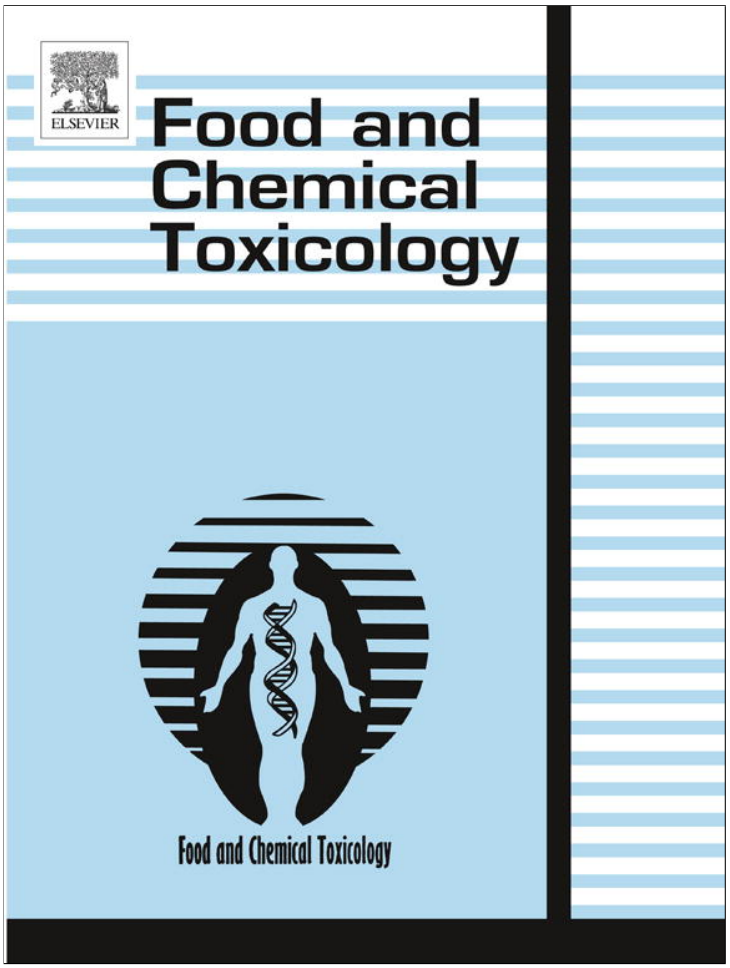

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A soy-based product fermented by *Enterococcus faecium* and *Lactobacillus helveticus* inhibits the development of murine breast adenocarcinoma

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ABSTRACT

Purpose: Soy and its fermented products are considered functional foods. The study objective was to assess three functional food – a non-fermented soy product (NFP), fermented soy product (FSP), fermented soy product enriched with isoflavones (FI) – in terms of their ability to reduce the development of adenocarcinoma in mice, as well their ability on modulating immune system.
Methods: It was observed tumor volume and to verify correlations with the immune system it was measured levels of the cytokines IL-1β and TNF-α produced by macrophages as well as IFN-γ produced by lymphocytes using ELISA test, and nitric oxide production by macrophages using Griess reagent.
Results: All products showed immunological activity, but FSP showed the most effective tumor containment, resulting in smallest tumor volumes. FI animals expressed larger amounts of nitric oxide and IL-1β and exhibited larger tumor sizes than FSP and NFP animals.
Conclusions: The results suggested that the ingestion of FSP was most efficient in tumor containment, possibly due to a positive modulation of the immune system by when *Enterococcus faecium* and *Lactobacillus helveticus* are added to the soy product.

1. Introduction

Cancer represents a group of diseases characterized by uncontrolled proliferation of cells, which spreads from the original site to other parts of the body, resulting in destruction of those areas (Woyengo et al., 2009). Breast cancer is a common form of malignancy and its risk is highly modifiable by diet.

Cancer-associated inflammation generated by may promote survivor, implantation and tumor growth in a late stage. Tumor-associated macrophages may produce nitric oxide (NO), cytokines such as tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1), and factors that promote angiogenesis (Allavena et al., 2008; Mantovani et al., 2008). Inflammation-derived signaling initiates an adaptive immune response. Innate immunity may trigger a protective adaptive immune response, with interferon-γ (IFN-γ) production, or not (Raulet and Guerra, 2009).

