Impact of fermented cow milk versus soy milk supplemented with water-soluble curcumin on inhibition of Ehrlich ascites carcinoma in female Swiss mice

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Research Article

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Abstract

Background

Fermented dietary products have been found to increase anti-carcinogenic activity and modulate immunological and physiological functions. This study assessed the preventive effect of fermented cow and soy milk supplemented with water-soluble curcumin against Ehrlich ascites tumor.

Methods

Both types of milk were prepared in the presence of Lactobacillus plantarum EMCC 1027. Ninety-five grams of basal diet mixed with 5g of either fermented cow or soy milk and supplemented with 150 mg of soluble curcumin. Induction of Ehrlich tumor-bearing mice synergized with the feeding of a prepared special diet for 20 days.

Results

Both fermented cow and soy milk increased the survival rate to 117% while the supplementation with curcumin improved the survival rate to 150% combined with soy milk. The decreased concentration of glutathione and superoxide dismutase in Ehrlich tumor-bearing was markedly increased after feeding by any kind of a special diet. In Ehrlich tumor-bearing mice, fermented soy milk plus curcumin showed the highest reduction levels of the augmented levels of tumor necrosis factor (TNF)-α or interleukin-6. The relative gene expression TNF-α was significantly ($P<0.05$) down-regulated in all treated Ehrlich tumor-bearing while fermented soy milk plus curcumin demonstrated the highest reduced levels. Fermented cow and soy milk with or without curcumin significantly ($P<0.05$) caused a rise in the viable count of lactobacilli and bifidobacteria.

Conclusions

Collectively, the dietary feeding with fermented cow or soy milk displayed beneficially protective effects, and the best results were recorded in combination with soluble curcumin.

Background

Generally, the global occurrence of cancer has increased. In 2018, about 9.6 million cancer-related deaths were recorded [1]. In Egypt, Carcinoma of the breast is the most prevalent cancer among Egyptian women representing 29% of National Cancer Institute cases [2]. The occurrence of cancer is due to several key factors such as unhealthy lifestyle, aging, and socio-economic development [3].
Probiotic fermented milk and soy milk had shown an inhibitory effect against different cancer models. In this context, it has been demonstrated that yogurt and soy yogurt containing *bifidobacteria* could inhibit the proliferation of Ehrlich ascites tumor cells [4]. Different probiotic strains had anticarcinogenic activities [5, 6]. Also, yogurt manufactured by *L. helveticus* R389 suppressed tumor growth and modulated the inflammatory system positively [7]. Furthermore, Kefir grains could inhibit Ehrlich ascites tumor cells through induction of apoptosis and immunomodulation [8].

Curcumin as a functional ingredient has a high content of polyphenols and has been used extensively in Ayurvedic medicine for centuries. Curcumin has shown anticarcinogenic activity through inhibition of different inflammatory pathways as well as the NF-κB pathway [9, 10]. Recently, the fortification of probiotic fermented milk and soy milk with water-soluble curcumin had an enhancement effect on anticarcinogenic activity using *in vitro* model [11]. However, there is still a gap in knowledge in the mechanism of fortification of fermented milk with curcumin. Therefore, the present study aimed at exploring the possible mechanism of Ehrlich ascites carcinoma inhibition by probiotic fermented milk and soy milk supplanted with curcumin.

**Methods**

**Materials**

*Lactobacillus (L.) plantarum* EMCC 1027 was obtained from the Egyptian Microbial Culture Collection, Cairo, Egypt. Water-soluble curcumin was purchased from DolCasTenshi Bioceuticals Co. (New Jersey, USA). All microbiological media were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ehrlich tumor cells were obtained from the National Cancer Institute, Cairo University. Female Swiss albino mice were purchased from Ophthalmology Institute, Giza, Egypt.

**Propagation of L. plantarum Cultures**

*L. plantarum* EMCC 1027 was sub-cultured in an MRS broth medium and incubated aerobically at 37°C for 18 h.

**Preparation of fermented cow and soy milk supplemented with curcumin**

Fermented cow and soy milk supplemented with water-soluble curcumin were prepared as previously described by [11].

