ABSTRACT: Lactic acid bacteria (LAB) when used as probiotics can promote stimulation of immune system through increase of the pathogen phagocytosis and also the cytokines production. The purpose of the present work was to evaluate cytokines production by peritoneal macrophages and splenic lymphocytes in rats with colon cancer induced 1,2 dimethylhydrazine (DMH) after treatment with soy product fermented with Enterococcus faecium CRL 183 supplemented with or without calcium. A total of 30 male Wistar SPF rats, randomly allocated to 6 groups (n=10), received the following treatments: Group 1- control; Group 2 -Positive control, rats with colon cancer induced with DMH; Group 3 – DMH rats treated with fermented soy product; Group 4- DMH rats treated with fermented soy and calcium; Group 5- rats treated with fermented soy alone; 6 Group- rats treated with fermented soy and calcium. The groups of induced animals received, subcutaneously, 20 mg/kg body weight of DMH in a weekly dose for 14 weeks. The non-induced animals were inoculated, subcutaneously, with 1mM EDTA (pH 6.5). At the end of the forty-second week the animals were euthanized in a CO2 chamber. Thioglycollate-elicited peritoneal exudates cells (PEC) were harvested from Wistar SPF rat using 5.0 mL of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were washed twice by centrifugation at 200 g for 5 minutes at 4 °C and resuspended in appropriate medium for each test (H2O2, NO, TNF-α, IL-6, IFN-γ and IL-4). Results showed that in colon cancer animals, which were ingested fermented soy product there is a higher immune system stimulation observed in increased of the H2O2, TNF-α, IFN-γ and IL4 production. Results suggest that ingestion of fermented soy product supplemented or not with calcium favor antitumoral response stimulating the cytokines produced by peritoneal macrophages and splenics lymphocytes.

KEY WORDS: Colon Cancer, Cytokines, Hydrogen Peroxide, Macrophages, Nitric Oxide, Soy Product.

INTRODUCTION

Colorectal cancer is one of the most common fatal malignancies worldwide and affects men and women equally and with approximately equal frequency (Weir et al., 2003). Established risk factors include a low consumption of fiber and folate, high consumption of animal fat, red meat and a family history of colon cancer ([Potter, 1981; Hardman, 2003; Linch & Chapelle, 2003].

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the consumers (Homayouni, 2008). The best known is Lactobacillus, Bifidobacterium, and Streptococcus spp., which collaborate synergistically in milk or soy fermented product and are widely used as major starter bacteria for the production of soy fermented product (Schaafsma, 1996). Several lines of evidence have established the benefits of certain probiotics to reduce signs and symptoms of lactose intolerance, prevention and treatment of certain diarrheal diseases, reduction of bacterial enzyme activities, stimulation of the immune system, retarding colon carcinogenesis (Sanders, 1993; Roberfroid et al, 1995).

Enterococci are used as starter and probiotic cultures in foods (Klein, 2003), and they occur as natural food contaminants. The genus Enterococcus is of increased significance as a cause of nosocomial infections, and this trend is exacerbated by the development of antibiotic resistance (Coque et al., 1995). According Heikens et al., (2009) E. faecium strains were generally free of virulence determinants, with notable exceptions. The enterococcal surface protein Esp, identified as a potential virulence
factor, is specifically linked to nosocomial clonal lineages that are genetically distinct from indigenous E. faecium strains. According Eaton et al., (2001) Esp is not essential for Caco-2 cell adherence and intestinal colonization or translocation of E. faecium in mice.

Currently, many researchers seek microorganisms that have probiotic properties, as is the case, for example, Enterococcus faecium CRL 183. In a recent study, our research group verified that E. faecium CRL 183 has the capacity to survive in and colonize the gastrointestinal tract of rats (Sivieri et al., 2008), one of the prerequisites for it to be considered probiotic (Lund et al., 2002), since the viability of lactic acid bacteria (LAB) can be lost on exposure to gastric acid and to bile salts (Bezkorovainy, 2001). Our group observed that the consumption of 200 mL/day of soy fermented with E. faecium CRL 183 and Lactobacillus helveticus subsp. jugurti 416 by normocholesterolemic adult men, for a period of 6 weeks, reduced the levels of total cholesterol and of the LDL fraction and led to an increase of about 10% in HDL-C levels (Rossi et al., 2003). Other need results have also been achieved with a combination of E. faecium CRL 183 and Lactobacillus helveticus subsp. jugurti 416, partial inhibition of breast cancer (Kinouchi, 2006) and prevention of osteoporosis (Bedani et al., 2006, Shiguemoto et al., 2007).

