ISSN: 2347-467X, Vol. 09, No. (3) 2021, Pg. 961-969

Current Research in Nutrition and Food Science

www.foodandnutritionjournal.org

Enhancement of Selected Health Benefits in Fermented Cow and Soy Milk Supplemented with Water Soluble Curcumin

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Abstract

Different formulates of fermented cow and soy milk by *Lactobacillus plantarum* EMCC 1027 with 50, 100, and 150 mg/100mLcurcumin were investigated for antibacterial, antioxidant, anti-colonic cancer, and anti-inflammation activities. Also, the viability of *L. plantarum* was monitored during cold storage period. Our results showed that values of antibacterial, antioxidant, anti-colonic cancer, and anti-inflammation activities in crude extracts of fermented soy milk were significantly increased in comparison with crude extracts of fermented cow milk. The addition of different concentrations (100 and 150 mg/100mL) of curcumin had a significant enhancement effect for all selected health benefits properties. The increase inantioxidant capacity of different crude extracts was in a good correlation with their polyphenols content. Addition of water soluble curcumin did not have any adverse effect on the viability of *L. plantarum* during fifteen days of cold storage. Therefore,the synergistic effect between fermented cow/soy milk and water solublecurcumin could be recommended. Indeed, extensive research is still needed in order to investigate the molecular mechanisms of health a benefit of different formulates of fermented cow/soy milk and watersoluble curcumin.

Article History
Received: 20 July 2021
Accepted: 26 October 2021

Keywords
Curcumin;
Fermented Soy Milk;
Functional Dairy Foods;
Lactobacillus plantarum;
Synergistic Effect

Introduction

Over the last decade, functional foods, in other words, healthy foods, has a great impact on the health of consumers with economic benefits in both grow and industrialized countries.[1] Fermented milk as functional food are firstly commercialized as probiotics carrier foods by Danisco.[2] Several researches illustrated that different probiotic strains could grow in milk and survive well in fermented milk.[3,4,5] Soy milk as a plant-based non-dairy alternative milk is useful as a dietary adjunct for lactose intolerance patients.[6] It is also rich source offlavonoids and oligosaccharides. Therefore,soy milk ismore preferable for growth of probiotics than cow milk.[7]

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Doi: http://dx.doi.org/10.12944/CRNFSJ.9.3.23

A probiotic is defined as "a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract".[8,9] Probiotics have wider range of beneficial effects such as lowering plasma cholesterol level.[10] anticarcinogenic, and antioxidantactivities.[11,12,13] Genera of *Lactobacillus* and *Bifidobacterium* are used as probiotics.[14] *L. plantarum* had many applications in different fermented foods[15] with different probiotic properties.[16]

Curcumin as a functional ingredient with yellow pigment isisolated from turmeric. It is well known to possess desire health benefits; antimicrobial, anticancer, antioxidant and it could act as a complementary therapy for the treatment of Alzheimer disease.[17] Gutierres *et al*[18] illustrated that supplementation of yogurt with curcumin could enhance the glycemic index in diabetic rats. On the other hand, supplementation of yogurt with 1% curcumin decreased the overall acceptability.[19]

The health effect of the synergistic effect of curcumin-fermented milk still needs further exploration and the consumer acceptability represents a challenge. Therefore, the aim of our study is to assess the effect of supplementation of fermented milk by *L. plantarum* EMCC 1027 with curcumin on its viability as well as enhancement of antibacterial, antioxidant, anticarcinogenic, and inflammatory activities of these products.

Materials and Methods

Materials

L. plantarum EMCC 1027 was obtained from the Egyptian Microbial Culture Collection, Cairo, Egypt. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. (St. Louis, MO,USA).Water soluble curcumin was purchased from DolCas-Tenshi Bioceuticals Co. (New Jersey, USA). De Man Rogosa and Sharpe (MRS, pH 5.3) agar medium (Merck Co., Darmstadt, Germany). Fresh cow milk was obtained from the herd of Faculty of Agriculture, Cairo University. Soybean was purchased from local market.

Preparation of Fermented Cow and Soy Milk

Soy milk (12% total solids) was prepared according to Sumarna[20] then soy milk was inoculated with freeze-dried *L. plantarum* culture at 0.02% and incubated at 37ºC. Fermented cow milk (12% total solids) was prepared according to Donkor *et al*.[21] The fermentation of both soy and cow milk was ended when the pH was reached to 5.0 then curcumin was added at 0, 50, 100, and 150 mg/100mL for both types of milk. The viable count of *L. plantarum* was monitored during fifteen days of cold storage period using MRS agar medium (pH 5.3). Plates of *L. plantarum* were incubated aerobically at 37ºC for 72 days.