**Animals and feeding protocol**

The female Swiss albino mice were maintained in an air-conditioned room at a temperature of 22 ± 1°C and humidity of 55 ± 1% with a 12 h light/dark cycle and had *ad libitum* access to water. All animals were kept under normal healthy conditions and fed a basal diet for 1 week. After this adaptation period, all mice were divided randomly into six experimental groups each of 10 animals as the following:
Group 1 (Normal group): mice were fed a basal diet. The composition of the basal diet was as previously described by [12].

Group 2 (Control group): mice were fed a basal diet and injected intraperitoneally with $1 \times 10^6$ Ehrlich ascites tumor cells in PBS (0.2 ml PBS/mouse).

Group 3 (Cow milk): mice were fed a 95g basal diet mixed with 5g fermented cow milk and were injected intraperitoneally with $1 \times 10^6$ Ehrlich ascites tumor cells.

Group 4 (Cow milk + cur): mice fed 95g basal diet mixed with 5g fermented cow milk supplemented with 150 mg curcumin and were injected intraperitoneally with $1 \times 10^6$ Ehrlich ascites tumor cells.

Group 5 (Soy milk): mice were fed a 95 g basal diet mixed with 5g fermented soy milk and were injected intraperitoneally with $1 \times 10^6$ Ehrlich ascites tumor cells.

Group 6 (Soy milk + cur): mice were fed a 95 g basal diet mixed with 5 g fermented soy milk supplemented with 150 mg curcumin and were injected intraperitoneally with $1 \times 10^6$ Ehrlich ascites tumor cells. The mice were allowed free access to diet and water. Feces were collected from each mouse for microbiological analysis at the end of the experiment (20 days).

**Blood collection from survived mice**

At the end of the experiment, the survival percent in each group was calculated as follows: survival percent = (number of survived animals/total number of animals) × 100. Mice were lightly anesthetized by isoflurane. Blood samples were collected from the retinal orbital plexus through heparinized capillary tubes into heparinized tubes, separated by centrifugation (3500 xg for 15 min), and stored at -20°C until analysis.

**Determination of biochemical parameters and cytokine levels**

Plasma glutathione and superoxide dismutase (Thermo Fisher Scientific, Passau, Germany) were determined using a spectrophotometer (SHIMADZU, Tokyo, Japan). Interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured by commercial ELISA kits (Genzyme Diagnostics, Cambridge, England) according to manufactured instructions. Optical density was measured at 450 nm.

**Gene detection of TNF- alpha**

The detection of the TNF-α gene in tumor tissues by reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously by [8, 13].

**Enumeration of fecal lactobacilli, bifidobacteria, and Enterobacteriaceae**

Fecal samples of mice were freshly collected and transferred into the anaerobic tube (Clinical Supply, Gifu, Japan). Samples were examined for total lactobacilli using MRS medium pH 5.3, bifidobacteria
using TOS-MUP medium, and total Enterobacteriaceae according to the methods [14].

**Statistical analysis**

Results were statistically analyzed with the SAS statics package (version 9.4, SAS Institute, Cary, NC). One–way ANOVA was used to assess the significance of differences between groups with \( P < 0.05 \) being considered significant. Data were expressed as mean ± standard deviation (SD). Duncan test and one-way analysis of variance were used.

**Results**

**Survival rate**

The mean survival rate of Ehrlich tumor-bearing mice was found to be 60%. Feeding basal diet either with cow or soy milk significantly \((P < 0.05)\) increased the survival rate of Ehrlich tumor-bearing mice to 117%. Cow milk plus curcumin diet caused an increase in the survival rate to 133% while Soy milk plus curcumin resulted in an increase in the survival rate to 150% (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>N (day0)</th>
<th>N (day20)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Ehrlich-tumor</td>
<td>10</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>Cow milk</td>
<td>10</td>
<td>7</td>
<td>117</td>
</tr>
<tr>
<td>Cow milk + cur</td>
<td>10</td>
<td>8</td>
<td>133</td>
</tr>
<tr>
<td>Soy milk</td>
<td>10</td>
<td>7</td>
<td>117</td>
</tr>
<tr>
<td>Soy milk + cur</td>
<td>10</td>
<td>9</td>
<td>150</td>
</tr>
</tbody>
</table>

\( N: \) number of animals; mice were fed a basal diet (Normal); mice were injected intraperitoneally with \(1 \times 10^6\) Ehrlich ascites tumor cells (Ehrlich-tumor); Ehrlich-tumor bearing mice were fed 95g basal diet mixed with 5 g fermented cow milk (Cow milk), 5 g fermented cow milk supplemented with 150 mg curcumin (Cow milk + cur); 5 g fermented soy milk (Soy milk), 5 g fermented soy milk supplemented with 150 mg curcumin (Cow milk + cur).

