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Inhibitory effect of yoghurt and soya yoghurt containing bifidobacteria on the proliferation of Ehrlich ascites tumour cells *in vitro* and *in vivo* in a mouse tumour model

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The effect of yoghurt and soya yoghurt containing *Bifidobacterium lactis* Bb-12 or *B. longum* Bb-46 on Ehrlich ascites tumour cell proliferation was investigated *in vitro* and *in vivo*. Tumour cells were incubated with *B. lactis* Bb-12 or *B. longum* Bb-46 cultivated in de Mann Rogosa Sharpe (MRS) broth medium, or with their centrifuged supernatant fractions or sediments, for 2 h at 37°C. Treatment resulted in the inhibition of tumour cell proliferation by 85·42 (SD 0·78) and 85·10 (SD 1·28) % by intact micro-organisms, 77·61 (SD 0·29) and 71·43 (SD 1·75) % by their supernatant fractions, but only 4·00 (SD 0·19) and 9·09 (SD 1·24) % by the two sedimented bacteria, respectively. The incubation of tumour cells with yoghurt and soya yoghurt containing Bb-12 for 2 h resulted in 83·01 (SD 0·11) and 88·23 (SD 0·06) % inhibition, respectively, while it was 83·82 (SD 0·24) and 86·36 (SD 0·06) %, respectively for the same products containing Bb-46. Corresponding values for plain yoghurt and soya milk (without bifidobacteria) were 32·81 (SD 0·14) and 5·55 (SD 0·12) %, respectively. The differences between yoghurt or soya yoghurt containing Bb-12 or Bb-46 and plain yoghurt, soya milk or control treatments were statistically significant (n 3; $P<0·05$). Female Swiss albino mice were injected intraperitoneally with the same tumour cells. The lifespan of mice fed diets supplemented with yoghurt or soya yoghurt containing Bb-12 or Bb-46 was prolonged by 16, 23, 34 and 39 %, respectively compared with that of the positive control group (n 6; $P<0·05$). The lifespan of groups fed plain yoghurt or soya milk was prolonged by 15 and 8 %, respectively. Prolongation of lifespan was positively correlated with faeces bifidobacterial count in the groups fed yoghurt or soya yoghurt containing bifidobacteria (r 0·917; $P<0·05$).

Yoghurt: Soya yoghurt: Ehrlich ascites tumour cells: Bifidobacteria

Functional foods hold great promise for human nutrition, but relatively little objective evidence exists for their beneficial effects. Since 1986, yoghurt cultures have been supplemented or replaced by *Bifidobacterium* spp. with the intention of enhancing their therapeutic value (Kailaspathy & Rybka, 1997). Most probiotic foods are derived from milk fermentation and the possibility of using other protein-rich substrates such as soya milk to make such foods has not been adequately considered. The potential role of dietary soya in the prevention and treatment of chronic diseases, in particular heart disease and cancer, has been recognised for a long time (Nagata *et al.* 1982; Anthony *et al.* 1996). Recently, soya yoghurt has been prepared by the fermentation of soya milk using lactic acid bacteria or bifidobacteria (Abd El-Gawad *et al.* 1998; Chou & Hou, 2000).

Although various studies have addressed the health effects of yoghurt, relatively little is known about the potential anti-tumour effects of yoghurt and soya yoghurt containing bifidobacteria.

Therefore, the aim of the present study was to explore the possible anti-tumour activity exerted by yoghurt and soya yoghurt containing bifidobacteria both *in vitro* and *in vivo*.

Materials and methods

Preparation of yoghurt

Low-fat buffalo milk (1·5 %) was inoculated with a 3 % (v/v) liquid culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (Chr. Hansen Laboratories, Copenhagen, Denmark), and then divided into three portions. No bifidobacteria were added to the first portion (plain yoghurt). To the second portion, a 0·07 % (w/v) standardised freeze-dried culture of *Bifidobacterium lactis* Bb-12 was added. To the third portion, a 0·07 % (w/v) standardised freeze-dried culture of *B. longum* Bb-46 was added. The two strains were obtained from Chr. Hansen Laboratories (Copenhagen, Denmark). After incubation the yoghurts were stored at 4 ± 1°C.

