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Lactoferrin's potential application in enhancing yoghurt's microbial and sensory qualities, with emphasis on the starter culture activity

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Research Article

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Abstract

This research paper aimed to examine the antibacterial activity of lactoferrin (LF) as a potential natural alternative in the dairy sector, by measuring its minimum inhibitory concentration (MIC) against a number of common food-borne pathogens as well as *Pseudomonas aeruginosa*, one of the major dairy product spoiling microorganisms. Additionally, a viability experiment was applied to laboratory-manufactured set yoghurt to assess its impact on the activity of starter culture, sensory properties and STEC survivability. The findings demonstrated that LF exhibited significant antimicrobial activity, particularly against *E. coli* and *S. typhimurium* with MIC values of 0.0001 and 0.01 mg/ml, respectively. However, *P. aeruginosa* and *B. cereus* were quite resistant to LF requiring higher concentrations for MIC (2.5 mg/ml). By the third day of storage, LF at 0.0001 and 0.001 mg/ml significantly reduced the survivability of Shiga toxin-producing *E. coli* STEC by 70 and 91.6%, respectively, in the lab-manufactured yoghurt. Furthermore, LF enhanced the sensory properties of fortified yoghurt with a statistically significant difference in comparison to the control yoghurt group. There was no interference with the activity of the starter culture throughout the manufacturing process and the storage period. In conclusion, the potent antimicrobial effect of LF opens a new avenue for the dairy industry's potential applications of LF as a natural preservative without negatively influencing the sensory properties and starter culture activity of fermented products.

Despite significant advances in food safety research, foodborne illnesses continue to be one of the major public health concerns that lead to global morbidity and mortality (Jenkins *et al.*, 2022). Food poisoning and intoxication happen despite the application of several food preservation measures during dairy production processes as a result of microbial growth and their potential toxin production (Gonelimali *et al.*, 2018; Quinto *et al.*, 2019). In the United Kingdom, there were an estimated 2.4 million instances of food-borne gastroenteritis in 2018, with 16 300 cases requiring hospitalization and more than 180 deaths (Jenkins *et al.*, 2022). *S. aureus, E. coli,* Salmonella species, and *B. cereus* are the most prevalent microorganisms isolated in the previous studies (Abdel-Salam and Soliman, 2019; Atia *et al.*, 2020; Adam *et al.*, 2021; Halim *et al.*, 2022; Taher *et al.*, 2022; Nadi *et al.*, 2023). In addition, *P. aeruginosa* is the leading cause of spoiled dairy products; it releases thermo-tolerant proteolytic and lipolytic enzymes that impact dairy product quality and shelf life (Eleboudy *et al.*, 2015; Ahmed *et al.*, 2021). Food-borne illnesses linked to yoghurt consumption have been reported in many countries (Cutrim *et al.*, 2017). Contamination of yoghurt with pathogens occurs mainly because of the use of raw milk, improper processing, inadequate thermal treatment, post-processing contamination, mishandling and poor sanitation programs (Salih *et al.*, 2018; Atia *et al.*, 2020; Taher *et al.*, 2020; Adam *et al.*, 2021; Nadi *et al.*, 2023).

Natural preservatives are expected to become a more popular alternative to synthetic ones for ensuring food safety (Rybarczyk *et al.*, 2017; Quinto *et al.*, 2019). Lactoferrin (LF) is a promising antibacterial compound that has recently been used against foodborne pathogens in the food industry (Ombarak *et al.*, 2019). LF is an 80 kDa multifunctional iron-binding glycoprotein, a member of the transferrin family, found naturally in exocrine secretions such as milk, saliva, tears, serum and the granules of neutrophilic polymorph nuclear leukocytes (Niaz *et al.*, 2019). Its concentration in milk ranges from 0.02 to 0.20 mg/ml (Taha *et al.*, 2019). It was first included in infant formula in 1986 and has subsequently been utilized in a wide range of products like toothpaste, food supplements and cosmetics (Taha *et al.*, 2019; Wang *et al.*, 2019). Consumer acceptance of LF has steadily increased in recent years following its approval as a food ingredient by the FDA in 2000 and the European Commission in 2012 (Franco *et al.*, 2018). Additionally, LF is purported to have antiviral, anticancer, antioxidant, anti-inflammatory and cell growth-promoting actions, and enhances the growth of the commensal probiotic in the gut microbiome