**Levels of glutathione and superoxide dismutase**

The plasma levels of SOD and GSH in the Ehrlich tumor-bearing group showed a significant decrease compared to the normal group. All other treatments induced significantly higher levels \((P < 0.05)\) of SOD and GSH compared to the Ehrlich tumor-bearing group (Fig. 1).

**Plasma levels of IL-6 and TNF-α**
As demonstrated in Fig. 2, the mean level of IL-6 was significantly ($P < 0.05$) elevated in Ehrlich tumor-bearing group (205.52 ± 3.1) compared to the normal group (50.2 ± 2.58). Either cowmilk (105.63 ± 4.25) or soymilk (91.62 ± 3.52) groups showed a significant reduction in the plasma levels of IL-6 compared to Ehrlich tumor-bearing group (205.52 ± 3.1). Both cow milk plus curcumin (85.35 ± 5.01) and soy milk plus curcumin (69.25 ± 2.68) groups significantly revealed lower levels of IL-6 than those of the Ehrlich tumor-bearing group (205.52 ± 3.1). Regarding TNF-α (Fig. 2), the mean level was found to be 2.11 ± 0.4 in Ehrlich tumor-bearing group (2.11 ± 0.4) in comparison to undetected levels of plasma TNF-α in normal mice. Feeding basal diet either with cow milk (1.61 ± 0.56) or soy milk (1.25 ± 0.35) significantly decreased ($P < 0.05$) the levels of TNF-α compared to the Ehrlich tumor-bearing group (2.11 ± 0.4). Moreover, both cow milk plus curcumin (1.11 ± 0.62) and soy milk plus curcumin (0.82 ± 0.52) groups exhibited significantly lower levels of TNF-α than those of the Ehrlich tumor-bearing group (205.52 ± 3.1).

**Gene expression of TNF-α**

On the gene level, the relative gene expression of TNF-α was (8.51 ± 1.25) significantly increased ($P < 0.05$) in Ehrlich tumor-bearing group compared to the normal group. The gene expression of TNF-α was significantly reduced in either cow milk (95.35 ± 1.62) or soy milk (4.21 ± 1.15), groups compared to Ehrlich tumor-bearing group (8.51 ± 1.25). Both the groups of cow milk + cur (2.51 ± 0.85) and soy milk + cur (1.85 ± 0.62) displayed lower levels of TNF-α in comparison to Ehrlich tumor-bearing group (8.51 ± 1.25) (Fig. 3).

**Fecal examination**

The total viable count of fecal *enterobacteriaceae, lactobacilli*, and bifidobacteria was illustrated in Table 2. The mean viable count of *enterobacteriaceae* in the Ehrlich tumor-bearing group was significantly increased in comparison to the normal group. On the other hand, all other treated groups showed a lower count of *enterobacteriaceae* compared to Ehrlich tumor-bearing group. The mean viable count of *lactobacilli* and bifidobacterial was significantly decreased in Ehrlich tumor-bearing group compared to the normal group. All other treatments significantly elevated the viable count of *lactobacilli* and *bifidobacteria* compared to Ehrlich tumor-bearing group.
Table 2
Total viable count (log CFU/g) of fecal *enterobacteriaceae*, *lactobacilli*, and *bifidobacterial*