Immune system is responsible for the organism defense against pathogenic substances and some tumors that unchain a specific immune response in the attempt of suppressing its growth. Combined the macrophages performance, the consumption of products derived from soy is being very spread now, could reduce the risk certain diseases, including tumors, improving health condition (Gredel et al., 2008). One explanation for tumor suppression by LAB may be mediated through an immune response of the host. Sekine et al., (1985) suggested that B. infantis stimulates the host-mediated response, leading to tumour suppression or regression. In addition, there are studies to suggest that LAB play an important role and function in the host's immunoprotective system by increasing specific and nonspecific mechanisms to have an antitumor effect (Kato et al., 1984; De Simone et al., 1993; Schiffrin et al., 1995).

Soy-consuming Asian populations have been suggested to be protected not only against breast and prostate cancer incidence but also against colorectal tumors. These populations are exposed to phytoestrogens from birth, which may protect them against the growth-promoting action of sex hormones. In contrast, intermittent exposure to soy products such as those used for relief of postmenopausal symptoms in Western countries could have potentially hazardous effects. Whether the ingestion of soy constituents with estrogenic activity in addition to the presence of high serum estrogen levels in premenopausal women is harmful should be questioned. (Bises et al., 2007). Animal studies have suggested that calcium may be involved in the etiology of colon cancer (Vinas-Salas et al., 1998; Lipkin, 1999; Pence et al., 1988; 1993; 1996). Calcium can bind secondary bile acids and ionized fatty acids, which can promote epithelial cell proliferation in the colon (Van der Meer et al., 1991; Wargovich et al., 1994; Weir et al., 2003). Calcium may also directly decrease epithelial cell proliferation (Newmark et al., 1984).

The purpose of the present work was evaluated the cytokines produced by peritoneal macrophages and splenic lymphocytes in rats with colon cancer induced chemically with 1,2 dimethylhydrazine (DMH) that received soy product fermented with Enterococcus faecium CRL 183 supplemented or not with calcium.

**MATERIALS AND METHODS**

**Animals**

A total of 30 male, Wistar SPF rats, 4 weeks old and average weight 90 g (± 2 g) were obtained from the central animal facility at the State University of Campinas (CEMIB, UNICAMP-SP, Brazil) and randomly divided in six experimental groups, Group 1- Negative control, healthy animals; Group 2- Positive control, rats with colon cancer induced with DMH; Group 3- Colon cancer soy fermented product group, rats with colon cancer induced with DMH and intake of fermented soy product; Group 4- Colon cancer calcium group, rats with colon cancer induced with DMH and intake of fermented soy fermented product supplemented with calcium; Group 5- Healthy soy fermented product group, healthy rats and intake of fermented soy fermented product; 6 Group Healthy calcium group, healthy rats and intake of fermented soy fermented product supplemented with calcium.

The animals were housed for 8 weeks in boxes within a vivarium cabinet (Alesco®, Brazil) equipped with air filtration, controlled temperature (22±1 °C) and a dark, light cycle of 12:12h. The fermented soy product added or not with calcium was administered (3ml/kg) by gavage daily for 42 weeks.

All the animals had free access, every day, water and to rat chow, both sterilized. Standard rat chow (in pellet form) was provided by Purina (Purina®, Brazil), with the following composition, 23% protein; 49% carbohydrate; 4% fat; 5% fiber; 7% ashes and 6% vitamins. Starting 1 week after the beginning of the ingestion of fermented soy product, fermented soy product supplemented with calcium, the groups of initiated animals received, subcutaneously, 20 mg/kg body weight of 1,2-dimethylhydrazine (Sigma, St. Louis, USA) and 1mM (Lab Synth-Brazil) (pH 6.5), in a weekly dose for 14 weeks. The non-initiated animals were inoculated, subcutaneously, with 1mM EDTA (pH 6.5) (Sivieri et al.,
2008). At the end of the forty-second week the animals were euthanized in a CO₂ chamber. All groups were received DMH dose showed aberrant crypt foci (ACF) and tumors formation in the end of the experiment (forty-second week).

