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THE UNIVERSITY OF ALBERTA

The Effect of *Lactobacillus* spp. on the Proliferation of Morris Hepatoma 7777 Tumours in Buffalo Rats.

by

© Anna B. MacLeod

A THESIS

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ABSTRACT

Studies were undertaken to determine the antitumour effect of perorally administered Lactobacillus casei and Lactobacillus bulgaricus strains. Skim milk inoculated with various strains of bacteria was fermented, freeze-dried and fed to Buffalo rats with transplanted Morris hepatoma #7777 tumours. The experiment demonstrated that different strains of L. casei possess differing antitumour activities. A sex dependent effect was also observed with male rats being the most susceptible. For example, the inhibition ratios were 58% and 29% when male Buffalo rats were fed diets containing L. casei 03 and L. casei 44, respectively. In addition to the fermented skim milk, other dietary carriers for the bacteria were examined. In these experiments, the bacteria were grown in modified Rogosa media and supplemented to egg white protein, skim milk and casein. When the same number of bacteria was added to each of the above carriers no significant tumour inhibition was observed. The above suggests that the fermentation process itself and (or) the resultant coagulum acts synergistically to reduce tumour growth.

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1. INTRODUCTION

The immunoadjuvant and antitumour activity of some gram positive bacteria is well recognized, especially in the case of bacteria such as Propionibacterium acnes, Nocardia rubra and Mycobacterium tuberculosis BCG. The antitumour effect is thought to be due to stimulation of the immune system rather than direct cytotoxicity of the microorganisms. The literature concerning immunoadjuvant and antitumour activity sometimes overlaps because immune surveys are sometimes studied in conjunction with direct tumour assays.

The aforementioned activity has also been observed in nonpathogenic lactic acid bacteria. Classically, pathogenic and nonpathogenic bacterial preparations were injected in order to study their antitumour and (or) immunoadjuvant activity. When a dosage is administered by the parenteral route, the agent is delivered to the site of action without metabolic processing by the host. This is not the case in peroral administration. Hence, the primary concern with peroral treatment is the effect of the host's digestive system on the active agent(s). While viability of the microorganisms is not believed to be a prerequisite for antitumour activity degradation of the antitumour factor(s) may influence the activity.

Lactic acid bacteria, in addition to showing antitumour activity, have also been associated with general health benefits such as protection against invading pathogens and production of enzymes which aid in host metabolism. Lactobacilli are well suited for oral

administration because they are nonpathogenic and found in the gastrointestinal tract of healthy individuals.

Taking all of the above factors into account, the objectives of the research were: 1) to assess the antitumour activity of Lactobacillus casei and Lactobacillus bulgaricus strains against Morris hepatoma #7777 tumours in Buffalo rats; and 2) to compare the effectiveness of nonfermented and fermented skim milk, casein and egg white protein as dietary carriers of the lactobacilli.

2. LITERATURE REVIEW

2.1 General immunology

The immune system of animals protects against foreign agents such as bacteria, viruses and tumour cells. For ease of definition, the immune system can be divided into two broad classes: 1) humoral immunity; and 2) cell mediated reactions (Roitt et al. 1985). The term humoral immunity is used to describe responses where antibodies play a dominate role (Darnell et al. 1986). Antibodies (immunogens) are proteins which are produced in response to an antigen (any molecule which is capable of eliciting the production of antibodies). Cell mediated immunity, on the other hand, involves reactions where antibodies play a subordinate role (T cell immunity). Quite simply, cell mediated immunity cannot be transferred from animal to animal via the injection of antibodies. While convenient in terms of description, there is no clear cut interdependency between cell mediated and humoral immunity.