Propagation of Indicator Bacteria

Pure cultures of *Bacillus subtilis* EMCC 1121, *Staphylococcus aureus* EMCC 3114, *Escherichia coli* EMCC 7661, *Pseudomonas fluorescens* EMCC 1067 (as indicator bacteria) were purchased from Egyptian Microbial Culture Collection (EMCC), Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams University,Cairo, Egypt. Nutrient broth (Merck, Darmstadt, Germany) was used as propagation medium for indicator bacteria, whereas each pure culture was propagated twice in nutrient broth at 37ºC for 18 h.

Preparation of Crude Extract

Crude extract (whey) was prepared by centrifugation of each sample at 6,000 ×g for 20 min at 5 ºC and the supernatant was filtered (0.45μm Millipore Corp, Billerica, USA).

Antibacterial Activity

The antibacterial activity of collected whey fraction was assessed against tested indicator bacterial strains using agar well diffusion method as previously described by Varadaraj *et al*.[22]

Total Polyphenols Content

Total polyphenol content in each crude extract sample was measured according to Singleton and Rossi.[23] Briefly, crude sample (100 μL) was mixed with 900 μL of distilled water then stirred for two hours at room temperature. To this, 1 mL of Folin–Ciocalteu reagent (1:2 dilution) and 2 mL of 10% Na_2CO_3 was added. The mixture was centrifuged at 10,000 ×g for 30 min, the supernatant was collected and filtered using membrane filter 0.45μm (Millipore Corp, Billerica, MA, USA). The absorbance was measured at 760 nm using Jenway 6300 Spectrophotometer (Cole-Parmer Ltd, Staffordshire, UK). Gallic acid (Sigma-Aldrich, Inc., Missouri, USA) was used as a standard.

DPPH Free Radical Scavenging

The antioxidant activity of fermented milk, based on the scavenging activity of the stable DPPH free radical, was determined by the method described by Lee *et al*.,[24]

One mLof filtrated whey collected from each sample was added individually to the test tube. 1.0 mL of DPPH solution (0.2 mM in ethanol) was added to each glass tube then mixed well-using vortex (DD Biolab S.L., Barcelona, Spain) and incubated at 25 ºC for 30 min. Ascorbic acid solution (0.03%, w/v) was used as a positive control. The absorbance (A) of the solution was measured at 517 nm using Jenway 6300 Spectrophotometer.

Anti-colonic Cancer Activity

The human colon cancer cell lines HT-29 was honorably gifted from the National Institute of Piles, Cairo University, Egypt.HT-29 was routinely 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 using methylene blue assay.[25]

Anti-inflammatory Assay

The anti-inflammatory activity of each fraction was assessed using human epithelial cell line (Caco-2, National Institute of Piles, Cairo University, Egypt) and measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[26]

Data Analysis

Microsoft Excelsoftware (Microsoft Office 2013, License: Shareware N/A) was used for data analyses. The results represent the mean and standard deviations (±) of three independent replicates.Statistical analysis was carried out using analysis of variance (ANOVA) and Duncan test using MSTAT-C software (Michigan State University, East Lansing, Michigan, USA) at probability level of $P < 0.05$.

Table 1: Viability of *L. plantarum* during cold storage (5°C) period of different fermented cow and soy milk supplemented with curcumin

Samples	Viable Counts (Log CFU/g)	
	0	20 days
Fermented Soy milk (FS)	8.50±0.62^{a}	8.20±0.72^{a}
FS+50 mg/100mL curcumin	8.40±0.55^{a}	8.10±0.80^{a}
FS+100 mg/100mL curcumin	8.30±0.71^{a}	8.15±0.76^{a}
FS+150 mg/100mL curcumin	8.30±0.82^{a}	8.20±0.91^{a}
Fermented milk (FM)	8.30±0.62^{a}	7.60±0.91^{b}
FM+50 mg/100mL curcumin	8.50±0.70^{a}	7.40±0.78^{b}
FM+100 mg/100mL curcumin	8.40±0.91^{a}	7.30±1.05^{b}
FM+150 mg/100mL curcumin	8.20±0.68^{a}	7.10±0.85^{bc}

±: Standard deviation of three replicates. Means with the same superscripts letters do not differ significantly (P< 0.05).

