain was adjusted to 105- 106 cfu/ml (Murdock et al., 2007)

2.4. Antibacterial activity assay in lauria broth:

The antibacterial assay was performed according to Atef Yekta et al.(2010).one ml of each above strain of E. coli was transferred into sterile test tubes containing 1 ml of Lauria Bertani broth (LB) supplemented with different concentrations of lactoferrin (zero, 0.5, 1.0, 5.0, 10 and 20 mg/ml) and incubated at 37°C for 24, 48 and 72 h. Viable bacteria were counted by spread plating of appropriate bacterial serial dilutions onto EMB plates.

Antibacterial activity assay in pasteurized milk (Murdock and Matthews, 2002): Skim milk was laboratory pasteurized by heating at 63˚C for 30 min then rapid cooling to 4-5 ˚C in ice water bath. Pasteurized milk was divided into groups which inoculated with Strain O1 (previously isolated from Kariesh Cheese carry stx2 and hly gene) and strain O26 (previously isolated from plain yogurt carry hly gene). The concentrations of LF tested were (10 and 20 mg/ml) and last group was free from lactoferrin as a control. Samples were preserved in refrigerator at 4±2°Cfor periodical counting. Tenfold serial dilution was done then, 0.1 ml was spread plated onto EMB agar, incubated at 37°C for 24 h and colonies was enumerated. The samples examined daily until deterioration of milk was detected according to (GSO,2005 and EOS,2008).The experiment was

repeated 3 times and the results were expressed as the mean± Standard error "SE".

2.5. Statistical analysis:

The effect of different concentrations of the lactoferrin on strains of E. coli non O157 was analyzed using one-way analysis of variance and repeated measures. A p-value of <0.05 was considered significant.

3. RESULTS

3.1. Antibacterial activity of LF against E. coli O1and O26 in broth.

In lauria broth, E. coli O1 was decreased from 6 x 105 at zero time to 3 x 104,2 x104,3 x104,2 x 103 and 9 x 102 after 72 h at the concentration of lactoferrin 0.5,1,5,10 and 20 mg/ml respectively as illustrated in Figure (1).
The effect of lactoferrin on growth of E.coli O26 in broth was illustrated in Figure (2). The count decreased from 8 x 105 at zero time to 7 x 104, 1 x 104, 9 x 103,6 x 103 and 4 x 103 after 72 h at the concentration of lactoferrin 0.5,1,5,10 and 20 mg/ml respectively.

3.2. Antibacterial activity of LF against E. coli O1 and O26 in pasteurized milk:

Regarding pasteurized milk, the mean count of E. coli O1 increased from 7 x 106 at zero time to 2 x 107 by the 4th day in the sample control. while, the count decreased to 9 x 105 and 1 x 105 at the concentration of 10 and 20 mg/ml lactoferrin, respectively (Table 1). In the same context, the mean count of E. coli O26 increased from 4 x 106 to 6 x 107 by the 4th day in the sample control. while, the count decreased to 3 x 106 at the concentration of 10 and 20 mg/ml lactoferrin by the 3rd day. The count increased to 6 x 106 by the 4th day at both concentration of lactoferrin (10 and 20 mg/ml) (Table 2).

Figure (1): Effect of lactoferrin on growth of *E.coli* O1 in broth

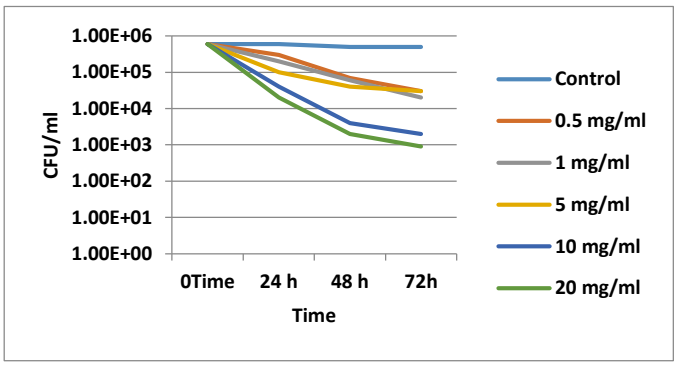

Figure (2): Effect of lactoferrin on growth of *E.coli* O26 in broth

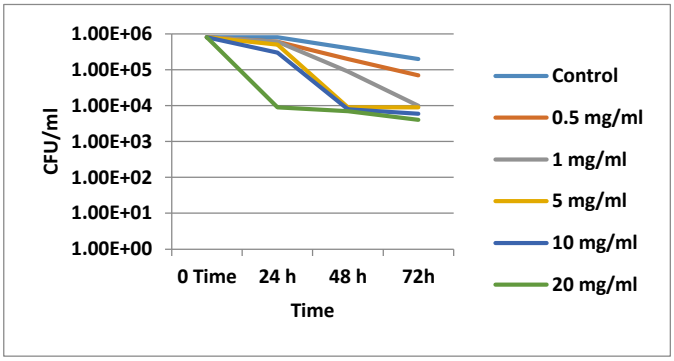

Table (1): Effect of lactoferrin on *E. coli* O1 carry *stx2* and *hly* gene in pasteurized milk