Abbreviation: MRS, de Mann Rogosa Sharpe.

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Fresh and non-beany-flavour soya milk was prepared according to Tanteeratarm *et al.* (1993) as described by Abd El-Gawad *et al.* (1998). Soya yoghurt was prepared from soya milk using 0·07 % (w/v) *B. lactis* Bb-12 or *B. longum* Bb-46 according to El-Sayed *et al.* (1998).

Ehrlich ascites tumour cells were obtained from the National Cancer Institute, Cairo University and maintained by weekly intraperitoneal injection of 0·1 ml ascitic fluid in female Swiss albino mice.

Test materials

The test materials were bifidobacterial cultures in de Mann Rogosa Sharpe (MRS) medium and their centrifuged fractions (at 11 400 ***g*** and 5°C for 15 min) as well as six different products: plain yoghurt, yoghurt containing Bb-12, yoghurt containing Bb-46, soya milk, soya yoghurt containing Bb-12 and soya yoghurt containing Bb-46.

Bacterial quantification

Bacterial counts of *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in yoghurt were determined according to Lee *et al.* (1973), in which the yoghurt sample was added to Lee's agar and incubated at 43°C for 3 d. Bifidobacteria were enumerated in soya yoghurt by a poured plate method using Lactobacilli MRS-agar medium as described by Samona & Robinson (1991). Bifidobacteria were enumerated in yoghurt containing Bb-12 or Bb-46 according to the method of Dinakar & Mistry (1994), in which a mixture of antibiotics, including 2 g paromomycin sulfate, 0·3 g nalidixic acid, and 60 g lithium chloride, was dissolved in 1 litre distilled water, filter-sterilised (0·2 μm) and stored at 4°C until use. The antibiotic mixture (5 ml) was added to 100 ml MRS-agar medium. L-Cysteine-HCl 0·5 % (Sigma Chemical Co., St Louis, MO, USA) was also added to decrease the redox potential of the medium. Plates were incubated at 37°C for 48 h anaerobically. The viable counts of yoghurt cultures and bifidobacteria in the test products are shown in Table 1.

In vitro *determination of anti-tumour activity*

At 7 d after the injection of tumour cells, 2×10^6 Ehrlich ascites tumour cells were taken from the peritoneal cavity of the mice and mixed with the RPMI 1640 medium, then inoculated with 10 μl of each test material for 2 and 24 h at 37°C. Each test material was added to the tumour cells with and without heating to 85°C, followed by cooling to 37°C. After incubation, 30 μl cell suspension were mixed with 5 μl trypan blue dye. The number of dead tumour cells was measured according to the method of Bennett *et al.* (1976) using a haemocytometer slide under microscope examination.

In vivo *determination of anti-tumour activity*

The female Swiss albino mice were housed in cages with a screen bottom in a temperature- and humidity-controlled room. All animals were kept under normal healthy conditions and fed a basal diet (Table 2) for 1 week. After this adaptation period, the mice were divided randomly into eight experimental groups each of six animals. Two groups received the basal diet throughout the experimental period (control groups), the other six groups were fed a basal diet supplemented with plain yoghurt, yoghurt Bb-12, yoghurt Bb-46, soya milk, soya yoghurt Bb-12 or soya yoghurt Bb-46 throughout the experimental period. After 3 weeks, these six groups and one of the two control groups were injected intraperitoneally with 2×10^6 Ehrlich asites tumour cells. The injected control group served as a positive control, while the other control group without injection served as a negative control. The mice were allowed free access to the experimental diet and water. Every 3 d, faeces were collected from each mouse and kept frozen until analysis. Whenever a mouse died the time of its death was recorded immediately. Faecal bifidobacteria were quantified by a poured plate method on Lactobacilli MRS-agar medium as described by Chen *et al.* (1999).