2.1.1 Tumour immunology

The mechanism(s) that the immune system uses to kill tumour cells has long been sought after. The research done to date, although promising, is riddled with problems inherent in oncological research. There are several proposed mechanisms for the destruction

of tumour cells: specific and nonspecific cytotoxic macrophages, natural killer cells (NK), cytotoxic T cells (CTL), specific antibodies, natural cytotoxic antibodies, antibody dependent cytotoxicity (ADCC), and various combinations of the above (Roitt et al. 1985). According to Den Otter (1986) the restrictions that inhibit the interpretation of tumour surveys are: 1) tumours are a very heterogenous population; 2) immune cells may have more than one function; 3) cytotoxic cells can only have an effect on tumour cells if they are closely associated with the tumours; 4) immune reactions to transplanted tumours are transient; and 5) effector cells (those lymphocytes and phagocytes which cause tumour cell death) are only important in regressing tumours.

2.1.2 Host resistance to tumours

It was implied above that every experimental tumour model is different in terms of its general characteristics and hence its response to an attack by the immune system. In the discussion to follow, immunoadjuvant and (or) antitumour activity, induced by bacteria, will be the focus. Macrophages, natural killer cells, and T cells are thought to play the most important role in this type of activation.

Macrophages are large mononuclear cells which are derived from blood monocytes. Blood monocytes are manufactured in the bone marrow and become macrophages once they are in the tissue. Macrophage may be

involved in the initial stages of an immune response or act as cytotoxic cells. In the first case, macrophages capture and engulf immunogens and present them to antigen-reactive lymphocytes. In their role as cytotoxic cells, macrophages act as final effectors, i.e. they bring about removal or destruction of the antigens (Adams et al. 1982; Hoerl 1984). For macrophages to be effective they must be in close association with the antigen (tumour cell) and be activated. Macrophages are activated by large molecules called lymphokines (i.e. macrophage activating factor - MAF, migration inhibition factor - MIF, and chemotactic factor for macrophage - MCF) which are produced by T cells (Brock 1979).

Natural killer cells (NK) are leucocytes which have the ability to recognize surface changes on virally infected cells and tumour cells (Roitt et al. 1985). NK cells, as the name implies are cytotoxic. Interferons are proteins capable of modifying the immune response and activating NK cells.

T cells are lymphoctes which are manufactured by bone marrow stem cells and differentiated in the thymus. T cells are associated with many immune reactions: 1) they produce cell mediated immunity; 2) produce lymphokines; 3) show delayed-type hypersensitivity (DTH); 4) act as helpers to B cells in the production of antibodies; 5) react against intracellular pathogens (viruses, parasites and bacteria); and 6) act as killer T lymphocytes (Brock 1979). DTH is defined as type IV Hypersensitivy and is characterized by antigen-sensitized T cells

producing lymphokines when they are reintroduced to the antigen. This is important as it is one of the proposed mechanisms in which bacteria are able to stimulate the immune system and therefore, the theory behind bacterial immunoadjuvants.

2.2 Macrophage activity in tumour bearing hosts

Both specific and nonspecific macrophage activity have been associated with host resistance to tumour proliferation. Activated macrophages are able to distinguish neoplastic cells from normal cells and mediate their cytotoxicity in a nonspecific manner (Bloksma et al. 1981; Currie 1976; Hibbs 1974). Meltzer and Stevenson (1978) found that there was a dissociation of phagocytic and chemotactic responsiveness in male mice bearing subcutaneous tumours (tumour 1038). Decreased chemotactic response was true for both lymphocyte and complement-derived factors. The increased phagocytic activity of the peritoneal macrophages was transient and only persisted for 12 days. Other indexes of macrophage stimulation such as increased DNA synthesis were also observed by Meltzer and Stevenson (1978). Although the macrophages were activated, decreased chemotaxis would render them less effective as they must be closely associated with the neoplastic antigen.

In agreement with Meltzer and Stevenson's work (1978), Cheung et al. (1979), Iannello et al. (1984a) and Rhodes et al. (1979) all reported decreased chemotaxis in macrophages from tumour bearing

animals (phagocytic activity was not assessed in these studies). The decreased activity was determined to be due to a low molecular weight factor produced by all of the tumours studied. Cheung et al. (1979) stated that the above factor was unique to tumour tissues, while Rhodes et al. (1979) suggested that it was similiar to an inhibitory factor found in normal serum. The normal physiological inhibitor of macrophage is the migration inhibition factor (MIF) produced by T cells. Despite the disagreement over the factor itself, the impact is still the same: a factor is present which may allow tumours to escape normal immune surveillance.