Results

Viability of *L. plantarum*in Fermented Milk during Cold Storage Period

Data in Table 1 show the viability of *L. plantarum* EMCC 1027 in fermented cow and soy milk supplemented with different concentrations of curcumin after fifteen days of cold storage (5 ºC). At the end of cold storage, the viable counts of *L. plantarum* were significantly decreased in fermented cow milk in comparison with fermented soy milk. Furthermore, the addition of curcumin did not have a negative effect on the viability of *L. plantarum* during cold storage.

Antibacterial Activity

The diameter of inhibition zone (mm), as an indicator for antibacterial activity, was significantly increased in fermented soy milk than fermented cow milk as shown in Table 2. Furthermore, the addition of different concentrations of curcumin resulted in a significant enhancement of the antibacterial activity against Gram-positive indicator bacteria (*B. subtilis* and *S. aureus*) as well as Gram-negative bacteria (*E. coli* and *P. fluorescens*).

Table 2: Antibacterial activity of fresh crude extract of different fermented cow and soy milk supplemented with curcumin

Samples	Diameter of Inhibition Zone (mm)			
	B. subtilis	***S. aureus***	***E.coli***	***P. fluorescens***
Fermented Soy milk (FS)	10.50±0.81^{d}	11.10±1.20^{de}	7.50±1.05^{e}	8.20±0.65^{d}
FS+50 mg/100mL curcumin	11.20±1.05^{c}	12.00±0.95^{c}	8.20±1.10^{c}	9.10±1.25^{c}
FS+100 mg/100mL curcumin	12.10±1.20^{b}	12.90±1.10^{b}	9.10±0.91^{b}	9.80±0.72^{b}
FS+150 mg/100mL curcumin	12.90±0.70^{a}	14.20±1.40^{a}	10.30±1.00^{a}	10.50±0.86^{a}
Fermented milk (FM)	9.50±0.85^{e}	10.30±1.31^{f}	6.50±0.77^{f}	7.30±0.78^{f}
FM+50 mg/100mL curcumin	10.70±0.61$_{d}$	10.80±0.80^{e}	7.10±1.40^{e}	7.90±0.67^{e}
FM+100 mg/100mL curcumin	11.40±1.25^{c}	11.60±0.63^{d}	7.90±1.20^{cd}	8.50±1.26^{d}
FM+150 mg/100mL curcumin	12.00±1.30^{b}	12.30±0.71^{c}	8.40±0.68^{c}	9.30±1.03^{b}

±: Standard deviation of three replicates. Means with the same superscripts letters do not differ significantly ($P< 0.05$).

Table 3: Total polyphenols content (mg/100mL) and DPPH free radical scavenging activity of fresh crude extract of different fermented cow and soymilk treatments

Samples	Total polyphenols content (mg/100 mL)	DPPH Scavenging (%)
Fermented Soy milk (FS)	30.21±0.65^{d}	78.25±1.05^{c}
FS+50 mg/100mL curcumin	42.15±0.72^{c}	81.92±1.30^{c}
FS+100 mg/100mL curcumin	50.32±0.36^{b}	89.60±0.81^{b}
FS+150 mg/100mL curcumin	58.63±0.53^{a}	92.05±1.56^{a}
Fermented milk (FM)	20.35±0.74^{e}	68.41+0.86^{f}
FM+50 mg/100mL curcumin	33.11±0.24^{d}	74.60±0.91^{e}
FM+100 mg/100mL curcumin	43.72±0.36^{c}	80.75±0.56^{c}
FM+150 mg/100mL curcumin	52.80±0.82^{b}	85.05±1.02^{d}
Ascorbic acid	-	94.32±0.06^{a}

±: Standard deviation of three replicates. Means with the same superscripts letters do not differ significantly ($P< 0.05$).

Polyphenols Content and DPPH Free Radical Scavenging

Crude extracts of fermented soymilk had higher amounts of polyphenols than crude extract of fermented milk. Furthermore, the addition of different concentrations of curcumin enhanced the polyphenols content of different crude extract types (Table 3). Data in Table 3 show an improvement of scavenging activity of DPPH free radical when water soluble curcumin was supplemented. The DPPH scavenging activity of crude extract of different fermented soy milk treatments was significantly increased in comparison with crude extract of different fermented cow milk treatments. The scavenging activity of DPPH by different fermented milk treatments was in a good correlation with their polyphenols content.