All procedures involving animals followed the recommendations of the Brazilian College of Animal Experimentation (COBEA), and were granted approval by Research Ethics Committee of the School of Pharmaceutical Sciences at Araraquara/Unesp (Protocol n° 46/2004) and the experiments adhered to the guiding principles in the care and use of animals.

Preparation and administration of fermented soy product

The fermented soy product was processed by the method described in Rossi et al. (2000). Fermented soy product supplement with calcium was produced through fermented product improved with 600mg calcium/L (Lab Synth-Brazil) (Umbelino et al., 2001).

Peritoneal macrophages

Thioglycollate-elicited peritoneal exudates cells (PEC) were harvested from Wistar rats using 5.0 mL of PBS (Sigma, St. Louis, USA) pH 7.4. The cells were washed twice by centrifugation at 200 g during 5 minutes at 4°C and re-suspended in RPMI-1640 medium (Sigma, St. Louis, USA) with 100U/mL penicillin (Sigma, St. Louis, USA), 100 μg/mL streptomycin (Sigma, St. Louis, USA), 5.10-2 M mercaptoethanol (Sigma, St. Louis, USA) and 5% inactivated fetal calf serum (Sigma, St. Louis, USA). The adherent cells were obtained by incubation for 1 h at 37°C in an atmosphere of air/CO₂ (95,5, v/v) and incubated with LPS (Cutilab-Brazil), or RPMI-1640 medium (Buset et al., 1986).

Splenic cells

The spleens were collected aseptically and placed on a Petri dish containing 3,0 mL RPMI-1640 medium. The cell suspension was obtained by tweezing the spleen and washing three times with RPMI-1640. The cells were resuspended to a concentration of 5x10⁶ cell/mL of RPMI-1640 medium and cultured with Concanavalin-A (Con-A) (Corning Inc., NY) or RPMI-1640 medium (Buset et al., 1986).

H₂O₂ assay

PEC (2x10⁶cells/mL) were suspended in a solution containing 140 mmol NaCl (Lab Synth-Brazil), 10 mmol potassium phosphate buffer (Lab Synth-Brazil) (pH 7.0), 5.5 mmol dextrose, 0.56 mmol phenol red (Merck, Brazil) and 0.01mg/mL type II horseradish peroxidase (Santa Cruz biotechnology, USA). Next 100μL of this suspension was added to each of the wells of a 96-well flat-bottom tissue culture plate and exposed to 50μL of suspension. A solution of zymosan (Biosinth, USA) was used as a positive control. The cells were incubated for 1h. The reaction was stopped with 10μL of 4N NaOH (Lab Synth-Brazil) and the absorbance of the samples were read at 620nm with a Multiskan Ascent ELISA (Multiskan Ascent, Labsystems) reader against a blank containing phenol red solution and 4N NaOH. The results were expressed as nanomols of H₂O₂/2x10⁵ peritoneal cells, from a standard curve established in each test consisting of known molar concentrations of H₂O₂ in buffered phenol red (Pick et al., 1981).

Nitric oxide assay

Nitric oxide synthesis was determined by assaying culture supernatants for nitrite, the stable reaction product of NO and molecular oxygen. Nitrite concentration was determined by the Griess reaction. Briefly, 50 μL of culture supernatants were incubated with equal volumes of the Griess reagent (1% sulfanilamide (Promega, USA), 0,1% N- (1-naphthyl)-ethylenediinedihydrochloride (Merck, USA), 2,5% H₃PO₄ (Lab Synth-Brazil) at room temperature for 10 minutes and absorbance (540 nm) was determined in a Multiskan Ascent ELISA reader. The results were reported as μmols NO/5x10⁵ peritoneal cells quantified from the standard curve (Pick and Mizel, 1981).