2.3 Bacterial immune modulators

2.3.1 Effect on macrophage activity

It is known that the activated macrophage not only has the ability to kill pathogens but also acts against foreign tissue cells. Specific antigens that differ from that of the host can be noted in both foreign tissue and tumour cells. It is hypothesized that protection from tumour proliferation can result from the "immunization" of the host with bacteria, inducing a delayed-type hypersensitivity reaction. It is thought that there is an interaction between the immunogens (bacteria) and a small number of lymphocytes. These lymphocytes in turn release soluble factors which cause macrophages to accumulate (Zbar et al. 1970). The above type of protection is nonspecific in nature but macrophages have been shown to be tumouricidal as well (Freedman et al. 1980; Ogura et al. 1979).

Microorganisms such as BCG (bacillus of Calmette-Guerin) a nonpathogenic strain of Mycobacterium tuberculosis, Nocardia rubra, Corynebacterium parvum, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus plantarum and commercial microbial preparations such as Buccalin (Diplococcus pneumoniae I-II-III, Streptococcus haemolyticus, Staphylococcus aureus and Haemophilus influenzae), Bioflorin (Streptococcus faecium) and Liobifar (Bacillus subtilis) have all been associated with macrophage activation (Azuma et al. 1976; Bloksma et al. 1979; Freedman et al. 1980; Hashimoto et al. 1984; Iannello et al. 1984b; Kato et al. 1983; Nanno et al. 1986; Ogura et al. 1979; Perdigon et al. 1986; Potter and Moore 1980; and Zbar et al. 1970). The above group includes both gramnegative and gram positive microorganisms. In general, gram negative microorganisms are thought to induce lipopolysaccharide type immunity (i.e. B cell mitogen), while the immunity derived from gram positive bacteria is thought to be due to their peptidoglycan structure (Namba et al. 1981; Nishimura et al. 1985; and Roitt et al. 1985). Gram positive microorganisms, which include BCG and lactic acid bacteria are the most important in terms of the discussion that follows.

2.3.2 Effect on NK and T cells

Up to this point, the discussion has focussed on the effect of bacteria on macrophage activity. As immune cells rarely act independently, it is also important to examine how other tumour retarding cells are enhanced. Hanna and Fidler (1980) examined the

role of NK cells on the destruction of bloodborne tumour cells and found that they inhibited metastasis formation. Kasai et al. (1979) selected NK cells from non-immune mice using Ly5-surface components as the criterion for selection. They determined that these cells could inhibit the proliferation of lymphomas in mice.

These studies show that natural killer cells are indeed involved in nonspecific protection against some tumours. Work has also been completed that links bacterial immunopotentiators and enhanced NK activity in vivo and in vitro (Kato et al. 1984; Ojo et al. 1978; and Oshimi et al. 1980). The mechanism whereby NK activity is augmented has not been elucidated. Ojo et al. (1979) found that the route of administration is critial in terms of NK activation. Intravenous administration caused a decrease in NK activity, while intraperitoneal injection of Corynebacterium parvum caused a sharp increase in activity.

Studies which concentrate on bacterial induced T cells in tumour bearing mice and rats have also been done (Fujimoto et al. 1976, 1978; and Kawase et al. 1981). Once again the mechanisms involved are unknown, however, T cell mediated cytotoxicity is thought to play a role in the overall antitumour effect. It should be noted that all of the studies mentioned have based their conclusions on results from experiments using different: 1) animal models; 2) tumours; 3) modes of administration; and 4) immune surveillance tests. The assumptions from each study add to the information in the area, however, the

results vary depending upon the exact model used.

2.4 Adjuvant Administration

The classic example of immune modulation using bacteria involves BCG administered by the parenteral route (Potter and Moore 1980). The advantage to this mode of administration is that the adjuvant is placed directly at the site of action. Oral administration is indirect and the agent is subjected to digestive processing by the host. This may result in inactivation of the active component(s). Despite the above theoretical disadvantage, Iannello et al. (1984b), Friend et al. (1982) and Perdigon et al. (1986) reported effective oral administration of polyvalent vaccine, Lactobacillus casei and Lactobacillus bulgaricus respectively.