Table 4: Proliferative inhibition (%) of human colon cell lines treated with 100 µg of fresh crude extract of different fermented cow and soymilk treatments

Samples	Inhibition (%)
Fermented Soy milk (FS)	23.75±0.60[e]
FS+50 mg/100mL curcumin	32.21±1.15[c]
FS+100 mg/100mL curcumin	38.30±2.05[b]
FS+150 mg/100mL curcumin	41.05±0.35[a]
Fermented milk (FM)	18.55±1.17[f]
FM+50 mg/100mL curcumin	22.10±1.11[e]
FM+100 mg/100mL curcumin	29.50±0.65[d]
FM+150 mg/100mL curcumin	32.12±0.40[c]

±: Standard deviation of three replicates.Means with the same superscripts letters do not differ significantly (P< 0.05).

Anti-colonic Cancer Activity of Crude Extract of Different Fermented Cow and Soy Milk Treatments

A significant increase in levels of proliferative inhibition (%) of human colon cell lines (HT-29) was observed in fermented soy milk compared to fermented cow milk using *L. plantarum* EMCC 1027. Furthermore, by increasing levels of curcumin, levels of proliferative inhibition (%)of HT-29 cell line were significantly increased as shown in Table 4.

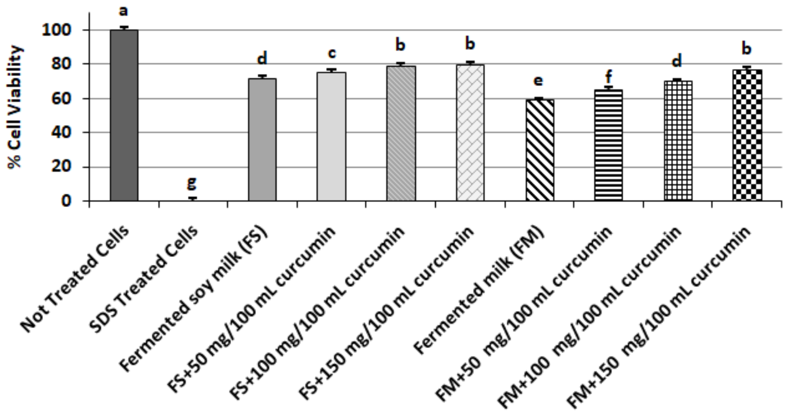

Fig.1: Anti-inflammatory activity (measured as % cell viability) of fresh crude extract of different fermented cowand soymilk treatments

Anti-Inflammatory

The addition of different concentrations of curcumin enhanced the viability of Caco-2 cell line after SDS treatment in either crude extract of fermented soy milk or cow milk. Also, the percentage of viable cells was significantly enhanced in the crude extract of fermented soy milk compared to the crude extract of cow milk as presented in Figure 1.

Discussion

The viability of different probiotic strains in fermented milk plays an essential role in their health benefits.

In this regard, the viable count of *L. plantarum* was significantly enhanced in fermented soy milk in comparison with fermented cow milk after fifteen days of cold storageas shown in Table 1. The enhancement of viability of *L. casei* Zhang in fermented soy milk was observed by Wang *et al*.,[7] during fermentation and cold storage compared to cow milk, probably because the soy milk had buffering capacity as well as oligo-saccharides more than cow milk. Also, a significant increase in the viable counts of commercial probiotic starter *Bifidobacterium*animalis Bb-12 and *L. casei* Shirota strain were observed during fermentation of soy milk.[42] Furthermore,*L. plantarum* is used for the production of different curcumin beverages indicating that curcumin is a good carrier for it.[27] Finally, in all treatments counts of *L. plantarum* were above the recommended level of probiotic 10^6 CFU/mL[43] at the end of cold storage.

Antibacterial activity of different probiotic strains plays an important role in enhancement the shelf life of different types of food and it also confers health benefits through modulation of host gut microbiota towards healthier composition. Our findings in Table 2 show an increase in the antibacterial activity of crude extracts collected from different fermented soy milk treatments in comparison with fermented cow milk. Crude extract of different fermented milk contains different organic acids, bacteriocins,and other metabolites which enhance the efficacy of antimicrobial activity.[28] The growth of different probiotic strains and organic acid production (mainly lactic and acetic acids) were increased in fermented soy milk compared to fermented cow milk as previously reported by different researchers.[7,29,30] Consequently, the antibacterial activity of crude extract from fermented soy milk was higher than crude extract from fermented milk (Table 2). Furthermore, a significant increase in levels of bioactive components with antibacterial activity was observed when soy milk was fermented with different commercial probiotic strains.[42] Curcumin has a wide spectrum of antimicrobial activity against Gram-positive indicator bacteria e.g. *B. subtilis* ATCC 6633 and *S. aureus* ATCC 43300, methicillin-resistant *S. aureus, S.epidermidis* and Gram-negative bacteria e.g. *E. coli* ATCC 25922, and P. aeruginosaATCC 27853 as reported previously by Gunes *et al*.,[31] Liu *et al*.[44] and Mun *et al*[45] respectively.This antimicrobial activity of curcumin depends on its solubilityand the degree of suppression of cytokines.[31] Therefore, in this research, watersoluble curcumin was used.