(Kell *et al.*, 2020). The antibacterial activity of LF has been explained by two mechanisms; (i) iron-dependency, by depletion of the microorganism's main food source, iron and (ii) iron-independent, where both Gram-negative lipopolysaccharide (LPS) and Gram-positive lipoteichoic acid (LTA) have been shown to interact specifically with LF, resulting in disruption of pathogen cell membranes, proteolysis of virulence factors and inhibition of their ability to adhere to the host cells through binding with glycosaminoglycans (GAGs: Taha *et al.*, 2019).

The application of LF in the dairy industry may face some challenges, such as its purity, iron saturation level, heat processing of the milk, presence of various chelating substances, water activity, pH, dairy product constituents (lipid, protein, and carbohydrate) and cations (Mg^{2+} and Ca^{2+}: Rybarczyk *et al.,* 2017). Some studies have reported that LF can promote the population growth of some lactic acid bacteria, although the mechanism of action has not yet been fully understood (Inay *et al.*, 2012). Hence, its effect on the starter culture is still not understood and requires further investigation. Therefore, this study aimed to investigate the antimicrobial effect of LF on the foodborne pathogens *S. aureus*, *E. coli*, STEC, *S. typhimurium* and *B. cereus,* in addition to *P. aeruginosa,* as one of the most common spoilage microorganisms in dairy products. Moreover, two in vitro experimental lab-manufactured set yoghurt were prepared, one to evaluate the LF effect on starter culture activity and sensory properties and the other a challenged model with Shiga toxin-producing *E. coli* to evaluate the LF effect on its survivability over a 14-day cold storage period.

Material and methods

Determination of the minimum inhibitory concentration (MIC) of LF: preparation of the bacterial strains

Antimicrobial activity was assessed using *S. aureus* ATCC25923, *E. coli* 25922, *S. typhimurium*14028, *B. cereus* 10876 and *P. aeruginosa* 27853 which were obtained from National Research Institute of Dokki, Egypt as well as a Shiga toxin-producing *E. coli* (STEC) of dairy origin previously isolated and identified by our research team (Fahim *et al.*, 2016). A pure culture of each bacterial strain was grown overnight in nutrient broth (Oxoid, USA) containing 0.6% yeast extract (Hi-media, UK) at 37°C. A ten-fold serial dilution was prepared, then a viable colony count of each strain was applied on their specific media (*S. aureus*; Baird-Parker (Hi-media, UK), *E. coli*; eosin methylene blue (Hi-media, UK), *S. typhimurium*; MacConkey agar (Hi-media, UK), *B. cereus*; mannitol egg yolk polymyxin agar (Hi-media, UK), *P. aeruginosa*; Pseudomonas agar base (Hi-media, UK) following the method described by Ahmed *et al.* (2021).

Preparation of LF concentrations

Different concentrations of LF (Sigma Aldrich, USA) were prepared using sterile distilled water (0.0001–0.001–0.01–0.1–1–2.5–5 mg/ml). The freshly prepared concentrations were used in the experiment.

Broth micro dilution method to determine the MIC of LF

MIC of the LF against the tested strains (*S. aureus* at 1.3×10^9 cfu/ml, *E. coli* at 2.6×10^9 cfu/ml, STEC at 2.79×10^{12} cfu/ml, *S. typhimurium* at 3.7×10^9 cfu/ml, *B. cereus* at 1.8×10^9 cfu/ml and *P. aeruginosa* at 85×10^7 cfu/ml) was performed using the broth micro dilution method modified by Habty and Ali (2022).