Immune system can be modulated by diet. Immune tissues affected by diet includes those present on intestinal mucosa, which contain macrophages, dendritic cells and lymphocytes B and T in contact with food that is being digested (Wershil and Furuta, 2008). Those cells can influence systemic immune response, modulating cells dispersed on lymph nodes and spleen (Adolfsson et al., 2004).

Functional foods present bioactive components, capable of modulating the organism physiology (Hakkak et al., 2000). The consumption of soy (*Glycine max* Merr.) and soy-derived foods is related to a 50% reduction in the risk of breast and prostate cancer and of heart disease (Linton and Harder, 2007). Isoflavones are polyphenolic compounds present in soy, and they can be present in the form of β-glycosides, such as genistin, daidzin and glycitin, or in the form of aglycones, such as genistein, daidzein and glycitein (Kudou et al., 1991), and those substances appear to have an anticancer action (Su and Simmen, 2009). Isoflavones are more present in the form of β-glycosides in soy, and aglycones isoflavones can be better absorbed on human intestine. Conversion from

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β-glycoside form to aglycone is performed by bacteria (Tsuchihashi et al., 2008). As such, fermented soy products are the ones that may contain the highest levels of aglycones isoflavones.

When associated to microorganisms, or microbial fermented stimulants (*Bifidobacterium, Lactobacillus*), functional food are denominated probiotics (Urtreger et al., 1997). Once ingested, probiotic bacteria may persist on intestinal tract for some time, but they do not become a permanent member of the intestinal mycrobiota (Corthésy et al., 2007). The probiotics produce several therapeutic effects in human and animal health such as: infection resistance, allergy reduction (Wolowczuk et al., 2008) and immune-stimulant effect, activating macrophages and leading to release of cytotoxic mediators and cytokines, such as TNF-α, IFN-γ. Those effects might cause death of circulating tumor cells (Queiroz and Batista, 1999; Dranoff, 2004; Wolowczuk et al., 2008).

Considering the importance of obtaining soy-based products that can help controlling and/or preventing breast cancer, Rossi et al. (1999) developed a soy product fermented by *Enterococcus faecium* CRL 183 and *Lactobacillus helveticus* ssp. jugurti 416. The objective of the present study was to evaluate the susceptibility to breast cancer of animals ingesting different soy products, to correlate the effects observed with the immune response by production of nitric oxide (NO) and levels of cytokines IL-1β, TNF-α and IFN-γ.

2. Methods and materials

2.1. Animals

Female BALB/c mice weighing 18–25 g were obtained from CEMIB (Centro Multidisciplinar para Investigação Biológica), UNICAMP, Campinas, SP, Brazil. The animals were maintained in cages in groups of 5 under controlled environment (temperature 23 ± 2 °C, relative humidity 56 ± 2%, and 12:12 h light/dark cycles). The animals had free access to water and commercial mouse chow (Purina). The study was approved by Research Ethics Committee, of School of Pharmaceutical Sciences – UNESP, Brazil (08/2009 CEP/FCF/CAr).

2.2. Tumor implantation

The murine adenocarcinoma LM3 lineage was provided by Dr. Elisa Bal de Kier Joffé, Institute of Oncology Angel H. Roffo, Buenos Aires, Argentina. This cell lineage does not have estrogen receptors and it was obtained from primary subcultures of murine mammary cell adenocarcinoma (Urtreger et al., 1997). LM3 cells were maintained in culture in MEM medium (Sigma–Aldrich, United States) supplemented with inactivated 10% fetal bovine serum (Sigma–Aldrich, United States). All animals were inoculated subcutaneously on the right side of the abdomen with 1.25×10^4 cells in 250 μL of culture medium; that inoculum was set by previous tries.

2.3. Diets

The bacteria *L. helveticus* ssp. *jugurti* 416 were obtained from the culture collection of the Institute of Food Technology (Araraquara, SP, Brazil) and the strain *E. faecium* CRL 183 was obtained from the culture collection of the Reference Center for Lactobacilli (Buenos Aires, Argentina).

The fermented soy product (FSP) was prepared weekly by the method of Rossi et al. (1999) and had the following basic composition: soybean aqueous extract with 8.0% sucrose, 1.0% soy oil, 1.0% lactose and 0.5% gelatin. The product was inoculated with mixed cultures of *E. faecium* and *L. helveticus* at 1.5% each (v/v), incubated at 37 °C until pH 4.5 was reached, and stored at 4 °C until used. Viable counts (Rossi et al., 1999) were performed on fermented products with specific media for cocci (Agar M-17, Difco) and for bacilli (Agar MRS, Difco). Fermented product was considered as having at least 10^8 colony-forming unit of each strain. The non-fermented soy product (NFP) was of the same composition as the fermented product but without microorganisms, and it was acidified by the addition of lactic acid until pH 4.5 was reached.