The crude extract (whey) after milk fermentation by different probiotic strains contains different peptides with antioxidant activities.[32] Crude extract of fermented soy milk had antioxidant activity more than crude extract of fermented cow milk as shown in Table 3 because (1) protein hydrolysis might be higher in fermented soy milk than cow milk and (2) soy milk is rich source in polyphenols with antioxidant capacity.[33] Recently, fermented soybean milk with *L. plantarum* CQPC01 enhanced levels of antioxidant biomarkers in mice.[34] Curcumin is well known as antioxidant agent in many food systems and this effect is due to its content of polyphenols and flavonoids as reported by Tanvir *et al*.[35] Therefore, supplementation of either fermented soy or cow milk with watersoluble curcumin enhanced their antioxidant capacity.Levels of antioxidant activity (as % DPPH scavenging) of fermented soymilk supplemented with curcumin were more than fermented cow milk supplemented with the same concentration of curcumin indicating types of milk play an essential role in the antioxidant activity (Table 3).

The inhibition of proliferation of HT-29 depends on the concentration of curcumin in fresh crude extract of either fermented soy or cow milk (Table 4). Fermented soy milk contains isoflavones with antioxidant and anti-carcinogenic properties as previously observed by Qian *et a.l*[25] The mechanism suggested by the same author was that fermented soy milk by *L. casei* 16 had high levels of total phenolic components and isoflavones which could suppress the proliferation of HT-29 cell lines. Abd El-Gawad *et al*[11] found that both fermented cow and soy milk had anti-breast cancer activity and this activity is strain specific. In HT-29 cell line model, curcumin (an active ingredient of turmeric) has anti-carcinogenic activity through its antioxidant capacity[36,37,38] and it could activate p53 and down-regulating pro-caspase-3 and 9 proteins as apoptosis-related proteinsand it also could down-regulate cyclooxygenase-2 and matrix metalloproteinase-2 expressions.[39] Furthermore, different curcumin-related compounds had anti-cancer activity through stimulation of cancer cells apoptosis.[40]

Sodium dodecyl sulfate (SDS) was used as an irritant agent was in order to induce the inflammation in human epithelial cell line (Caco-2) whereas it was toxic for it as shown in Figure 1. The addition of different crude extracts of fermented soy milk or cow milk supplemented with curcumin could protect cells from damage caused by SDS whereas the viability of cells was enhanced. Results obtained by Jiang *et al*[41] showed the protective role of *L. plantarum* WLPL04 against SDS induced inflammation in cell line model whereas *L. plantarum* could suppress the production of cytokines (interleukins 6 and 8). Finally, our results illustrated that fermented milk was more irritated than fermented soy milk. This might be due to fermented milk had lower polyphenols content and antioxidant capacity than fermented soy milk (Table3).

Conclusion

This work represents a report to investigate the influence of the synergistic effect of fermented soy/ cow milk and curcumin on the enhancement of some beneficial properties of fermented soy/cow milk. Our data show that the addition of curcumin to cow milk or soy milk did not negatively effect on the viability of *L. plantarum* strain during fermentation or cold storage period. Also, the addition of water soluble curcumin enhances the antibacterial activity, antioxidant capacity through enhancement free radical scavenging activity of crude extract of fermented soy and cow milk. Enhancement of anti-colonic cancer and anti-inflammation activities was in a good correlation with the enhancement of the antioxidant capacity of different crude extract samples. Indeed, the molecular mechanism by which anti-colonic cancer activity of different crude extract samples of fermented soy and cow milk fortified with water soluble curcumin is needed for further research.

Acknowledgement

The authors are grateful to Cairo University research park (CURP), Faculty of Agriculture, Cairo University for the technical support.

Funding

This research was funded by Cairo University (CU).

Conflict of Interest

The authors declare no conflict of interestincluding financial, personal, or other relationships with people or organizations that could influence the work.

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