Impact of the different concentrations of LF on the activity of starter culture and sensory properties of laboratory manufactured set yoghurt

Raw buffalo milk was obtained from the dairy production unit, Faculty of Agriculture, Cairo University, Egypt. Raw milk was tested and confirmed to be free from any inhibitory substances following the method described by Ahmed *et al.* (2021). Raw milk was laboratory pasteurized at 80°C for 10 min, then cooled immediately in an ice bath to the inoculation temperature (44.5 ± 0.5°C) according to Oktavia *et al.* (2016). The amount of starter culture (Yo-Flex, UK) was added to the milk following the manufacturer's instructions with thorough mixing. Following that, milk was divided into six equal portions for the five treatments, which were derived from MIC concentrations; 0.0001% LF (treatment 1), 0.001% LF (treatment 2), 0.01% LF (treatment 3), 0.1% LF (treatment 4), 2.5% LF (treatment 5) as well as a control group without LF (treatment 6). The treated milk samples were thoroughly mixed and placed into sterile cups (200 g capacity) and incubated in a water bath at 44.5 ± 0.5°C for 3–4 h (until complete coagulation of the yoghurt), then transferred to a refrigerator (4°C). Samples were examined at zero time (end of yoghurt manufacturing), after 24, 72 h and every 3 d until the end of the storage period (14 d/4°C) for titratable acidity% according to APHA (2004). Sensory evaluation was done according to Zakaria *et al.* (2020) for treatments 1 and 2, these being the concentrations used in the viability study. A total of 21 panelists participated in the evaluation, 10 women and 11 men from the students and staff of the Faculty of Veterinary Medicine, Cairo University, ranging in age from 20 to 40 years. They received a training session for the yoghurt descriptive profile of sensory parameters: appearance (10), body and texture (30), flavor (45), packaging (5), and taste (10).

The activity of yoghurt starter culture was defined by its ability to ferment milk lactose and produce the acid that is responsible for the formation of yoghurt. Therefore, we depended on measuring the amount of lactic acid produced during the fermentation step rather than counting the starter culture.

Survivability of STEC in inoculated fortified lab-manufactured set yoghurt

Lab-pasteurized milk was inoculated with 4–6 $\log_{10}$ cfu/ml STEC followed by the addition of yoghurt starter culture according to the manufacturer's instructions. The inoculated milk was divided into three groups; the first was fortified with 0.0001% LF (treatment 1), the second with 0.001% LF (treatment 2), and the third was left as a control without the addition of LF. Both treatments and control groups were completed as described before. Samples were examined for total STEC count at zero-time (after complete manufacture of yoghurt), after 24, 72 h and every 3 d until the end of the storage period (14 d/4°C) following the method described by Silva *et al.* (2018).

A detailed account of the full materials and methods is provided in the online Supplementary File.

Statistical analysis

All experiments were carried out in triplicate and the average results were calculated and recorded using SPSS Version 26.0 software. Comparisons of sensory evaluation, titratable acidity and the viability study between the fortified and control groups and between the different LF concentrations were done using one-way analysis of variance (ANOVA), Kruskal–Wallis H and Mann–Whitney U tests. Significant results were set at *P*-value < 0.05.

Results

Determination of MIC of LF

Antimicrobial activity of the different LF concentrations (from 0.0001 to 5 mg/ml) against foodborne pathogens and spoilage microorganisms was tested using the micro dilution method. Concentrations were chosen based on previous studies and to determine the minimum effective concentration that could be used at the industrial level without affecting the starter culture activity. The results shown in Table 1 revealed that LF could affect all tested strains, of which *E. coli* and STEC were the most sensitive microorganisms with MIC values of 0.0001 mg/ml. However, *P. aeruginosa* and *B. cereus* were quite resistant, with an MIC of 2.5 mg/ml whilst *S. typhimurium* and *S. aureus* showed moderate susceptibility with MIC values of 0.01 and 0.1 mg/ml (Table 1).

Impact of different concentrations of LF on the activity of yoghurt starter culture

The onset of milk coagulation and the time required for making fortified yoghurt in both treated and control samples were observed and the titratable acidity percentage (TA%) was assessed throughout the processing and storage period (Fig. 1). Results revealed that there was no statistical significant difference between the control and fortified groups (*P* > 0.05). At the end of the storage period, acidity % of yoghurt samples reached 0.99, 1, 1.18, 1.2 and 1.22% in treatments T1 to T5, respectively. The value of this parameter increased over storage time, and the increase was non-significantly associated with increased LF concentrations.