Animals ingesting this product or PFS ingested 0.05 mg of isoflavones/day (Rossi et al., 2004). Fermented soy product enriched with isoflavones (FI) was prepared by using FSP and enriched with 1.125 g/L of Isoflavin Beta® (Galena, Brazil), which lead to a 0.09 mg ingestion of isoflavones per day (Rossi et al., 2004).

The commercial mouse chow used (Purina #5001) has a closed formula, but it contains 810 ± 10 μg of total isoflavones per gram of chow, detected by reverse-phase HPLC chromatographic, as reported elsewhere (Brown and Setchell, 2001).

2.4. Groups and time of study

The mice received the various treatments by gavage, 1 mL/day, for 10 days before the implantation of the tumor (item 2.2) and continuously thereafter for 30 days, when they were euthanized in a CO_2 chamber. The animals were divided into four groups containing 5 animals each: non-fermented product group (NFP group): receiving NFP; fermented soy product group (FSP group): receiving FSP; fermented soy product enriched with isoflavones group (FI group): receiving FI; water group (W group): receiving only sterile water. All tests were repeated in three separate experiments, and then combined.

2.5. Tumor volume

After sacrifice, the tumors were removed and measured for length, height and width, using a Mitutoyo Digimatic caliper. Tumor volume was calculated by the following formula (Zhou et al., 1998):

$$\text{Volume}(\text{mm}^3) = 0.523 \times \text{length}(\text{mm}) \times \text{width}(\text{mm}) \times \text{height}(\text{mm})$$

2.6. Peritoneal exudate cells (PEC) and supernatants

Animals received a 3 mL-thioglycollate injection intraperitoneally 3 days before sacrifice. Thioglycollate-elicited peritoneal exudate cells (PEC) were harvested from Balb/c mice in 5.0 mL of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were cultivated in RPMI-1640 culture medium containing 2×10^{-5}M 2β-mercaptoethanol, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine, and 5% fetal bovine serum (denoted RPMI-1640-C) (all from Sigma–Aldrich, United States) at a concentration of 5×10^6 cells. Cells were added to 24-well tissue culture plates (Corning Incorporated, United States) and incubated for 60 min in an incubator at 37 °C, 5% CO_2. After incubation, non-adherent cells were removed by washing with RPMI-1640-C culture medium. To the macrophages that adhered to the plate, a volume of RPMI-1640-C and LPS (10 μg/mL) equal to the initial volume was added, or only RPMI-1640-C was added as a cell control. The plates were again incubated at 37 °C, 5% CO_2, for an additional 24 h and then the content of wells was centrifuged at 4 °C for 10 min at 14000*g*. The culture supernatants were collected and stored at −80 °C.

2.7. Spleen cells

The spleens were collected aseptically and placed on a Petri dish containing 3.0 mL RPMI-1640 culture medium (Sigma). The cell suspension was obtained by tweezing the spleen. A culture at concentration of 5×10^6 cells/mL in RPMI-1640-C was plated with Concanavalin-A (Con-A) (0.5 μg/mL) or only RPMI-1640 medium (cell control) for 24 h at 37 °C in a constant 5% CO_2. After this incubation, the content of wells was centrifuged at 4 °C for 10 min at 14000*g* and the supernatants were collected and stored at −80 °C.

2.8. NO measurement

From PEC culture supernatant, 50 μL aliquots were mixed with 50 μL of Griess reagent (Green et al., 1982) (1% w/v sulfanylamide, 0.1% w/v naphthylethylenediamine and 3% H_3PO_4) and incubated at room temperature for 10 min on dark, and the color reaction was determined at 540 nm with a Multiskan Ascent ELISA reader. Results were reported as the mean ± SD nitrite concentration.

2.9. Determination of IL-1β, TNF-α and IFN-γ

Cytokine determination was made on PEC culture supernatant for IL-1β and TNF-α, and on splenic cells culture supernatant for IFN-γ. The determination was made using BD Biosciences ready-to-use kits, according to instructions. Absorbance was read at 450 nm in a microplate reader (Multiskan Ascent, Labsystems), and cytokine concentrations were calculated from curves of known concentrations of IL-1β, TNF-α or IFN-γ standards. Results were reported as picograms/mL.