<table>
<thead>
<tr>
<th>Groups</th>
<th>enterobacteriaceae</th>
<th>lactobacilli</th>
<th>bifidobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.30 ± 0.65b</td>
<td>8.35 ± 0.57a</td>
<td>7.65 ± 0.80a</td>
</tr>
<tr>
<td>Ehrlich-tumor</td>
<td>6.85 ± 0.95a</td>
<td>5.21 ± 0.63d</td>
<td>4.23 ± 0.75c</td>
</tr>
<tr>
<td>Cow milk</td>
<td>5.72 ± 1.30b</td>
<td>6.30 ± 0.92c</td>
<td>6.35 ± 0.68b</td>
</tr>
<tr>
<td>Cow milk + cur</td>
<td>4.51 ± 1.25c</td>
<td>7.40 ± 1.05b</td>
<td>6.80 ± 0.71b</td>
</tr>
<tr>
<td>Soy milk</td>
<td>4.58 ± 0.95c</td>
<td>8.15 ± 0.85a</td>
<td>7.41 ± 0.62a</td>
</tr>
<tr>
<td>Soy milk + cur</td>
<td>4.15 ± 1.15c</td>
<td>8.21 ± 0.93a</td>
<td>7.60 ± 1.01a</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (SD). In the same column, mean values marked with different superscript letters are significantly different (*P* < 0.05). Data were expressed as a mean ± SD. Mice were fed a basal diet (Normal); mice were injected intraperitoneally with 1x10⁶ Ehrlich ascites tumor cells (Ehrlich-tumor); Ehrlich-tumor bearing mice were fed 95g basal diet mixed with 5 g fermented cow milk (Cow milk), 5 g fermented cow milk supplemented with 150 mg curcumin (Cow milk + cur); 5 g fermented soy milk (Soy milk), 5 g fermented soy milk supplemented with 150 mg curcumin (Cow milk + cur).

**Discussion**

The present study assumed that a fermented feed diet has beneficial health effects. A comparison between a fermented food of animal origin like cow milk and another one of plant origin like soy milk was assessed against the Ehrlich tumor. Additionally, the protective impact of fermented cow milk and soy milk against tumor formation had compared to the supplement of curcumin in its water-soluble form.

Here, dietary feeding with supplements of fermented soy milk and cow milk increased the survival rate of Ehrlich ascites-bearing mice to 117% after 20 days of tumor cells injection. This survival rate was higher than reported by a study that used soy milk without fermentation, reaching a survival rate of 108% [15]. Thus, the process of fermentation presented a more beneficial impact than the unfermented form.

Moreover, the antitumor activity of fermented milk against HT-29 and Caco-2 cells has been documented through suppression of cell proliferation and reduction of lung metastasis by supplementation of fermented milk [16]. The progression of breast tumors was suppressed in the rats fed a fermented soymilk diet [17] due to the antioxidant activity of fermented milk [18].

Curcumin is one of these phytochemicals that has received a lot of attention due to its effectiveness against a variety of cancer types. It has antioxidant, anti-inflammatory, and cancer cells anti-proliferative activity against cancer cells [19]. In the present study, the use of formulated water-soluble curcumin resolved the previous poor water solubility of curcumin. Providing diet pellets with soluble curcumin enhanced the higher survival rate of Ehrlich ascites-bearing mice to 133% in the case of fermented cow milk and 150% in the case of fermented cow milk. This result agreed with previous studies that prevent
colorectal cancer proliferation by inhibiting the cell cycle and provoking apoptosis [20, 21]. An eighty percent reduction of ascites cells accompanied oral administration of curcumin in the mouse mammary Ehrlich ascites model [22].

Fermentation is an effective strategy for enhancing the supply of natural antioxidants because it boosts antioxidative activity by increasing the release of phenolic compounds from plant-based diets. Antioxidant activity in soy milk was increased after fermentation [23]. As a result of many metabolic activities, reactive oxygen species (ROS) are continuously created that cause harm to DNA, proteins, lipids, and carbohydrates, contributing to multiple diseases such as cancer, atherosclerosis, and arthritis. Antioxidants serve a critical function in scavenging ROS. SOD and GSH performed an important antioxidant defense that converts superoxide radicals into molecular oxygen and hydrogen peroxide, protecting tissues from oxidative damage. The Ehrlich tumor-bearing mice that were supplemented with fermented soy milk exhibited higher levels of both SOD and GSH than those supplemented with fermented cow milk. This finding is matched with previous research that found soya milk has a much stronger antioxidant activity than cow milk [24]. The same finding of higher levels of antioxidant enzymes was also observed in the Ehrlich tumor-bearing mice supplemented with fermented cow or soy milk plus curcumin. According to the study by [25], the phenol content of turmeric-fortified soya milk was higher than that of turmeric-fortified cow milk.

