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Antibacterial and Anti-colon Cancer Potentials of Fermented Acidophilus Milk Supplemented with Water Soluble Curcumin

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ABSTRACT

Background: Different beneficials of fermented milk products-containing *Lactobacillus acidophilus* to improve health status were investigated.

Methods: Fermented acidophilus milk samples were prepared with freeze dried *Lb. acidophilus* La-5 and 0, 50, 100 and 150 mg/100 mL of water-soluble curcumin were added at the end of fermentation. Crude extracts (whey) of different fermented acidophilus milk formulates with curcumin were prepared and each extract was tested for its antibacterial activity, total phenolic content, DPPH free radical scavenging activity, anti-colonic cancer activity and fermentation profile (measured as lactic acid concentration). Further the sensory evaluation for each sample was conducted.

Result: The results showed that no effect of the addition of curcumin on the viability of *Lb. acidophilus* La-5 either fresh or after 20 days of cold storage. However, the higher the quantity of curcumin added, the higher the antibacterial activity. The highest antibacterial effect was against *Staph. aureus* NRRL B-314. The total phenolic content and the DPPH free radical scavenging activity increased with the increase in curcumin. The higher the proliferative inhibition was achieved at higher level of curcumin. The addition of curcumin to fermented acidophilus milk samples help increase the quality of the product with negative effect on the sensory evaluation at higher level of curcumin. Finally, supplementation of fermented acidophilus milk with curcumin has no effect on the lactic acid production in fermented acidophilus milk samples. Therefore, the synergistic effect of water-soluble curcumin and *Lb. acidophilus* could be recommended for enhancing the functional properties of acidophilus milk.

Key words: Acidophilus milk, Antibacterial activity, Anticancer activity, Antioxidant activity, *Lb. acidophilus* La-5.

INTRODUCTION

Fermented milk is a famous dairy product that are produced by fermentation of milk using different types of starter cultures (Aryana and Olson, 2017). Globally, there is an increasing awareness among the consumers towards consumption of more healthy dairy foods. There was also an interest in the health potentials of the normal microbiota that are live in the gastro-intestinal tract such as Lactobacilli (Mitsuoka, 1992). This type of bacteria that capable of exerting health benefits to the host is called probiotic bacteria (Wood, 1992; Darsanaki *et al*., 2014). Currently, a lot of fermented milk products-containing *Lactobacillus acidophilus*-which sometimes called *Lactobacillus* fermented milk (FAM)-is commercially available in the global market (Mortazavian *et al*., 2006). Moreover, several investigators found of different beneficials of FAM such as its ability to improve health status (Ojha *et al*., 2022), prophylactic effects (Sarkar and Misra, 2010), nutritional status improvement of young children (Silva *et al*., 2008), reduction of bad cholesterol levels in hypercholesterolemic patients (Andrade and Borges 2009), protects against *Salmonella typhimurium* (Adriani *et al*., 2024) and anti-cancer characteristics (Abdelrazik *et al*., 2022).

However, fermented milk products with probiotic bacteria showed a short-term suitability as they are perishable due to its short shelf-life. The problem is that the most common process to improve the shelf-life of fermented milk products is a heat treatment that is negatively affected the viability of probiotic bacteria (Behare and Prajapati, 2007). In addition, the increased interest of consumers to search for dairy products that are natural with no artificially synthesized additives has encourage the researchers to find a natural antimicrobial food component to be added to the probiotic fermented milk to increase its shelf-life by affecting the undesirable microorganisms with no effect on probiotic bacteria (Batiha *et al*., 2021).

Curcumin is a polyphenolic derivative compound from turmeric (Chainani-Wu, 2003). It is highly important to choose water-soluble form of curcumin as several curcuminoid extracts with low solubility are precipitated when added to