2.10. Statistical analysis

Data were analyzed statistically using the GraphPad Instat statistical program. Analysis of variance was used, with the level of significance set at $p < 0.05$ and multiple comparisons were carried out using the Tukey–Krammer test. All experiments were carried out in triplicate on groups of 10 animals each.

3. Results

3.1. Tumor volume

In the Fig. 1, tumor volume differed significantly between groups NFP, FSP, FI and W, being considerably smaller in the

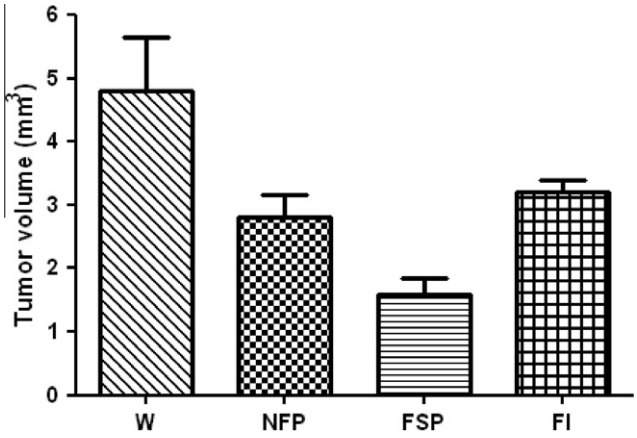

Fig. 1. Final tumor volume. Tumors from animals were removed after sacrifice, and volume was calculated. FSP (fermented soy product) was found to be statistically different from W (water), NFP (non-fermented soy product), and FI (fermented soy product enriched by isoflavones) ($p < 0.001$).

FSP group. A comparison of the four groups shows that tumor volume increased in the order: FSP (1.6 ± 0.5 mm^3) < NFP (2.8 ± 0.8 mm^3) < FI (3.2 ± 0.4 mm^3) < W (4.8 ± 1.9 mm^3).

3.2. NO production

The reactive intermediates of nitrogen are currently considered important in physiological and pathological processes. In the Fig. 2, the macrophages were cultured in the presence of LPS, a stimulus for NO production. In tumor-bearing animals, the FSP group showed the lowest production ($p < 0.001$) compared to the NFP, FI and W groups.

3.3. Determination of IL-1β, TNF-α and IFN-γ

In the Fig. 3A, the production of IL-1β in the tumor-bearing animals of the FI group showed the highest IL-1β level, and it was statistically different ($p < 0.001$) when compared to all others groups. Fig. 3B shows that animals of groups NFP and FSP presented no significant difference in the amount of TNF-α released ($p > 0.05$). The FI group, producing the largest amount of this mediator, presented significative difference when compared to NFP and FSP ($p < 0.001$).

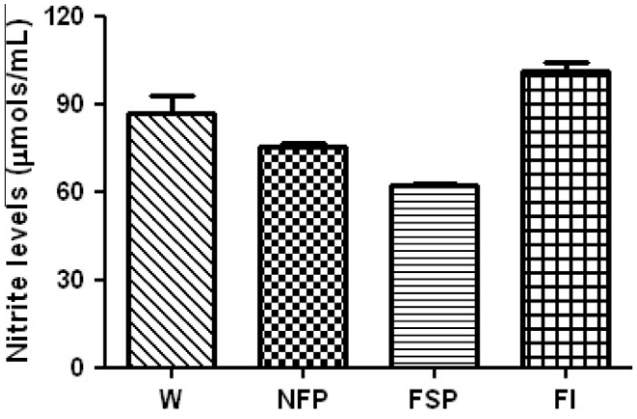

Fig. 2. Nitric oxide (NO) production by peritoneal macrophages from BALB/c mice. Macrophages were harvested from animals of each group. The FSP (fermented soy product) group showed the lowest NO production ($p < 0.001$) compared to the NFP (non-fermented soy product), FI (fermented soy product enriched by isoflavones) and W (water) groups.