Influence of LF on the sensory properties of lab-manufactured set yoghurt

The lab-manufactured set yoghurt fortified with two concentrations of LF (0.0001 and 0.001 mg/ml) and the control group (without fortification) were sensory evaluated and as seen in Table 2 both showed a statistically significant difference (improvement) in comparison to the control group (*P < 0.05*) with no difference between them. The fortified samples scored grade A concerning the overall acceptability throughout the storage period of 14 d/4°C, whilst the control samples had grade A during the first day only and then dropped to grade B until the end of the storage period. Briefly, flavor and body and texture scores of LF-fortified yoghurt achieved excellent scores throughout the storage period, while the control group achieved excellent scores during the first day then the score decreased to very good till the end of the storage period (Table 2).

Table 1. MIC values of LF against the examined microorganisms

Tested strains	MIC (mg/ml)
Staphylococcus aureus	0.1
Echerichia coli	0.0001
Shiga toxin producing E. coli	0.0001
Salmonella typhimurium	0.01
Bacillus cereus	2.5
Pseudomonas aeruginosa	2.5

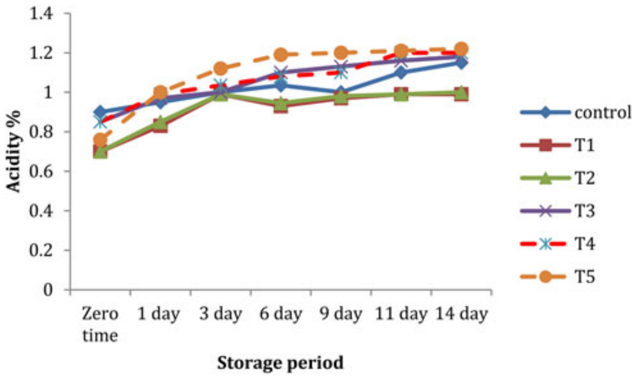

Figure 1. Titratable acidity % of lab-manufactured fortified set yoghurt with different concentrations of the lactoferrin (LF) over the storage period of 14 d at 4°C. Control, (without Lf); T1, (0.0001 mg/ml); T2, (0.001 mg/ml); T3, (0.01 mg/ml); T4, (0.1 mg/ml); T5, (2.5 mg/ml).

Survivability of STEC in inoculated fortified lab-manufactured set yoghurt

The data are shown in Fig. 2. After 72 h of storage STEC survivability was reduced by 70, 91.6 and 56% in T1 (0.0001 mg/ml LF), T2 (0.001 mg/ml LF) and control (without LF) samples, respectively. In T1 and T2 this decline continued until the inoculated strain completely disappeared by the end of the storage period, while STEC remained viable (at 10^3 cfu/g) in the control group until the end of the storage period (Fig. 2).

Discussion

Foodborne illness causes economic losses and puts the general public's health at risk (Jenkins *et al*., 2022). Consumer awareness of the hazards linked to the use of synthetic chemical preservatives has grown significantly in recent years. Furthermore, food producers confront significant hurdles in producing food that is both safe and of high quality in terms of nutritional benefits and sensory attributes (Ahmed *et al*., 2021). LF has significant antibacterial activity as its iron binding capability is double that of transferrin, and this bond is strong enough to withstand the low pH values of fermented dairy products (Duran, 2021). Therefore, it is considered a great natural alternative to chemical preservatives, especially after the FDA certification as a food additive (Franco *et al*., 2018).

Our results confirmed the antibacterial activity of LF and showed that gram-negative pathogens were more susceptible than Gram-positive ones. This result could be attributed to the interaction of LF with the anionic structure of LPS in the bacterial membrane, causing membrane instability, detachment of LPS and bacterial death (Hafez *et al*., 2013; Sijbrandij *et al*., 2017). Likewise, Kutila *et al*. (2003) revealed that the most effective inhibitory activity of LF was against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) rather than Gram-positive (*S. aureus* and coagulase-negative *S. aureus*). However, Jahani *et al*. (2015) observed the opposite, that bactericidal effects were more pronounced against Gram-positive bacteria (*S. epidermidis, B. cereus*) than Gram-negative bacteria (*C. jejuni,* and Salmonella). Moreover, Karam-Allah *et al*. (2022) recorded that LF was more