Another concern is the viability of the microorganisms administered. Freedman et al. (1980) used a heated killed preparation of BCG in their study and found that it stimulated mononuclear phagocyte activity. Bloksma et al. (1979) found that viable Lactobacillus plantarum stimulated delayed-type hypersensitivity exclusively, while heat-killed preparations of the same organism had adjuvant effects on antibody formation as well. Studies which use cell wall skeletons of Nocardia rubra as immune modulatory agents have also given positive results (Kawase et al. 1981; Ogura et al. 1979; and Yamawaki et al. 1978). Perdigon et al. (1986) found that viable and nonviable Lactobacillus casei and Lactobacillus bulgaricus were

able to activate macrophages in mice. Due to the above, it is thought that viability is not a requirement.

2.5 Macrophage mediated cytotoxicity

Nelson and Nelson (1978) demonstrated that macrophages are important in terms of obtaining an antitumour effect by using antimacrophage agents such as carrageenan and silica in vivo. Treated animals had less resistance to tumours than nontreated animals. It has been mentioned that macrophages must be activated to be effective and that bacteria such as lactobacilli are capable of causing immune stimulation (activation), however the direct cytotoxic effect of macrophage augmentation needs to be mentioned. The extracellular cytotoxic reactions of macrophages have been attributed to a variety of factors including: 1) oxygen radical production; 2) cytolytic factors (CF), e.g. serine protease; 3) thymidine, 4) complement fragment C3a; 5) arginase; and 6) tumour necrosis factor (TNF) (Drysdale et al. 1983).

Hashimoto et al. (1984) showed an increase in oxygen radical (OR) production by peritoneal macrophages when Lactobacillus casei (YIT 9018) was injected intraperitoneally in mice. To determine if OR production could be linked to tumour resistance, Meth A fibrosarcoma was also injected intraperitoneally. A significant correlation between YIT 9018 induced OR production and fibrosarcoma inhibition was observed.

Activated macrophages have also been shown to mediate tumour target cytolysis. Adams and Marino (1981) and Drysdale et al. (1983) indicate that macrophages bind neoplastic cells and then secrete a CF which has a molecular weight of approximately 45,000. Adams and Marino (1981) stated that binding and secretion of CF are not regulated together. This was demonstrated using bovine pancreatic trypsin inhibitor (BPTI). BPTI did not alter the binding of neoplastic cells but did inhibit cytolysis. The macrophages were activated by lipopolysaccharide (LPS) in the study done by Drysdale et al. (1983) and by BCG in Adams and Marino's (1981) work.

The nature of CF has been investigated because it is a necessary component in macrophage mediated cytolysis of tumour cells (inhibitors of CF also inhibit tumour cell lysis). Adams et al. (1980) showed that CF is extremely potent. A 1:200 dilution of macrophage supernatant lysed over 50,000 tumour cells. These above authors believe that CF is a neutral serine protease. The release of CF from macrophage elicited with Lactobacillus casei (YIT 9018) was also reported by Hashimoto et al. (1987).

Tumour necrosis factor (TNF) is a cytotoxin that has been associated with tumour regression (Mannel et al. 1980). TNF has more than one biological activity, however, the other functions are not relevant to tumour regression. Activated macrophages produce TNF and kill tumour cells by either cell-to-cell contact or by the release of the TNF secretory component (the exact mechanism is unknown) (Kriegler

et al. 1988).

The release of lysosomal and nonlysosomal enzymes by activated macrophages was shown by Perdigon et al. (1986). A study by Currie (1978) indicated that activated macrophages kill tumour cells by releasing arginase which causes arginine deprivation. Enzymes manufactured by macrophages therefore, appear to play a role in the overall cytotoxicity of the macrophage.