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fermented milk products (Gholipour *et al*., 2023). The curcumin itself showed several health potentials such as anti-Cryptosporidium (Ganai *et al*., 2023) anti-inflammatory, anticancer, antioxidant and immunomodulatory properties (Abd El-Hack *et al.* 2021; Veni *et al*., 2022), in both animals and humans (Haftcheshmeh *et al*., 2020). Apparently, oral administration of curcumin seems to have anti-gastrointestinal tract cancers potential (Stanić, 2017). Gholipour *et al.* (2023) found that curcumin increased the anti-cancer ability of probiotic bacteria due to induction of more apoptosis against cancer cells than probiotic bacteria alone. Moreover, it is well-known as a natural antimicrobial compound (Maurya *et al*., 2020). Therefore, several researchers found positive effects of supplementing fermented milk with curcumin. Gereltuya *et al* (2015) found a remarkable growth rate of lactic acid bacteria when curcumin was added on in milk during fermentation. Similarly, Buniowska-Olejnik *et al.* (2023) found a higher vital activity of the probiotic bacteria due to the addition of curcumin.

As it can be noticed from the previously published work, little number of studies focused on the health benefits of the combined effects of *Lb. acidophilus* and water-soluble curcumin. Therefore, the current study was designed to explore the antibacterial and anti-colon cancer potentials of *Lb. acidophilus*-fermented milk supplemented with water-soluble curcumin. In addition, the current study designed to examine the effect of different levels of water-soluble curcumin to better understand its positive combined effects. Moreover, the current work assessed the impact of curcumin levels on sensory characteristics.

MATERIALS AND METHODS

Materials

Lb. acidophilus La-5 (CHR-Hansen, Copenhagen, Denmark) was honorably gifted from MIFAD Co. Badr City, Cairo, Egypt. 1,1-diphenyl-2-picrylhydrazyl (DPPH), Water soluble curcumin (98.5% turmeric-oleoresin extract) and De Man Rogosa and Sharpe (MRS, pH 5.3) agar medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA), DolCas-Tenshi Bioceuticals Co. (New Jersey, USA) and Merck Co., Darmstadt, Germany, respectively.

Preparation of fermented acidophilus milk

Fermented acidophilus milk (FAM) was prepared in the laboratories of Department of Dairy Science, Faculty of Agriculture, Cairo University, Egypt, during June to October 2023. Fermented acidophilus milk (3.5% fat and 12% total solids) with freeze dried *Lb. acidophilus* La-5 was prepared according Kandylis *et al* (2016). Briefly, milk was heated to 95°C, cooled to 37°C, inoculated with *Lb. acidophilus* La-5and incubated at 37°C. Water soluble curcumin at 0, 50, 100and 150 mg/100mL was added at the end of fermentation (pH 4.4±0.02) then mixed with laboratory mixer (Silverson Mechines, Inc., East Longmeadow, USA) and finally stored at 5°C. The selection of curcumin levels was based on a recent report (Abdelrazik and Elshaghabee, 2021). Samples from the prepared fermented acidophilus milk was collected for further analysis when fresh and after 20 days of cold storage at 5°C.

Preparation of indicator bacteria

Pure cultures of *Bacillus stearothermophilus NRRL B*-4419, *Staphylococcus aureus* NRRL B-314*, Escherichia coli* NRRL B-3008 and *Pseudomonas fluorescens* NRRL B- 15132 were obtained from NRRL laboratory, Illinois, USA. All cultures were propagated twice in nutrient broth (Oxide, Yorkshire, UK) at 37°C for 18 h.

Preparation of crude extract

Crude extract (whey) was prepared by centrifugation of each sample as previously described by Abdelrazik and Elshaghabee (2021).

Antibacterial Activity

The antibacterial activity of each whey fraction was evaluated against tested indicator bacterial strains using agar well diffusion method as previously described by Varadaraj *et al.* (1993).

Total polyphenol content

The total polyphenol content of each sample was assessed using crude extract of different fermented acidophilus milk treatments according to Singleton and Rossi (1965). The method was briefly described by Abdelrazik and Elshaghabee (2021).

DPPH Free radical scavenging

The antioxidant activity of samples based on the scavenging activity of DPPH free radical, was measured using crude extract of different fermented acidophilus milk treatments according to the method that described by Lee *et al.* (2004).

Anti-colonic cancer activity

The human colon cancer cell lines HT-29 (National Institute of Piles, Cairo University, Egypt) was cultured at 37°C in a 5% CO_2 and 95% air atmosphere. HT-29 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL, Sigma-Aldrich, St. Louis, USA). The anti-human colon cancer cells proliferation was determined by treating cancer cells with 100 µg of fresh crude extract of different fermented acidophilus milk treatmentsand using methylene blue assay (Qian *et al*., 2020).