Determination of IL-6, TNF-α, IFN-γ and IL-4

The cytokines TNF-α and IL-6 release in the supernatant of macrophage cell culture and the cytokines IFN-γ and IL-4 released in the supernatant of splenic cell culture were quantified by ELISA. The ELISA kit (Kit BD OptEIA®, using recombinant rat TNF-α, IL-6, IFN-γ and IL-4 as standard, was used to measure respectively TNF-α, IL-6, IFN-γ and IL-4 secretion in culture supernatants following the manufactures instructions.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), using a statistical program SigmaStat® Statistical Analysis System for Windows Version 5.0 (Jandel Scientific, Erkrath, Germany). The differences among the groups were analyzed with a post hoc TuKey test. Significance was declared at P<0.05.

RESULTS

Cytokine production (see Table 1)

The data presented in Table show that there was a significant (p<0.05) increase in levels of IL1, but not IL4, IL6 or IFN-γ in the rats treated with DMH to induce colon cancer. The treatment of non-DMH treated control rats with either fermented soy alone or in combination with calcium also increased levels of IL1, IL6 and IFN-γ, but not IL4. Treatment of DMH rats with fermented soy
TABLE 1: Effect of treatment with fermented soy and calcium on cytokine levels in control and rats with colon cancer. The data are presented as means ± SD; Means with the same letter do not differ statistically (P<0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>IL1 (pg/ml)</th>
<th>IL4 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1616.25± 431.68</td>
<td>5.07± 0.60</td>
<td>1746.30± 597.40</td>
<td>5551.11± 466.70</td>
</tr>
<tr>
<td>2</td>
<td>DMH</td>
<td>2534.31± 71.31</td>
<td>7.60± 3.40</td>
<td>1612.74± 853.21</td>
<td>5654.25± 1428.41</td>
</tr>
<tr>
<td>3</td>
<td>DMH + Fermented Soy</td>
<td>1216.10± 431.68</td>
<td>15.49± 7.76</td>
<td>2230.53± 752.74</td>
<td>5952.12± 783.85</td>
</tr>
<tr>
<td>4</td>
<td>DMH + Fermented Soy + Calcium</td>
<td>1494.55± 473.61</td>
<td>18.47± 5.49</td>
<td>2819.27± 282.79</td>
<td>5195.53± 787.69</td>
</tr>
<tr>
<td>5</td>
<td>Fermented Soy</td>
<td>2595.97± 308.41</td>
<td>8.07± 1.41</td>
<td>784.50± 179.25</td>
<td>6627.86± 130.75</td>
</tr>
<tr>
<td>6</td>
<td>Fermented Soy + Calcium</td>
<td>2401.90± 68.64</td>
<td>8.31± 3.68</td>
<td>517.50± 11.66</td>
<td>7323.78± 130.75</td>
</tr>
</tbody>
</table>

TABLE 2. Effect of treatment with fermented soy and calcium on TNF-α, Nitric oxide and H2O2 levels in control and rats with colon cancer. The data are presented as means ± SD; Means with the same letter do not differ statistically (P<0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>TNF-α (pg/ml)</th>
<th>NO (micromols NO/5x10⁵ cells)</th>
<th>H₂O₂ (nanomols H₂O₂/2x10⁵ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1853.31± 36.49</td>
<td>174.50± 12.89</td>
<td>247.87± 96.38</td>
</tr>
<tr>
<td>2</td>
<td>DMH</td>
<td>1927.94± 42.64</td>
<td>155.52± 16.62</td>
<td>326.14± 25.01</td>
</tr>
<tr>
<td>3</td>
<td>DMH + Fermented Soy</td>
<td>2040.48± 34.00</td>
<td>101.68± 2.61</td>
<td>548.98± 18.14</td>
</tr>
<tr>
<td>4</td>
<td>DMH + Fermented Soy + Calcium</td>
<td>1922.19± 15.02</td>
<td>66.68± 7.16</td>
<td>511.00± 71.24</td>
</tr>
<tr>
<td>5</td>
<td>Fermented Soy</td>
<td>1982.90± 30.41</td>
<td>182.23± 0.39</td>
<td>525.16± 67.98</td>
</tr>
<tr>
<td>6</td>
<td>Fermented Soy + Calcium</td>
<td>1813.14± 101.92</td>
<td>175.22± 7.72</td>
<td>544.38± 13.52</td>
</tr>
</tbody>
</table>