2.6 Antitumour activity

Bacterial induced antitumour activity is thought to be due to stimulation of the immune system rather than direct cytotoxicity of e microorganisms. Studies which deal with immune modulation often use tumour cells to determine the overall activation of the immune system. On the other hand, antitumour studies use DNA counts, tumour size (weight and diameter), tumour metastasis, accumulation of ascites fluid, survival time and macrophage activation to assess an effect. While the above two fields of research can be divided for purposes of definition, they do, in most cases, support the same general hypothesis that bacteria modulate the immune system.

2.6.1 Bacteria

BCG, mycobacteria related Nocardia rubra, and Propionibacterium acnes are all gram positive bacteria which have been cited as

antitumour agents. The antimetastatic activity of BCG preparations after intradermal treatment was investigated by Kelly et al. (1978). Lagrange and Gheorghiu (1981) found BCG preparations to be effective against Lewis lung carcinoma. Ogura et al. (1979) treated rats injected with syngenic fibrosarcoma with Nocardia rubra cell wall skeletons (CWS). They found that intraperitoneal injections of the CWS completely prevented the accumulation of ascites fluid and prolonged the survival period of the animals. Yamawaki et al. (1978) tested Nocardia rubra CWS suspended in squalene (a "vaccine") against EL4 leukemia, Meth-A and MC104 tumours. They found that the preparation was effective and more stable than CWS treated with Drakeol 6VR (mineral oil). Intramoral and intravenous injections of Propionibacterium acnes CWS was reported by Tanio et al. (1984) to have antimetastic effects against Lewis lung carcinoma. The above research was chosen not to represent all of the work done in this area, but rather to point out the diversity of the studies.

2.6.2 Lactic acid bacteria

In recent years, bacterial mediated antitumour research has shifted away from mycobacterial related organisms to the use of lactobacilli. Lactic acid bacteria are gram positive, nonsporeforming, rod shaped, nonmotile microorganisms which are ubiquitous (Boyd 1984). Lactobacilli are normal inhabitants of the gastrointestinal tract of humans. According to Gilliland (1979), the lactobacilli which are most often referred to as potential dietary

adjuncts are Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus bifidus (Bifidobacterium bifidum).

The aforementioned microorganisms are considered candidates for antitumour research because: 1) they are present in the gastrointestinal tract of healthy individuals; 2) they are able to survive digestive processes i.e. exposure to bile acids, enzymes and hydrochloric acid; and 3) they are able to survive and grow in the lower intestinal tract (Gilliland, 1979).

Besides the specific antitumour activity of lactobacilli, they have also been associated with general health benefits: 1) protection against invading pathogens due to direct competition and production of antimicrobial substances (lactic acid, hydrogen peroxide and antibiotics); 2) bile deconjugation; 3) elaboration of enzymes which aid in host metabolism; 4) reduction of serum cholesterol levels; and 5) synthesis of B vitamins (Fernandes et al. 1987; Goldin et al. 1977; Perdigon et al. 1986; Shahani and Ayebo 1980; and Sandine 1979). Although there is no consensus regarding some of the above research (i.e. anticholesteremic effect), lactobacilli are generally considered to have at least some therapeutic value.

Oral administration of Lactobacillus acidophilus has been shown to decrease the activity of Beta-glucuronidase, nitroreductase and azoreductase (fecal bacterial enzymes) in rats and humans (Ayebo et al. 1980; Goldin et al. 1977, 1980; Goldin and Gorbach 1980, 1984). These enzymes play an important role in converting procarcinogens to

carcinogens and are therefore associated with cancer induction. Although an alteration in enzyme activity is a direct rather than adjuvant effect, the overall potential of lactobacilli supplementation can be appreciated from the above.

Lactobacilli differ in their anticancer and (or) antitumour activity. Shimizu et al. (1987) examined the antitumour activities of 23 lactobacilli (13 species; 23 strains) and found vast differences. It was determined that the effect varies not only due to species but also strain; Lactobacillus casei YIT (Yakult Institute of Tokyo) 9018 was found to be most effective while Lactobacillus casei YIT 0105 had little or no effect. Solid and ascites forms of methylcholanthrene-induced (Meth A) fibrosarcoma were used to assess the potential of the microorganisms. The above observation that Lactobacillus casei YIT 9018 is effective against Meth A fibrosarcoma has been confirmed by Kato et al. (1985) and Yasutake et al. (1984a, 1985).