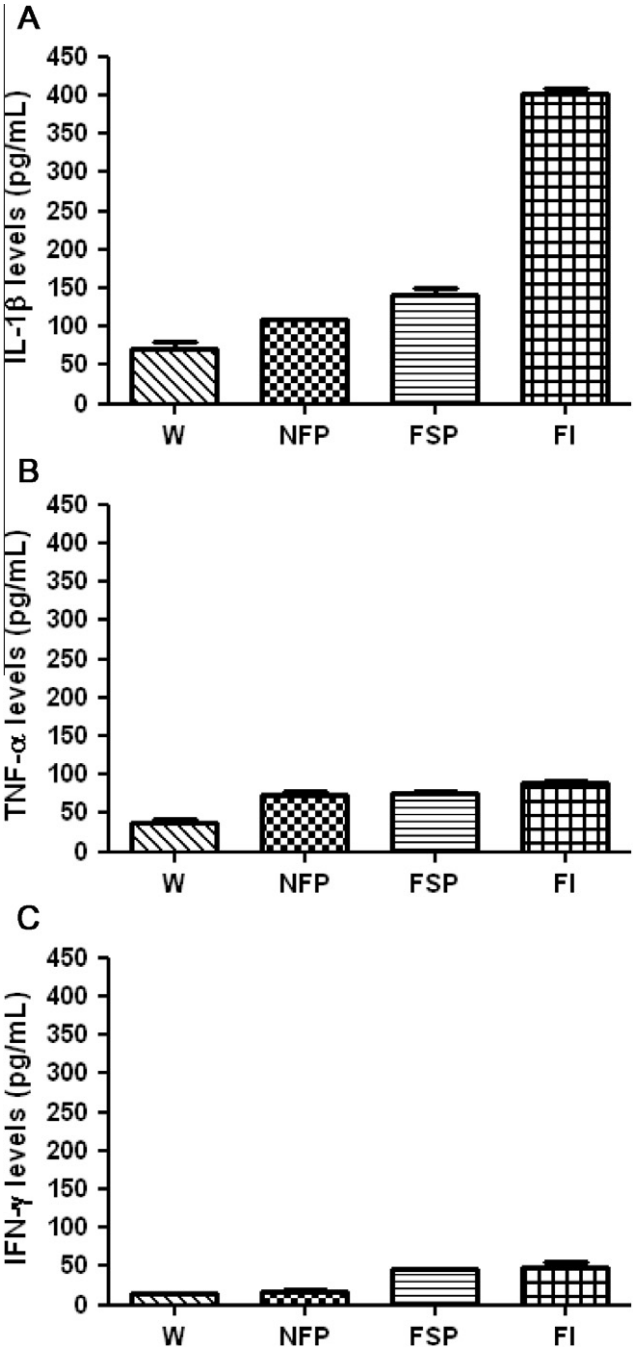

Fig. 3. Levels of interleukin (IL)-1β (A), TNF-α (B) and IFN-γ (C). Cytokines IL-1β and TNF-α were of peritoneal macrophages, while IFN-γ was released into the culture supernatants of splenic cell culture of mice of each group. (A) Significant difference ($p < 0.001$) was observed when FI (fermented soy product enriched by isoflavones) was compared to W (water), NFP (non-fermented soy product) and FSP (fermented soy product) groups. (B) Significant difference ($p < 0.001$) was observed only when FI was compared to W group. (C) FSP and FI groups are statistically similar ($p > 0.05$) but both are significantly different from NFP and W groups ($p < 0.001$).

In the Fig. 3C, the concentration of IFN-γ of group NFP showed different values from FSP and FI ($p < 0.001$).

4. Discussion

A dietary strategy that may prevent the development of breast cancer, especially for the estrogen receptors-negative breast cancer, is of great interest. The mice used in this study were subjected

to the various soy-based treatments for a period of 30 days. After 10 days of ingestion, LM3 tumor cells, which does not present estrogen receptors, were inoculated subcutaneously and the animals were monitored daily. All animals continued to receive their respective treatment until the time of sacrifice.

During the course of study, animals ingesting soy-based treatments developed smaller tumors than control group, receiving only water, even though only FSP group presented a statistical difference compared to water group (Fig. 1). This indicates that soy compounds may be influencing the tumor microenvironment or acting directly on tumor cells, as it can be corroborated by some studies (Zhou et al., 1998; Yihai et al., 2002), and it is known that some tumor cells may be influenced by genistein (Privat et al., 2009). Also, probiotic bacteria may also have a role in this notable volume difference. In one study using chemical induction for experimental colon cancer, *E. faecium* CRL 183 ingestion diminished polypus formation (Sivieri et al., 2008), although this effect could not be observed when animals were ingesting the soy-based probiotic product (Silva et al., 2009).