Storage period	Mean *E. coli* O1 in pasteurized milk ± SE*(cfu/ml)		
	Control	10 mg/ml	20 mg/ml
0 time	$7 \times 10^6 \pm 3 \times 10^5$	$7 \times 10^6 \pm 3 \times 10^5$	$7 \times 10^6 \pm 3 \times 10^5$
1st day	$1 \times 10^7 \pm 5 \times 10^6$	$1 \times 10^6 \pm 6 \times 10^5$	$7 \times 10^5 \pm 6 \times 10^5$
2nd day	$6 \times 10^6 \pm 2 \times 10^6$	$3 \times 10^6 \pm 2 \times 10^6$	$3 \times 10^5 \pm 2 \times 10^5$
3rd day	$1 \times 10^7 \pm 5 \times 10^6$	$3 \times 10^5 \pm 2 \times 10^5$	$7 \times 10^5 \pm 6 \times 10^5$
4th day	$2 \times 10^7 \pm 4 \times 10^6$	$9 \times 10^5 \pm 6 \times 10^5$	$1 \times 10^5 \pm 6 \times 10^4$

*The values indicated were the mean of three trials± SE(Stanndard Error).

Table (2): Effect of lactoferrin on *E. coli* O26 carry *hly* gene in pasteurized milk

Storage period	Mean *E. coli* O26 in pasteurized milk ± SE*(cfu/ml)		
	Control	10 mg/ml	20 mg/ml
0 time	$4 \times 10^6 \pm 2 \times 10^6$	$4 \times 10^6 \pm 2 \times 10^6$	$4 \times 10^6 \pm 2 \times 10^6$
1st day	$3 \times 10^7 \pm 2 \times 10^7$	$3 \times 10^6 \pm 6 \times 10^5$	$1 \times 10^6 \pm 5 \times 10^5$
2nd day	$2 \times 10^7 \pm 6 \times 10^6$	$4 \times 10^6 \pm 2 \times 10^6$	$3 \times 10^6 \pm 2 \times 10^6$
3rd day	$5 \times 10^7 \pm 2 \times 10^7$	$3 \times 10^6 \pm 2 \times 10^6$	$3 \times 10^6 \pm 2 \times 10^6$
4th day	$6 \times 10^7 \pm 2 \times 10^7$	$6 \times 10^6 \pm 2 \times 10^6$	$6 \times 10^6 \pm 3 \times 10^6$

*The values indicated were the mean of three trials± SE(Stanndard Error).

4. DISCUSSION

Lactoferrin has recently been tested for food applications due to its significant antibacterial and antifungal activities, combined with a wide safety profile. Now it is interesting to apply LF in food preservation for protection from both spoilage and pathogenic bacteria beside fungi. This strategy may allow to reduce the use of chemical preservatives (Bruni et al., 2016).

Lactoferrin has been shown to inhibit the growth of a number of pathogenic bacteria including E. coli in both in vitro and in vivo studies (Yen et al., 2011). Lactoferrin clearly has two major effects on bacterial enter-opathogens. It binds iron and limits growth under low iron conditions and it disrupts surface expressed virulence proteins, typically causing their loss and degradation (Ochoa and cleary, 2009).

The current study evaluated the antibacterial activity of lactoferrin in lauria Bertani broth (LB) inoculated by two strains of E. coli (O1 carry stx2 and hly gene and O26 carry hly gene) with different concentrations of lactoferrin (zero, 0.5, 1, 5, 10 and 20 mg/ml).

Significantly different antibacterial activities were observed relative to the control for all the tested concentrations.

All concentrations of LF cause significant decrease $P < 0.05$ on growth of E. coli O1 isolated from Kariesh cheese carry Stx2 and hly gene in broth. The count decreased from 6 x 105 at zero time to 2 x 103 and 9 x 102 after 72 h at the concentration of 10 and 20 mg/ml lactoferrin , respectively (Figure1) .Another study, Atef Yekta et al. (2010) found that Escherichia coli O157:H7 growth in broth was significantly inhibited from three to six hours post incubation using 0.5 to 10 mg/ml and 0.1 to 10 mg/ml of human or bovine LF, respectively. Ochoa et al.(2006) reported that bovine lactoferrin inhibited enteroaggregative E.coli (EAEC) at a concentration of 1.0 and 0.1 mg/ mL. They revealed that Lactoferrin inhibited EAEC biofilm formation and increased autoagglutination. Lactoferrin blocks EAEC adherence by inducing release and degradation of aggregative adherence fimbria, a key element of EAEC pathogenesis. They hypothesized that lactoferrin binding to lipid A of lipopolysaccharide disrupts the virulence proteins anchored to the bacterial outer membrane. In another study, ninteen strains of enterotoxigenic E.coli were studied for their sensitivity for inhibition by LF using Bacto Synthetic Broth (BSB)in vitro. Both apo and native LF at 1.0 mg/ml inhibited growth in all strains and no significant difference ($P > .01$) in activity occurred between native and apo LF for the 19 strains tested (Dionysius et al., 1993).