Lactic acid concentration

Levels of lactic acid (µg/mL) as main metabolite in different fermented acidophilus milk treatments (when fresh and after 20 days of cold storage) was assessed using high performance liquid chromatography as previously described by Elshaghabee *et al.* (2016).

Sensory evaluation

Fresh samples of fermented acidophilus supplemented with curcumin were sensorily assessed using a 9-point hedonic scale from 1; dislike extremely to 9 like extremely (Junaid *et al.*, 2013). The sensory evaluation was conducted by 10 experienced panelists from the Department of Dairy Science, Faculty of Agriculture, Cairo University, Egypt. The samples' size were 120 g each (cooled at 10°C) and served at room temperature.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by assessment of differences by Tukey's post-hoc test. For sensory evaluation, Dunnett post-hoc test was used to perform multiple comparisons with control. All statistical calculations were performed using SPSS version 22.0. Results were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Viability of *Lb. acidophilus* La-5

Table 1 is showing the viability of *Lb. acidophilus* La-5 (Log CFU/g) during cold storage at 5°C of different fermented acidophilus milk supplemented with curcumin. The viability of *Lb. acidophilus* La-5 (Log CFU/g) showed no significant differences ($P>0.05$) among all treatments. The range of the counts of *Lb. acidophilus* La-5 is between 8.15±0.57 to 8.40±0.53 Log CFU/g. After 20 days of storage period all treatment showed less viability compared to fresh treatment. However, there was no significant difference on the viability among all treatment after 20 days of *Lb. acidophilus* La-5 (Log CFU/g). The results showed that no effect of the addition of curcumin on the viability of *Lb. acidophilus* La-5 (Log CFU/g) either fresh or after 20 days of cold storage.

Table 1: Viability of *Lb. acidophilus* La-5 (Log CFU/g) during cold storage (5°C) period of different fermented acidophilus milk treatments supplemented with curcumin.

Treatments	Fresh	20 days
FAM	8.40±0.53^{a}	7.50±0.75^{b}
FAMC50	8.32±0.61^{a}	7.30±0.80^{b}
FAMC100	8.30±0.91^{a}	7.40±1.01^{b}
FAMC150	8.15±0.57^{a}	7.42±0.92^{b}

FAM: Fermented acidophilus milk, FAMC50: Fermented acidophilus milk supplemented with 50 mg/100 mL curcumin, FAMC100: Fermented acidophilus milk supplemented with 100 mg/100 mL curcumin, FAMC150: Fermented acidophilus milk supplemented with 150 mg/100 mL curcumin. Data are expressed as means ± SD (n=3). Mean values in the same column bearing the same superscript do not differ significantly ($P>0.05$).

Antibacterial activity

The antibacterial activities of fresh crude extract of different fermented acidophilus milk treatments supplemented with curcumin are presented in Table 2. As can be noticed, the highest antibacterial effect of all treatments was shown against *Staph. aureus* NRRL B-314 followed by *B. stearothermophilus NRRL B*-4419, *Ps. fluorescens* NRRL B-15132 and *E. coli* NRRL B-3008, respectively. The antibacterial effect was higher at samples supplemented with 150 mg of curcumin followed by sample supplemented with 100, 50 mg curcumin. The sample with no added curcumin showed the lowest antibacterial activities. It can be concluded that the higher the quantity of curcumin added, the higher the antibacterial activity.

Similarly, curcumin improved antibacterial activity (Singh and Singh, 2016; Gholipour *et al.*, 2023). In addition, water soluble curcumin showed the highest antibacterial activity in low-fat yoghurt (Buniowska-Olejnik *et al.*, 2023) and in fermented cow or soy milk (Abdelrazik and Elshaghabee, 2021). The proposed mechanism of this antibacterial effect of curcumin is it able to prevent the biofilm formation by harmful bacteria through many interactions and transduction pathways (Mun *et al.*, 2013; Zheng *et al.*, 2020).