TNF-α, H₂O₂ and NO production (see Table 2)

The data presented in Table show that there was a significant (p<0.05) increase in H₂O₂ production, but not NO or TNF-α in the rats treated with DMH to induce colon cancer. The treatment of non-DMH treated control rats with either fermented soy alone or in combination with calcium also increased levels H₂O₂ production, but not NO or TNF-α. Treatment of DMH rats with fermented soy alone or in combination with calcium lowered NO, raised H₂O₂ production, but did not alter TNF-α.

DISCUSSION

Oral administration of LAB has proved efficacious in the reduction of DNA damage by carcinogenic chemical agents in the mucosa of rats. Certain strains of LAB have also been found capable of preventing putative preneoplastic lesions induced by carcinogens (Pool-Zobel et al., 1996). Probiotic characteristics of E. faecium CRL 183 have been the target of investigations by our research group and some positive effects so far include reduction in the development of breast cancer (Kinochii, 2006); hypocholesterolemic effect (Rossi et al., 1993); prevention of osteoporosis (Shiguemoto et al., 2007).

As reported by Valdez et al., (1991) tumor growth selectively induces an increase in neutrophils in peripheral blood, with a decrease in the number of monocytes and lymphocytes. Rachid et al., (2006) observed that, as a consequence of treatment with milk fermented with L. helveticus, hematological improvement occurred in circulating blood.

It is known that the oral administration of antigens can induce mucosal stimulation, not only of the immune cells associated with the gut, but also of others belonging to the common mucosal immune system (Moldeveanu et al., 1995). The stimulation of mucosal immune cells involves the release of many cytokines, such as IL-1, IL-6, IL-4, IL-5, TNFα, INFγ, transforming tumor growth factor-α (TGFα), TGFβ and prostaglandins (Matsuura et al., 1993).

The present study was inspired by the abundant data from experimental models indicating the consistent anticancer efficacy of probiotics, however at this moment, there are few studies which have been investigated the effects of oral administration of soy product fermented with Enterococcus faecium CRL 183 and supplemented or not with calcium on cytokines produced by macrophages and lymphocytes population in chemically-induced colon cancer in rats. IL1 showed smaller production in Groups 3 than compared with the negative control group. The results found with IL1 agreement with Ueda et al., (1994) were investigated serum levels of cytokines in patients with colorectal cancer, this cytokines was not elevated in this patients. In relation to IL-4 we observed that treatment with the fermented soy product (group 3) and supplemented with calcium (Group 4) induced a higher production of this cytokine in animal with colon cancer.

It was important observe in IL-6 production that groups were investigated serum levels of cytokines in patients with colorectal cancer, this cytokines was not elevated in this patients. In relation to IL-6 we observed that treatment with the fermented soy product (group 3) and supplemented with calcium (Group 4) induced a higher production of this cytokine in animal with colon cancer.

In the present study, we investigated serum levels of cytokines in patients with colorectal cancer, this cytokine was not elevated in this patients. In relation to IL-6 we observed that treatment with the fermented soy product (group 3) and supplemented with calcium (Group 4) induced a higher production of this cytokine in animal with colon cancer.