Yasutake et al. (1984b) also compared the antitumour activity of intralesionally (i.l.) and intravenously (i.v.) administered Lactobacillus casei YIT 9018 (LC 9018) Corynebacterium parvum and Mycobacterium bovis bacillus of Calmette-Guerin (BCG). They found that all three microorganisms were equally effective against Meth A fibrosarcoma and Kirsten murine sarcoma. Kato et al. (1981) determined that LC 9018 markedly inhibited sarcoma-180 and prolonged the lifespan of mice with L1210 leukemia. Intraplueral (i.pl.) injections of LC 9018 inhibited the metastasis of Lewis lung carcinoma and variant B16

melanoma (Matsuzake and Yokokura 1987; and Matsuzake et al. 1987). Matsuzake et al. (1985) determined that that i.pl. injections of LC 9018 were effective for regression and induction of systemic tumour immunity in strain-2 guinea pigs.

While LC 9018 has been studied most extensi there have been positive results for the antitumour activity of other lactobacilli. Bogdanov and Dalev (1975) reported that a single intravenous dose of a Lactobacillus bulgaricus preparation showed antitumour activity against sarcoma S-180. In 1987, Ketlinsky et al. noted that repeated administration of Lactobacillus bulgaricus (strain 51) markedly inhibited the growth of sarcoma S-180 while a single dose was ineffective. The conflicting observations may be due to strain differences.

A yogurt preparation containing Lactobacillus bulgaricus and Streptococcus thermophilus fed orally to mice inhibited Ehrlich ascites tumour cell proliferation (Friend et al. 1982; Shahani et al. 1983). Reddy et al. (1983) examined this observation further and found that initial tumour cell proliferation was inhibited, however, there was no increase in the overall lifespan of the mice. The antitumour activity was derived from the solid fraction (which contains the microorganisms) rather than the supernatant fluid. Friend et al. (1982) also established that fluid milk and lactic acid administered alone had no antitumour effect. It was concluded that milk may stabilize the antitumour agent(s) when administered

orally.

2.7 Isolation of antitumour agents

It was previously noted that: 1) the microorganisms are thought to induce antitumour activity by stimulating the host immune system and not through direct cytotoxicity; 2) viability of the microorganisms is not believed to be necessary; 3) immune modulation has been accomplished using gram positive bacteria and; 4) there are strain as well as species dependent factors. These factors led some authors to believe that antitumour activity is cell wall mediated. Goguel et al. (1982) assessed the antitumour activity of peptidoglycans from 4 different gram positive bacteria (Bacillus megaterium, Staphylococcus aureus, Micrococcus lysodeikticus and Corynebacterium poinsettiae). Subcutaneous administration of Bacillus megaterium and Staphylococcus aureus peptidoglycans enhanced resistance to chemically induced fibrosarcomas, while the peptidoglycans of the other two microorganisms did not.

Studies on synthetic peptidoglycan derivatives indicated that structure is of critical importance, especially in terms of the first two amino acids (Chedid et al. 1978). Bacillus megaterium and Staphylococcus aureus have peptide sequences which begin with -LAla-DGlu and -LAla-DGluNH$_2$, respectively (Goguel et al. 1982). Ellouz et al. (1974) determined that the minimal effective structure (smallest fraction able to induce an antitumour effect) from

mycobacterium cell walls was N-acetylmuramyl-L-alanyl-D-isoglutamire.

Namba et al. (1981) concluded from their experiments that bacterial lysozyme may cause the release of absorbable peptidoglycan fragments from Bifidobacterium longum, thus causing an immune response. In contrast, Vacheron et al. (1983) found a decrease in stimulation (measured by interleukin 1 production) if the peptidoglycans from Bacillus megaterium and Staphylococcus aureus were solubilized with bacterial lysozyme. The apparent contradiction may be due to: 1) the Vacheron study was in vitro, while guinea pigs were utilized by Namba et al. (1981); and 2) different species of bacteria were being examined. Immune stimulation is a very complex event, therefore in vitro results do not necessarily reflect in vivo responses. The effect of lysozyme aside, evidence does suggest that the peptidoglycan fraction is at least partially responsible for gram positive induced modulation.