Total phenolic compounds and DPPH free radical scavenging activity

The total polyphenols content and DPPH free radical scavenging activity of fresh crude extract of different fermented acidophilus milk treatments are presented in Table 3. As appears from the table the content of total phenolic compounds is increased with the increased quantity of added curcumin. On the other hand, the DPPH free radical scavenging activity increased with the increased addition

Table 2: Antibacterial activity of fresh crude extract of different fermented acidophilus milk treatments supplemented with curcumin.

Treatments	Diameter of inhibition zone, mm			
	B. stearothermophilus	*Staph. aureus*	*E.coli* NRRL	*Ps.fluorescens*
	NRRL B-4419	NRRL B-314	B-3008	NRRL B- 15132
FAM	8.30±0.35^{d}	9.35±0.51^{c}	5.30±0.38^{c}	6.40±0.38^{c}
FAMC50	9.75±0.57^{c}	11.25±0.25^{b}	7.32±0.45^{b}	7.20±0.25^{b}
FAMC100	10.72±1.03^{b}	12.10±0.42^{a}	8.01±0.63^{a}	8.10±0.45^{a}
FAMC150	11.65±0.56^{a}	12.80±0.35^{a}	8.73±0.58^{a}	8.90±0.31^{a}

FAM: Fermented acidophilus milk, FAMC50: Fermented acidophilus milk supplemented with 50 mg/100 mL curcumin, FAMC100: Fermented acidophilus milk supplemented with 100 mg/100 mL curcumin, FAMC150: Fermented acidophilus milk supplemented with 150 mg/100 mL curcumin. Data are expressed as means ± SD (n=3). Mean values in the same column bearing the same superscript do not differ significantly ($P >0.05$).

of curcumin too. Moreover, the ascorbic acid which is the main organic acid produced by *Lb. acidophilus* La-5 showed the highest DPPH free radical scavenging activity. The DPPH scavenging activity was higher at samples supplemented with 150 mg of curcumin followed by sample supplemented with 100, 50 mg curcumin. The sample with no added curcumin showed the lowest DPPH scavenging activity.

It can be concluded that the higher the quantity of curcumin added, the higher the DPPH scavenging activity. The same trend was showed by the total phenolic content. The reason behind that is the addition of curcumin to fermented acidophilus milk; curcumin is naturally high in both total phenolic content and DPPH scavenging activity. Other studies showed similar results (Singh and Singh, 2016; Abdelrazik and Elshaghabee, 2021; Ricardo *et al.*, 2023).

Proliferative inhibition (%) of human colon cell lines

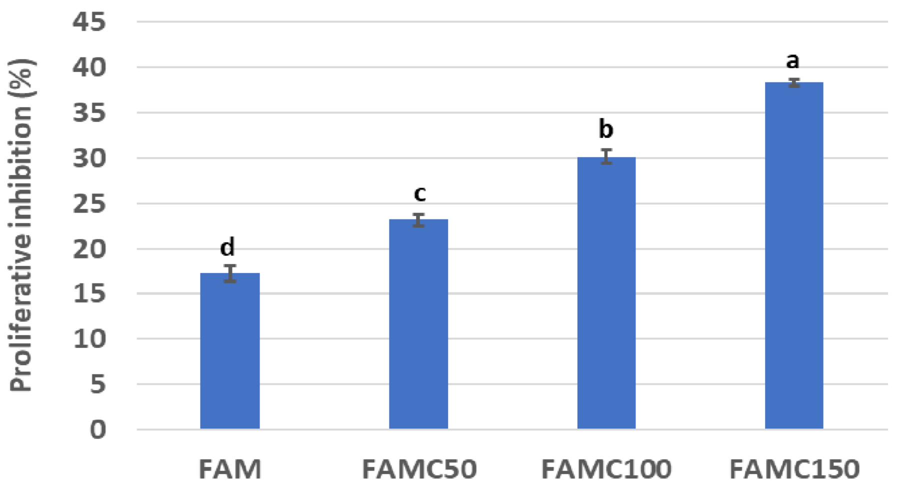
Fig 1 shows the proliferative inhibition (%) of human colon cell lines treated with 100 µg of fresh crude extract of different fermented acidophilus milk treatments. The proliferative inhibition was higher at samples supplemented with 150 mg of curcumin followed by sample supplemented with 100, 50 mg curcumin. The sample with no added curcumin showed the lowest proliferative inhibition. It can be concluded that the higher the quantity of curcumin added, the higher the proliferative inhibition. The addition of curcumin is clearly the reason behind the change in proliferative inhibition among the fermented acidophilus milk samples.