TNF-α, interferon (IFN)-γ, and interleukin (IL)-6, and are among the proinflammatory mediators produced during inflammation. Immune cells that produce too many pro-inflammatory mediators cause tissue harm as well as immune system failure. The excessive inflammatory reaction has related to cancer development [26]. Phenolic compounds are one of the important bioactive components which act as natural antioxidants and immunological modulators that are created during fermentation [27]. Dietary regulation of excessive inflammation is crucial for maintaining health. The elevated plasma levels of IL-6 and TNF-α in Ehrlich tumor-bearing mice were decreased by supplemented diet with fermented cow and soy milk. Also, the gene expression of TNF-α was downregulated after both treatments. A previous study reported that some fermented milk peptides stimulate lymphocyte proliferation and control the generation of pro-inflammatory cytokines such as IFN-γ and TNF-α in Balb/c mice [28]. Furthermore, feeding probiotic L. reuteri ATCC 6475 could reduce levels of IL-6 in mice [29]. Additional animal studies confirmed that oral administration of L. acidophilus had anti-breast cancer activity in mice models [30].

Enterobacteriaceae as a member of the gut microbiome plays a vital role in the development of breast cancer through increased secretion of β-glucuronidase [31, 32]. Our results in Table 3 showed a significant increase in the viable count of total Enterobacteriaceae in the Ehrlich tumor-bearing group. On the other hand, feeding mice with fermented cow milk or soy milk with /without curcumin lowered levels of Enterobacteriaceae and increased viable counts of lactobacilli and bifidobacteria in feces samples. The modulation of fecal microflora in mice may be due to the synergistic effect of both types of fermented milk and curcumin through their antimicrobial activities [11].

Declarations
Author contributions
T.E.A. and F.M.F.E. put the design of the project research. F.M.F.E. conducted the data analysis, interpreted the results, and wrote the main manuscript text. A.M.B. prepared the graphs, interpreted the results, and wrote the main manuscript text. All authors reviewed the manuscript and approved the final version.

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Availability of data and materials
The data provided in the manuscript will be available upon request pending approval by the authors.

Ethical Approval and Consent to participate
All animal procedures were approved by Cairo University-IACUC with approval Number: CU/I/F/29/21 and performed according to the Guide of animal care and use.

Competing interests
The authors have no conflicts of interest to declare.

Consent for publication
Not applicable

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References


Table 3

Table 3 is not available with this version.

Figures

**Fig. 1**

![Figure 1](image-url)
Plasma levels (U/ml) of glutathione (GSH) and superoxide dismutase (SOD). Mice were fed a basal diet (Normal); mice were injected intraperitoneally with 1x10^6 Ehrlich ascites tumor cells (Ehrlich-tumor); Ehrlich-tumor bearing mice were fed 95g basal diet mixed with 5 g fermented cow milk (Cow milk), 5 g fermented cow milk supplemented with 150 mg curcumin (Cow milk+cur); 5 g fermented soy milk (Soy milk), 5 g fermented soy milk supplemented with 150 mg curcumin (Cow milk+cur). Data were expressed as a mean ± SD. Mean values marked with different superscript letters are significantly different (P < 0.05). SD: ±Standard deviation

**Figure 2**

Plasma levels of IL-6 (pg/mL) and TNF- alpha (ng/mL) in all mice groups. Mice were fed a basal diet (Normal); mice were injected intraperitoneally with 1x10^6 Ehrlich ascites tumor cells (Ehrlich-tumor); Ehrlich-tumor bearing mice were fed 95g basal diet mixed with 5 g fermented cow milk (Cow milk), 5 g fermented cow milk supplemented with 150 mg curcumin (Cow milk+cur); 5 g fermented soy milk (Soy milk), 5 g fermented soy milk supplemented with 150 mg curcumin (Cow milk+cur). Data were expressed as a mean ± SD. Mean values marked with different superscript letters are significantly different (P < 0.05). SD: ±Standard deviation.
Figure 3

Gene expression of TNF alpha at the end of the experiment as measured by RT-PCR. Data were expressed as a mean ± SD. Mice were fed a basal diet (Normal); mice were injected intraperitoneally with $1 \times 10^6$ Ehrlich ascites tumor cells (Ehrlich-tumor); Ehrlich-tumor bearing mice were fed 95g basal diet mixed with 5 g fermented cow milk (Cow milk), 5 g fermented cow milk supplemented with 150 mg curcumin (Cow milk+cur); 5 g fermented soy milk (Soy milk), 5 g fermented soy milk supplemented with 150 mg
curcumin (Cow milk+cur). Data were expressed as a mean ± SD. Mean values marked with different superscript letters are significantly different ($P < 0.05$). SD: ±Standard deviation.