Statistical analysis

Data are presented as means and standard deviations. The significant differences among treatments (groups) were evaluated using the general linear model procedure of SAS (1993; SAS Institute, Inc., Cary, NC, USA) to analyse the biological examination data by least significant difference at $P < 0{\cdot}05$ (SAS Institute, Inc. 1990).

Table 1. Viable count of yoghurt culture and bifidobacteria in the test products

(Mean values of three replicates)

	Count (cfu × 10^8/ml)	
Test product	Yoghurt culture*	Bifidobacteria†
Plain yoghurt	1·9	–
Yoghurt Bb-12	1·6	2·7
Yoghurt Bb-46	2·9	1·7
Soya yoghurt Bb-12	–	5·9
Soya yoghurt Bb-46	–	9·9

cfu, Colony-forming units.
* *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*.
† *Bifidobacterium lactis* Bb-12 or *B. longum* Bb-46.

Table 2. Experimental mice groups and diets used in the trial treatments*

Group	Experimental diets
Non-injected by Ehrlich ascites tumour cells	
Negative control	100 g Basal diet† + 50 ml water
Injected by Ehrlich ascites tumour cells	
Positive control	100 g Basal diet + 50 ml water
Plain yoghurt	100 g Basal diet + 50 g plain yoghurt
Yoghurt Bb-12	100 g Basal diet + 50 g yoghurt Bb-12
Yoghurt Bb-46	100 g Basal diet + 50 g yoghurt Bb-46
Soya milk	100 g Basal diet + 50 g soya milk
Soya yoghurt Bb-12	100 g Basal diet + 50 g soya yoghurt Bb-12
Soya yoghurt Bb-46	100 g Basal diet + 50 g soya yoghurt Bb-46

* For details of the test products, see Table 1.
† Content (g/kg): casein, 150; maize oil, 100; cellulose, 100; mineral mix, 40; vitamin mix, 10; starch 600.

Table 3. Ehrlich ascites tumour cells proliferation as affected by bifidobacteria cultivated in MRS medium and its centrifuged fractions

(Mean values and standard deviations)

	Tumour cell death (%)			
	2 h Incubation		24 h Incubation	
Treatments	Mean	SD	Mean	SD
Control*	3·39^{f}	0·03	3·92^{f}	0·05
Bifidobacterium lactis Bb-12				
Bifidobacteria in MRS‡	85·42^{a}	0·78	94·74^{a}	0·05
Supernatant fraction	77·61^{c}	0·29	83·33^{c}	1·60
Precipitate	4·00^{f}	0·19	16·07^{d}	2·08
B. longum Bb-46				
Bifidobacteria in MRS†	85·10^{ab}	1·28	94·00^{a}	1·11
Supernatant fraction	71·43^{d}	1·75	88·06^{b}	1·79
Precipitate	9·09^{e}	1·24	11·11^{e}	0·80

^{a,b,c,d,e,f} Mean values (n 3) within a column with unlike superscript letters were significantly different ($P<0·05$).
* Saline solution without bifidobacteria.
† The viable count of bifidobacteria was not determined.

Results

In vitro *determination of anti-tumour activity*

Effect of bifidobacteria cultivated in MRS broth medium and its centrifuged fractions. *B. lactis* Bb-12 and *B. longum* Bb-46 cultivated in MRS broth medium and added at 0·07 % (w/v) inhibited tumour cell proliferation by 85·42 and 94·74 % and by 85·10 and 94·00 % after 2 and 24 h incubation, whereas their supernatant fractions inhibited by 77·61 and 83·33 %, and by 71·43 and 88·06 %, respectively (Table 3). In contrast, their sedimented fractions inhibited by only 4·00 and 16·07 %, and by 9·09 and 11·11 %. Data in Table 3 showed that the differences between the test and control treatments after 2 and 24 h incubation were statistically significant ($P<0·05$).