Recently, there is substantial interest in the anticancer effects of probiotic bacteria. For example, it was found that the supernatant of L. *rhamnosus* significantly decreased the growth of HT 29 cancer cells (Dehghani *et al.*, 2021). Moreover, the anticancer effect of L. *plantarum* against HT 29 cell line was significantly increased when bacterial cells treated with curcumin (Gholipour *et al.*, 2023). In addition, Abdelrazik and Elshaghabee (2021) reported an increased proliferative inhibitory activity of fermented soy milk

Table 3: Total polyphenols content and DPPH free radical scavenging activity of fresh crude extract of different fermented acidophilus milk treatments.

Treatments	Total polyphenols content	% DPPH scavenging
FAM	21.15±0.21[d]	63.25±0.81[d]
FAMC50	32.05±0.17[c]	74.31±0.56[c]
FAMC100	44.25±0.26[b]	82.75±0.61[b]
FAMC150	53.01±0.36[a]	89.01±0.84[a]
Ascorbic acid	-	94.01±0.06[a]

FAM: Fermented acidophilus milk, FAMC50: Fermented acidophilus milk supplemented with 50 mg/100 mL curcumin, FAMC100: Fermented acidophilus milk supplemented with 100 mg/100 mL curcumin, FAMC150: Fermented acidophilus milk supplemented with 150 mg/100 mL curcumin. Data are expressed as means ± SD (n=3). Mean values in the same column bearing the same superscript do not differ significantly (P>0.05).



FAM: Fermented acidophilus milk, FAMC50: fermented acidophilus milk supplemented with 50 mg/100 mL curcumin, FAMC100: Fermented acidophilus milk supplemented with 100 mg/100 mL curcumin, FAMC150: Fermented acidophilus milk supplemented with 150 mg/100 mL curcumin. Data are expressed as means ± SD (n = 3). Bars bearing the same superscript do not differ significantly (P>0.05).

Fig 1: Proliferative inhibition (%) of human colon cell lines treated with 100 µg of fresh crude extract of different fermented acidophilus milk treatments.

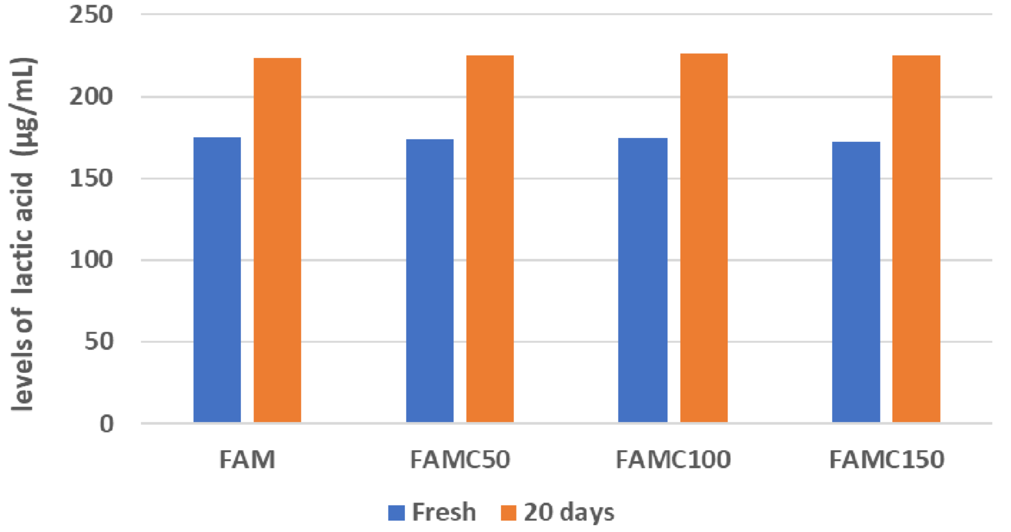
supplemented with curcumin against HT-29 human colon cell lines. The proposed mechanism of this anticancer effect is could be due to the produced fermentation metabolites by probiotic bacteria that directly inhibit the growth of cancer cells due to activation of caspase-3 protein (Baldwin *et al.*, 2010). When curcumin was added, remarkably higher rate of apoptosis in cancer cells was observed (Gholipour *et al.*, 2023).