Tumor volume can be modified by cells that are into the tumor, especially immune cells. The well-known tumor-associated inflammation is related to significant tumor-promoting effects, for instance, angiogenesis, metastasis and immunosuppression. The tumoricidal action of macrophages *in vitro* is a well-documented mechanism of host defense for the control and eradication of neoplastic disease. This macrophage activity on some tumor cells may occur by mechanisms involving the NO pathway (Ziegler, 2004). We determined NO levels in the culture supernatant of peritoneal macrophages from female Balb/c mice (Fig. 2). Comparing these results with those on tumor volume (Fig. 1) it seems that a smaller concentration of NO is produced in the group that developed smallest tumors, the FSP group. NO acts directly on tumor cells, inhibiting cell respiration, and indirectly, provoking damage to cellular DNA (Ziegler, 2004). In contrast to this tumoricidal activity, it has been proposed that NO is a mediator that favors tumor growth by acting on the initial stage. NO plays an important role in tumor progression by regulating the formation of new blood vessels (Dinarello, 2000; Green, 2003). This action may be dependent on the dose and the region NO is released. On normoxic regions, NO production may ensure cell survivor and generate angiogenic effects. However, on hypoxic regions, high doses of NO may reduce cell survivor (Weigert and Brüne, 2008). In our study, it seems that elevated NO levels may have contributed to tumor growth on W, NFP and FI groups, as reduced NO levels of FSP group was associated with diminished tumor volume.

IL-1β (Fig. 3A) may be associated with lower tumor development, because it may trigger the process of apoptosis and can inhibit cell growth (O'neill and Dinarello, 2000), although clinical reports suggest that this cytokine elevation is related to disease progression (Xue et al., 2010; Tsirakis et al., 2011). More recent studies point to a tumor-promoting and immunossupressive effect, as well as a chemoresistant action, which is strongly implicated in cancer progression (reviewed by Dunn et al., 2012). As such, higher levels of IL-1β found on FI group may help explaining why this group presented a different, larger, tumor volume than the other fermented soy-based treatment, FSP. Although genistein consumption seems to diminish IL-1β secretion in another disease model (Gao et al., 2009), the elevated IL-1β found in our study may also be generated by tumor progression per se, instead of because the higher isoflavone intake.

TNF-α (Fig. 3B) is one of the main pro-inflammatory cytokines and plays a central role in the initiation and regulation of the cytokine cascade during the inflammatory response. Although TNF-α may be responsible for the destruction of tumor cells by means of a cytotoxic action, this cytokine may promote tumor growth, invasion and metastasis if its levels are chronically elevated (Szlosarek et al., 2006). An interesting data was that group W released smaller amounts of TNF-α and was the group that also produced the lowest amount of IL-1β and the largest tumor volume. Although the difference among other groups regarding TNF-α production was not statically significant (Fig. 2), this suggests that an increase in TNF-α and IL-1β in the groups NFP and FSP may cross the line between inaction and cytotoxicity activity, and further, for IL-1β levels on FI group, towards to a cancer-promoting action.

IFN-γ (Fig. 3C) is a pleiotropic cytokine with a potent antitumor effect since it controls cell growth, increases apoptosis and reduces angiogenesis (Dranoff, 2004). Synergism between IFN-γ and TNF-α is important because it increases cellular apoptosis (Phillips et al., 1998) and affecting the adhesion and migration of endothelial cells (Guo et al., 2001). The increased production of IFN-γ in tumor-bearing FSP and FI groups give evidence of an attempt of containing the tumor. The high production of IFN-γ in the group FSP highlights this way the beneficial role of mediator.

5. Conclusions

Concluding, we verified that the treatments studied stimulate the immune system, but FSP was the most efficient in terms of tumor containment, possibly due to a positive modulation of the immune system when *E. faecium* and *L. helveticus* are added to the soy product. The NFP ingestion was also effective in preventing tumor development, reaffirming the properties of soy. Supplementation with isoflavones appeared to have contributed to tumor development in this concentration, possibly due to their estrogenic properties. Thus, the importance of the adaptation of the organism to this soy phytoestrogen should be emphasized. The FSP group exhibited more apoptosis and less necrosis of macrophages, a fact that may have contributed to the control of the inflammatory process. Thus, the present results show that the ingestion of the fermented soy product was more efficient in terms of tumor containment.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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