Effect of bifidobacteria-supplemented yoghurts. The inhibitory effects of yoghurt and soya yoghurt containing Bb-12 and Bb-46 in comparison with plain yoghurt and soya milk with and without heating at 85°C on the proliferation of tumour cells are presented in Table 4. Yoghurt and soya yoghurt containing Bb-12 and Bb-46 caused 83–98 % cell death. Plain-yoghurt treatment caused 32·81 and 63·89 % cell death after 2 and 24 h incubation, respectively. Soya milk and control treatments caused only 5·55 and 6·82 % and 3·39 and 3·92 % cell death after 2 and 24 h incubation, respectively. The differences between yoghurt or soya yoghurt containing Bb-12 or Bb-46 and plain yoghurt, soya milk or control treatments were statistically significant (n 3; $P<0·05$). The anti-tumour activity of all tested products markedly decreased upon heating at 85°C as compared with that of non-heated products (Table 4).

In vivo *determination of anti-tumour activity*

The effect of feeding of the basal diet supplemented with yoghurt and soya yoghurt containing Bb-12 or Bb-46 on the lifespan and survival rate of the mice injected with Ehrlich ascites tumour cells is presented in Table 5. The lifespan of mice fed the diets supplemented with soya yoghurt and yoghurt containing Bb-12 and Bb-46 was 39, 34, 16 and 23 % longer than those of the control group, respectively. The corresponding average prolongation of lifespan in the mice fed the diets containing plain yoghurt and soya milk was 15 and 8 %, respectively.

Table 6 illustrates the faecal bifidobacterial viable count of the experimental mice groups during the 9 d feeding period. Faecal bifidobacterial counts in the groups fed yoghurt and soya yoghurt containing Bb-12 and Bb-46 ranged between 1·5 and $3·0 \times 10^{4}$ colony-forming units/g at the end of the feeding period. In contrast, faecal bifidobacterial counts in the mice fed the diets containing soya milk and plain yoghurt were 3·2 and $2·1 \times 10^{3}$ colony-forming units/g, respectively; counts were lower still in the negative and positive control groups, reaching only 1·0 and $1·5 \times 10^{2}$ colony-forming units/g at the end of the feeding period, respectively.

The prolongation of lifespan was positively correlated with faecal bifidobacterial counts in the groups fed yoghurt and more particularly soya yoghurt containing bifidobacteria (r 0·917; $P<0·05$).

Table 4. Ehrlich ascites tumour cell death as affected by heat- and non-heat-treated test products after 2 and 24 h incubation

(Mean values and standard deviations)

	Tumour cell death (%)							
	Before heating				After heating			
	2 h Incubation		24 h Incubation		2 h Incubation		24 h Incubation	
Treatment group*	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control†	3·39^{ef}	0·03	3·92^{ef}	0·05	3·39^{ef}	0·03	3·92^{f}	0·05
Plain yoghurt	32·81^{d}	0·14	63·89^{d}	0·05	8·00^{ab}	0·13	36·62^{e}	0·75
Yoghurt Bb-12	83·01^{c}	0·11	92·31^{c}	0·07	8·51^{ab}	0·44	45·12^{c}	0·25
Yoghurt Bb-46	83·82^{c}	0·24	97·87^{a}	0·98	7·55^{ab}	0·44	48·68^{ab}	0·26
Soya milk	5·55^{e}	0·12	6·82^{e}	0·30	–		–	
Soya yoghurt Bb-12	88·23^{a}	0·06	98·12^{a}	1·17	8·16^{ab}	0·25	43·02^{cd}	1·02
Soya yoghurt Bb-46	86·36^{ab}	0·06	94·74^{ab}	0·75	11·67^{a}	0·27	54·17^{a}	1·09

^{a,b,c,d,e,f} Mean values (n 3) within a column with unlike superscript letters were significantly different ($P<0·05$).
* For details of bifidobacterial count in the test products, see Table 1.
† Saline solution without bifidobacteria.