Lactic acid content

Fig 2 presents the levels of lactic acid (µg/mL) as measured by HPLC method in different fermented acidophilus milk treatments during cold storage period. The lactic acid content increased significantly after 20 days of storage at cold temperature. However, it appears that the addition of curcumin to the fermented acidophilus milk samples has no effect on the lactic acid production. These results are in line with those found by Buniowska-Olejnik *et al.* (2023) who reported that the addition of curcumin was not affected the acid production in low fat yoghurt.

Sensory evaluation

Table 4 shows the sensory evaluation of different acidophilus fermented milk treatments. The consistency of all treatments was similar to that of the control. The evaluation of flavor and appearance was reduced with the increased amount of curcumin. As it can be noticed that the control (FAM) showed the highest overall acceptability (8.1±0.77). When the FAM was supplemented with curcumin, a reduction in the overall acceptability was appeared. The curcumin-supplemented treatments that showed a comparable overall acceptability with the control were the treatment with 50 and 100 mg/100 mL curcumin (overall acceptability of 7.9±0.57 and 7.8±0.89, respectively). Statistical analysis showed a significant reduction in the overall acceptability of FAM when curcumin was added with 100 mg/100mL ($P<0.05$) and 150 mg/100mL ($P<0.01$). Buniowska-Olejnik *et al.* (2023) who found similar organoleptic characteristics between control and curcumin supplemented low fat yoghurt samples ($P>0.05$).



FAM: Fermented acidophilus milk, FAMC50: Fermented acidophilus milk supplemented with 50 mg/100 mL curcumin, FAMC100: Fermented acidophilus milk supplemented with 100 mg/100 mL curcumin, FAMC150: Fermented acidophilus milk supplemented with 150 mg/100 mL curcumin.

Fig 2: Levels of lactic acid (µg/mL) as measured by HPLC method in different fermented acidophilus milk treatments during cold storage period.

Table 4: Sensory evaluation of different acidophilus fermented milk treatments.

Treatments	Flavor	Consistency	Appearance	Overall acceptability
FAM	8.1±0.57	8.2±0.63	8.1±0.74	8.1±0.77
FAMC50	7.5±0.53	8.1±0.74	7.7±0.82	7.9±0.57
FAMC100	7.2±0.43*	8.3±0.67	7.2±0.42*	7.8±0.89*
FAMC150	5.9±0.74**	7.4±0.97	6.2±0.92**	6.5±0.85**

FAM: fermented acidophilus milk, FAMC50: fermented acidophilus milk supplemented with 50 mg/100mL curcumin, FAMC100: fermented acidophilus milk supplemented with 100 mg/100mL curcumin, FAMC150: fermented acidophilus milk supplemented with 150 mg/100mL curcumin. Data are expressed as means ± SD (n=10 experienced panelists). Dunnett Post-hoc test was used for multiple comparisons with control. *: significantly differed from the FAM ($P<0.05$). **: significantly differed from the FAM ($P<0.01$).

Similarly, a gel-like curcuma suspension did not affect the sensory characterization of yoghurt (Guerra *et al.*, 2022).

CONCLUSION

Our results showed that addition of water soluble-curcumin did not negatively affect the viability of *Lb. acidophilus* La-5, either fresh or after 20 days of cold storage, because curcumin was added after milk fermentation by *Lb. acidophilus*. Furthermore, the higher the quantity of curcumin added, the higher the antibacterial activity. The highest antibacterial effect was against *Staph. aureus* NRRL B-314. Both the total phenolic content and the DPPH free radical scavenging activity increased with higher curcumin quantity. Proliferative inhibition was also more pronounced at higher level of curcumin. Based on the sensory evaluation results, the addition of curcumin to fermented acidophilus milk at levels of 50 or 100 mg/100 mL is recommended, as it could enhance selected functional characteristics of acidophilus milk while maintaining good level of consumer acceptability.

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Conflict of interest

The authors have no conflicts of interest to declare.

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