Experimental evidence suggests that resistance to the bacteriostatic effect of lactoferrin may be attributed to bacterial synthesis of iron chelators, which can compete with lactoferrin or transferrin for host iron. Moreover, lactoferrin resistance has not developed with even simple systems such as the adherence fimbria of enteroaggregative E coli; such fimbria are shed after exposure to lactoferrin despite the fact that such bacteria must have encountered lactoferrin many times over the years (Ochoa and cleary, 2009).

The effect of lactoferrin on growth of E.coli O26 isolated from Plain yoghurt carry hly gene in broth was studied. The concentration of lactoferrin 10 and 20 mg/ml cause significant inhibition compared with the control .The count decreased from8 x 105 at zero time to 6 x 103 and 4 x 103 after 72 h at the concentration of lactoferrin 10 and 20 mg/ml respectively (Figure2).

Xu et al. (2017) found that LF (0.5 mg/mL) was shown to have inhibition effects on E. coli O157:H7 at 8 and 24 h of incubation. The highest reduction was found to be 9 log cycles at the concentration of 2 mg/mL after 24 h and no microorganisms were observed after incubation for 8 h. At 8 and 24 h, For all the concentrations, the number of colonies increased significantly after 24 h, in comparison with those at 8 h.

Atef Yekta et al. (2010) revealed that at sub lethal concen¬trations, human and bovine lactoferrins acted bacteriostatically on E. coli O157:H7. The bacteria recovered and started to grow again. Also, Griffiths et al. (2003) examined the effect of the five Lf formulations on the in vitro growth of E. coli O157:H7 and stated that 66% iron-saturated bovine lactoferrin dramatic-ally slowed the growth of E. coli O157:H7 in single culture experiments, while 98% iron-saturated preparations had no effect. Furthermore, growth of E. coli O157:H7 was strongly inhibited starting at 4 hr and continuing through at least the 10-hr time. On the other hand, Murdock et al. (2007) revealed that up to 5000 µg/ml of lactoferrin was not inhibitory to E. coli O157:H7 using peptone yeast extract glucose medium.

Table (1 and 2) demonstrated the effect of lactoferrin on E. coli O1 isolated from Kariesh cheese carry stx2 and hly gene and E. coli O26 isolated from plain yoghurt carry hly gene in pasteurized milk for four days according to(GSO,2005 and EOS,2008). There was a Significant difference in comparison to control ($P < 0.05$) at the concentration of 10 and 20 mg/ml lactoferrin in pasteurized milk was noticed. Significant decrease have been observed on growth of E. coli O1.The count increased of E. coli O26 to 6 x 106 by the 4th day at both concentration of lactoferrin (10 and 20 mg/ml). E. coli might have also developed a bacterial defense system leading to blockage of lactoferrin (Atef Yekta et al., 2010).

The antimicrobial activity ascribed to lactoferrin and its peptides against E. coli showed vary between studies. This variation depends on lactoferrin purity, its iron saturation level, temperature, presence of different chelating compounds, water activity, pH, food components (lipid, protein and carbohydrate) and cations (Mg^{2+} and Ca^{2+}) (Rybarczyk et al., 2017). The ambient conditions such as pH, excessive iron content, calcium and phosphate-enhanced ionic environment reduce the antimicrobial activity of lactoferrin (Naidu, 2002). Also, blockage of LF could be due to LPS-mediated shielding of porins from LF interaction (Naidu et al., 1991) and/or to an interaction with a bacterial surface protein, as described by (Senkovich et al., 2007). Furthermore, Lactoferrin was hydrolysed with pepsin and the antimicrobial activity of the resulting hydrolysate was varied against enterohaemorrhagic Escherichia coli (Branen and Davidson, 2000).

5. CONCLUSION

Based on the current study, LF has varied inhibitory activity on E. coli non O157 in Lauria broth. In addition, there was significant difference in the count of E. coli O1and E. coli O26 at the concentrations of 10 and 20 mg/ml lactoferrin in pasteurized milk .Significant decrease have been observed on growth of E. coli O1. The count increased of E. coli O26 by the 4th day at both concentration of lactoferrin (10 and 20 mg/ml). lactoferrin could become a promising method to decrease growth of E. coli non O157 in pasteurized milk consequently decrease E.coli non O157 associated illness in humans.

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