Table 5. Effects of test products on survival of mice injected intraperitoneally with Ehrlich ascites tumour cells*

(Mean values and standard deviations)

	Lifespan		
Treatment group†	Mean	SD	Survival rate (%)‡
Positive control§	12·2[a]	0·22	100
Plain yoghurt	14·0[ab]	0·25	115
Yoghurt Bb-12	14·2[ab]	0·59	116
Yoghurt Bb-46	15·0[ab]	0·91	123
Soya milk	13·2[ab]	0·33	108
Soya yoghurt Bb-12	16·4[b]	0·46	134
Soya yoghurt Bb-46	17·0[b]	0·36	139

[a,b] Mean values (n 6) within a column with unlike superscript letters were significantly different ($P<0·05$).
* For details of the test products, see Tables 1 and 2.
† At the end of the experiment (17 d), the mice in the negative control group were killed by diethyl ether inhalation.
‡ Survival rate (%) = (mean life span of treated group/mean life span of positive control group) × 100.
§ Mice injected with tumour cells and fed the basal diet.

Discussion

The purpose of the present study was to find whether yoghurt and soya yoghurt containing *Bifidobacterium* spp. were effective in the inhibition of the proliferation of Ehrlich ascites tumour cells *in vitro* and *in vivo* and to compare their effects with those of plain yoghurt and soya milk.

B. lactis Bb-12 and *B. longum* Bb-46 cultures in MRS broth medium exhibited the greatest inhibitory effect on the proliferation of tumour cells, followed by their centrifugal supernatant fractions, while the sediments showed the lowest effect. These findings suggest that the tumour-suppressing activity of bifidobacteria was present mainly in the supernatant fraction. This is in agreement with Biffi *et al.* (1997), who reported that the inhibitory effect of bifidobacteria may be related to metabolites and cell-wall components (Sekine *et al.* 1994). Similarly Kohwi *et al.* (1978, 1982) found that an anti-tumour effect was associated with the cell wall and whole cells of *B. infantis* and the soluble fraction of *S. thermophilus* (Friend *et al.* 1982).

Yoghurt and soya yoghurt containing Bb-12 and Bb-46 had a greater anti-proliferation effect than plain yoghurt, while soya milk and control treatments had a weaker effect. A similar trend has been reported by Biffi *et al.* (1997), who found that the growth of MCF7 breast cancer cells was inhibited by cultured milk containing bifidobacteria. Baricault *et al.* (1995) found that *L. hilveticus* and *Bifidobacterium* were the most effective in decreasing the growth rate of HT-29 human colon cancer cells. The present data showed that the anti-tumour activity of all tested products decreased considerably after heating at 85°C (Table 4). These results are in agreement with the findings of De Simone *et al.* (1986), Perdigon *et al.* (1995) and Pool-Zobel *et al.* (1996).

All mice fed with the tested products showed prolonged survival compared with that of the positive control group (Table 5) but this increase was significant only in the case of the mice fed the diets supplemented with soya yoghurt containing Bb-12 or Bb-46. These results are in line with Takano *et al.* (1985) who found that the growth of Ehrlich ascites tumour cells was inhibited by 28 and 42 % in mice given yoghurt and cultured-milk diets compared with the control group, respectively. Friend *et al.* (1982) noticed a decrease in the occurrence of tumours by 25–30 % when feeding mice with yoghurt. Reddy *et al.* (1983) found that the consumption of yoghurt reduces the number of tumours by 20–32 % in mice implanted with Ehrlich tumour cells. Shackelford *et al.* (1983) found a significant prolongation in the lifespan of mice infected with colon tumours when fed with yoghurt. *B. longum*, a lactic acid-producing bacterium present in the human colon, has also been demonstrated to inhibit colon tumour development in experimental animals (Reddy & Riverson, 1993; Abdelali *et al.* 1995; Singh *et al.* 1997).