Table 2. Sensory evaluation of the fortified laboratory manufactured set yoghurt with the studied concentrations of LF

	Flavor (45)			Body and texture (30)			Taste (10)			Appearance (10)			Package (5)			Overall acceptability (100)		
	C	T1	T2	C	T1	T2	C	T1	T2	C	T1	T2	C	T1	T2	C	T1	T2
Zero time	44	44	44	30	30	30	9	10	10	9	10	10	5	5	5	97 (A)	99(A)	99(A)
1 d	40	44	43	27	30	30	8	10	10	9	10	10	5	5	5	89(A)	99(A)	98(A)
3 d	38	44	43	24	30	30	7	9	9	9	10	10	5	5	5	83(B)	98(A)	98(A)
6 d	38	43	42	24	30	30	7	9	9	9	10	10	5	5	5	83(B)	97(A)	96(A)
9 d	38	42	42	24	30	30	7	8	8	9	10	10	5	5	5	83(B)	95(A)	95(A)
11 d	38	42	42	24	30	30	7	8	8	9	10	10	5	5	5	83(B)	95(A)	95(A)
14 d	38	42	42	24	30	30	7	8	8	9	10	10	5	5	5	83(B)	95(A)	95(A)

C, control (without lactoferrin); T1, Treatment 1(0.0001 mg/ml LF); T2, Treatment 2 (0.001 mg/ml LF); acceptability grading as follows, Grade A (excellent), >86%; Grade B (very good), 73≤ 86%; Grade C (good), 60 ≤ 73%.

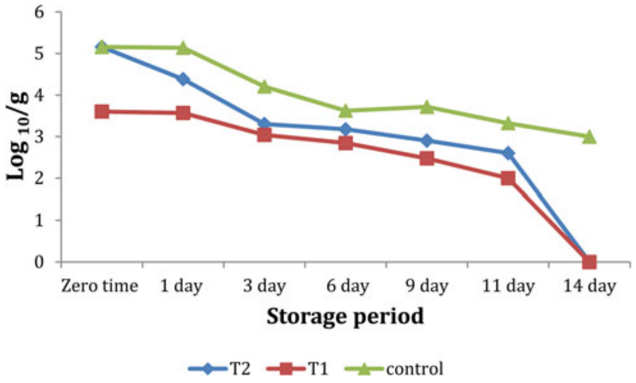

Figure 2. Counts of Shiga toxin producing *E. coli* ($\log_{10}$/g) during the storage period of inoculated fortified lab-manufactured set yoghurt (14 d/ 4°C).
Control, (without Lf); T1, (0.0001 mg/ml); T2, (0.001 mg/ml).

effective against Gram-positive (*S. aureus* and *B. cereus*) than Gram-negative (*E. coli*). In the present study, *E. coli* and STEC were the most susceptible to LF's effect, which may be attributed to the positively charged N-terminus of LF which hinders the interaction between LPS and bacterial cations (Ca^{2+} and Mg^{2+}) and interferes with aggregative proliferation in *E. coli* (Moradian *et al.*, 2014). On the other hand, *B. cereus* and *P. aeruginosa* were the most resistant to the effects of LF, which may be ascribed to their capacity to produce biofilm that protects them from the LF effect. Biofilm-associated bacteria are up to 1000 times more resistant to antimicrobial agents than planktonic bacteria (Majed *et al.*, 2016; Thi *et al.*, 2020). Our results were nearly similar to those obtained by Hafez *et al.* (2013) who reported that 3 mg/ml of the LF completely inhibited *E. coli* after 1 h of incubation, while the time required for *P. aeruginosa* suppression extended to 6 h and there was a slight inhibition of *S. aureus* compared to control. On the contrary, Embleton *et al.* (2013) reported that *P. aeruginosa* was more susceptible to LF effect than *E. coli.*