H₂O₂ and NO are important in cell signaling and are effectors molecules for the microbial and cytotoxic response.
of macrophage after stimulation (Salvemini et al., 1993). Intestinal epithelial tight junction (TJ) prevents the diffusion of potential injurious factors from the gastrointestinal lumen into the tissue. Disruption of TJ and elevated permeability to luminal toxins, allergens, and pathogens play a crucial role in the pathogenesis of a number of gastrointestinal diseases such as inflammatory bowel disease, celiac disease, and alcoholic liver disease. Proinflammatory factors such as reactive oxygen species, cytokines and toxins disrupt the TJ and compromise the barrier function of the intestinal epithelium. The factors that prevent this inflammation-mediated disruption of the TJ and barrier function may provide potential therapeutic benefit in the treatment of many gastrointestinal diseases. H\textsubscript{2}O\textsubscript{2} induces the redistribution of TJ and adherens junction (AJ) proteins, occludin, ZO-1, E-cadherin, and β-catenin, from the intercellular junctions into the intracellular compartments (Seth et al., 2008). Lower H\textsubscript{2}O\textsubscript{2} production in Groups 1 and 2 demonstrates that ingestion of fermented soy product or supplemented or not with calcium stimulates production of this molecule. It was possible observed that the higher H\textsubscript{2}O\textsubscript{2} was in Group 3, which animals received fermented soy product. According De Simone et al., (1993) the probiotic improve immune function and stimulate immunomodulatory cells, however the It is still unclear which mechanism or, more probably, which spectrum of mechanisms, is used by probiotics within the human gut microbiota to bring about improved health. One of the mechanisms is the produce toxic, e.g. hydrogen peroxide. On the other hand, according Seth et al., (2008) probiotic secretory proteins, LGG-s, p40, and p75, did not change the level of H\textsubscript{2}O\textsubscript{2}, indicating that the effect of probiotic was not mediated by an antioxidant effect.

The literature shows a increasing evidence links iNOS and COX-2 with tumor angiogenesis, and according Salvemini et al., (1993) both endogenous and exogenous NO plays a critical role in the release of PGE\textsubscript{2} by direct activation of COX in macrophages, however in our observation de NO decreased in induced animals and the lowest concentration of NO were found in animal were ingested fermented soy product with and without calcium. TNF-α acts as mediator of inflammatory response and in innate immunity; it’s a pro-inflammatory cytokine produced by activated macrophages. In relation TNF-α, just the ingestion of fermented soy product showed a significant TNF-α production. These results are agree with Sivieri et al., (2003), according this authors the animals that were colon cancer induced with DMH in the same condition that the present work and ingested a suspension of Enterococcus faecium CRL 183 showed the 40% of adenocarcinoma reduction and enhanced the immune response by increasing IL-4, TNF-α and IFN-γ.

In the analyses of the profiles of cytokines induced by some LAB, have been observed the most remarkable effect was the increase in the tumor necrosis factor alpha (TNF-α) in the probiotic strains assayed. This effect was obtained without increasing the inflammatory response and only a slight increase in the cellularity was found. However, the induction of TNF-α by the probiotic bacteria would be necessary to initiate the cross talk between the immune cells associated with the lamina propria and the intestinal epithelial cells. IFN-γ would also play a physiological role; it has been demonstrated that this cytokine is necessary for the maturation of some immune cells, such as dendritic cells, and also controls their cellular proliferation at the intestinal level (Galdeano et al., 2007). Higher production of IFN-γ occur in Groups 3 and 4. IFN-γ is involved in the induction of other cytokines, particularly IL4, IL5 and IL10. Because of its role in mediating macrophage and NK cell activation, IFN-γ is important in the host defense against intracellular pathogens, viruses and tumors (Kayser et al., 2000).

The results suggest that ingestion of the fermented soy product supplemented or not with calcium enhanced the some cytokines produced by peritoneal macrophages and splenics lymphocytes population (IL-4, IFN-γ and TNF-α). There are many articles showing positive correlations between probiotics and enhanced immune system (Galdeano et al., 2007; Seth et al., 2008; Sivieri et al., 2008). On the other hand, it is proved that dietary intake of soy products decreases tumor incidence of chemically induced colon cancer in rat models. However, to date the exact mechanism is not completely understood. The only thing already established is that, according to MacDonald et al., (2007), the soy products inhibit the cyclooxygenase-2 expression in mouse colon thereby providing a potential protective mechanism. Therefore, we can suggest the enhanced of the cytokines produced by peritoneal macrophages and splenics lymphocytes population, in our case, is probably due to Enterococcus faecium CRL 183 action, but additional studies need to be accomplished for understanding these correlations.

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