In the present study, there was a strong relationship between the bifidobacterial count in the tested products (Table 1) and its count in mice faeces (Table 5) and accordingly the intestinal flora in mice. The bifidobacterial count in the faeces of the mice fed soya yoghurt containing Bb-12 or Bb-46 was higher than for the mice fed yoghurt containing Bb-12 or Bb-46 as well as in the faeces of corresponding

Table 6. Faecal bifidobacterial counts of mice injected by Ehrlich ascites tumour cells as affected by feeding diets containing the test products*

(Mean values of three replicates)

	Bifidobacteria count (cfu/g faeces)				
			Feeding period (d)		
Treatment group	Before injection†	After injection‡	3	6	9
Negative control	$9·6 \times 10^2$	$4·4 \times 10^2$	$4·0 \times 10^2$	$5·1 \times 10^2$	$1·0 \times 10^2$
Positive control	$9·6 \times 10^2$	$5·7 \times 10^2$	$4·8 \times 10^2$	$4·5 \times 10^2$	$1·5 \times 10^2$
Plain yoghurt	$2·2 \times 10^2$	$3·0 \times 10^2$	$1·0 \times 10^3$	$5·0 \times 10^2$	$2·1 \times 10^3$
Yoghurt Bb-12	$1·2 \times 10^3$	$5·0 \times 10^3$	$3·0 \times 10^4$	$7·0 \times 10^2$	$1·5 \times 10^4$
Yoghurt Bb-46	$5·0 \times 10^3$	$8·3 \times 10^3$	$5·5 \times 10^3$	$2·5 \times 10^3$	$2·5 \times 10^4$
Soya milk	$2·5 \times 10^3$	$1·2 \times 10^3$	$1·4 \times 10^3$	$4·0 \times 10^2$	$3·2 \times 10^3$
Soya yoghurt Bb-12	$1·0 \times 10^4$	$3·0 \times 10^4$	$8·8 \times 10^3$	$1·2 \times 10^4$	$2·5 \times 10^4$
Soya yoghurt Bb-46	$3·1 \times 10^4$	$3·1 \times 10^4$	$6·5 \times 10^3$	$3·9 \times 10^4$	$3·0 \times 10^4$

cfu, Colony-forming units.
* For details of the test products, see Tables 1 and 2.
† At 3 d before injection with tumour cells.
‡ Immediately after injection.

mice groups (Table 6). The higher bifidobacterial count in soya yoghurt might explain the increase in lifespan of the mice fed soya yoghurt containing Bb-12 or Bb-46 compared with the other groups (Table 5).

Fernandes & Shahani (1990) suggested that the anti-carcinogenic effect of bifidobacteria may be the result of direct removal of procarcinogens, indirect removal of procarcinogens, or activation of the body's immune system. Bifidobacteria can greatly reduce the mutagenicity of nitrosamines by removing the source of procarcinogens or the enzymes which lead to their formation (Hosono *et al.* 1990). Alternatively Perdigon *et al.* (1998) suggested that one of the mechanisms by which yoghurt containing bifidobacteria exerts anti-tumour activity is through its immunomodulator activity by reducing the inflammatory immune response, which was markedly increased when the carcinogen was administered. Such reduction of the inflammatory immune response by bifidobacteria has been proposed potentially to prevent cancer (Sekine *et al.* 1985, 1994; Rafter, 1995). These anticancer effects are due to immune enhancements by the cells, cell-wall components and extracellular components of bifidobacteria (Abdelali *et al.* 1995; Rice *et al.* 1995; Pool-Zobel *et al.* 1996; McCarthy *et al.* 1997; Ohta *et al.* 2000).

In conclusion, both the *in vitro* and *in vivo* studies suggested that yoghurt and more particularly soya yoghurt containing large numbers of viable bifidobacteria may have anti-tumour effects. If such functional dairy foods that contain bifidobacteria have the same effect in human subjects they may have potential value in cancer prevention.

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