We examined the effect of LF on starter culture activity. The results (Fig. 1) revealed that the addition of LF to yoghurt had no effect on the yoghurt's onset of coagulation time or the starter culture's rate of growth throughout the processing stage, therefore it can be added safely in the fermented products. Numerous studies showed that the microbial growth-stimulating effect of LF may be linked to the presence of proteins that bind LF on the bacterial surface. Therefore, LF may be a pathway for acquiring iron if the bacteria (in this case, starter culture) have exterior membrane receptors capable of specifically attaching to the LF-iron complex, causing the internalization of the metal (Modun *et al.*, 2000; Kim *et al.*, 2004). Our data are in agreement with reports of Matijašić *et al.* (2020) and Duran (2021), who investigated the impact of various LF concentrations on the growth rate of lactic acid bacteria in raw milk and found that 5.0 mg/ml promoted the growth of lactic acid bacteria. On the other hand, Zakaria *et al.* (2020) reported that the titratable acidity% increase while processing yoghurt fortified with LF was slower than that of the control, which they attributed to the partial inhibition of lactic acid-producing microorganisms. Additionally, Franco *et al.* (2010) studied the effect of different concentrations of LF at 2 levels of iron saturation (holo –apo) on the fermentation process of milk and found LF-holo did not affect the fermentation of milk and its transformation into yoghurt, while the addition of LF-apo delayed milk acidification. Type, iron saturation level, and concentrations of LF are variables influencing LAB to varying degrees.

We examined the effect of LF on the sensory properties of fortified set yogurt (Table 2). LF is a component of milk, so it is expected that its presence in fortified dairy products would not have a negative impact on their organoleptic and sensory qualities, however, this has not previously been fully investigated. Results revealed a positive effect of LF on the sensory properties of fortified yoghurt. Similarly, Ombarak *et al.* (2019) demonstrated that adding LF could enhance the sensory qualities of cheese, with larger concentrations of LF producing the best results during the storage period. Furthermore, Zakaria *et al.* (2020) reported that LF-treated yoghurt was satisfactory and had no adverse effects on the yoghurt's taste or odor. These findings open the door for more applications of lactoferrin in other dairy products as they showed that adding LF to fermented dairy products would not only have antibacterial activity but could also improve their sensory attributes without disrupting the fermentation process.

STEC is one of the most prevalent pathogens affecting humans globally and causing serious infections such as hemorrhagic colitis, stomach pain, bloody diarrhea and hemolytic uremic syndrome. Moreover, it is an important cause of acute renal failure in children (Kieckens *et al.*, 2017). STEC could be isolated from several foods, including yoghurt which has an acidic pH (4.4: Fahim *et al.*, 2016). *E. coli* O157:H7 was found to survive for 10 d in inoculated yoghurt during a study conducted by Cutrim *et al.* (2017). Being highly acid resistant, the infectious dose of *E. coli* O157:H7 is very low, between 1 and 100 cfu/g, much lower than for most other entero-pathogens, which increases the risk of disease (Ababu *et al.*, 2020). Contamination of dairy products with such pathogenic organisms could be attributed to the poor hygienic conditions under which they were processed and/or stored. Its presence is an indicator of fecal contamination and suggests that other food-borne pathogens of fecal origin may also be present (Mohamed *et al.*, 2020). We examined the survivability of STEC in inoculated LF fortified yogurt. A viability study with two concentrations of LF (0.0001 and 0.001 mg/ml) demonstrated the presence of a statistically significant difference between the test and control groups as well as between the two LF concentrations, the higher being more effective ($P < 0.05$). Xu *et al.* (2017) and Ombarak *et al.* (2019) used higher concentrations of LF (0.5 and 4 mg/ml, respectively) to achieve the same effect against *E. coli* O157:H7, as did Hassan *et al.* (2022) in Tallega cheese (1 mg/ml LF in this case). On the other hand, Taha *et al.* (2019) reported that survivability of *E. coli* O26 was not affected by either 10 or 20 mg/ml LF, which they attributed to bacterial defense mechanisms developed by *E. coli* that prevented LF from binding with it. Positive effects of LF against inoculated *E. coli* could be attributed to its binding to ions which are crucial for microbial survival and growth, leading to inhibition of microbial proliferation and death. Since LF has significant levels of amylase, DNase, RNase and ATPase activity, it can kill bacteria by damaging their nucleic acids (Taha *et al.*, 2019).

In conclusion, this study emphasized the further potential applications of LF as a natural preservative alternative in fermented and non-fermented dairy products in the dairy industry.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000675

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