2.8 Conclusion

As microorganisms differ in their ability to affect tumour proliferation, tumours vary in their physical and metabolic properties, and inbred animals respond to treatment with considerable variability, no broad generalizations can be drawn from any one study. The investigation reported in this thesis was undertaken to select a strain of Lactobacillus that would exhibit an antitumour effect against Morris Hepatoma #7777 tumours in Buffalo rats when

administered orally. It was not only the overall antitumour activity that was of interest but also the experimental parameters, e.g. sex and initial weight of the test animals, the carrier for the microorganisms, and other variables.

The objectives of this research were to explore the diversity of antitumour responses exhibited by strains of lactobacilli, and examine the effect of media, sex, age, and other variables on the measurable tumour response.

3. MATERIALS AND METHODS

3.1 Bacterial cultures

3.1.1 Source

The lactic acid bacteria used in this study were strains of Lactobacillus casei and Lactobacillus bulgaricus donated by Mr. Roger Latta (National Research Council, Division of Biological Sciences, Ottawa, Ont.). The bacteria were received in lyophilized form. Table 1 indicates the original source and the National Research Council number for the microorganisms.

3.1.2 Media

Modified Rogosa broth and agar were used to culture and plate the lactic acid bacteria (LAB) in this study. It was determined that Modified Rogosa (RM) which was either formulated using the method of McDonald (1955) or obtained from Difco Laboratories (Detroit, MI) grew and maintained the lactobacilli equally well. pH adjustment of the media prior to autoclaving was carried out using 1.0 N NaOH or 1.0 N HCl.

Table 1. The Lactic Acid Bacteria used in the Study and Their Source.

Microorganism	NRC[1] Number	Source
Lactobacillus casei	13003	U. of Wiscosin
Lactobacillus casei	13028	U. B. C.[2]
Lactobacillus casei	13044	ATCC[3] 335
Lactobacillus casei subspecies rhamnosus	5749	ATCC[3] 10863
Lactobacillus bulgaricus	5747	ATCC[3] 11842
Lactobacillus bulgaricus	13035	USDA[4] 12

[1]National Research Council

[2]University of British Columbia

[3]American Type Culture Collection

[4]United States Department of Agriculture

3.1.3 Conditions for growth and maintenance

The lyophilized cultures were rehydrated using 0.5 mL of RM media. Growth was initiated by adding one drop of the above to 20 mL of RM broth. The LAB were grown at 37°C for 24 hours. The cultures were then streaked on RM agar to check for contamination and (or) atypical strains. All strains appeared normal and pure. Single colonies from the plates were subcultured three consecutive times at 16 hour intervals and then restreaked.

Resultant isolated colonies were used to inoculate broth which was subsequently used for preparation of frozen cultures. Freezing was done using either glycerol citrate carried on filter paper or pure glycerol. The preparations were frozen at -70°C (Maniatis et al. 1982). It was noted that the microorganisms frozen in pure glycerol grew rapidly and appeared as smooth colonies on RM agar (typical colonly morphology) while the cultures stored on filter paper were atypical (rough colonies). For this reason, only the glycerol cultures were used for experimentation.

3.2 Experimental animals

3.2.1 Breed

The animals used throughout this experiment were Buffalo rats. The Buffalo strain was inbred in the early 1940's for use in chemical carcinogen studies at the National Institute of Health (U.S.A.).

Transplantability of tumours induced by chemical carcinogens was one of the criteria considered important as a test of malignancy (Morris and Slaughter 1979). The tumour transplant studies began in the early 1940's however, it was not until the early 1950's that successful tumour transfers were made in this strain.

A breeding stock of Buffalo rats is maintained in the Department of Animal Science, University of Alberta. It is from this stock that animals were obtained for experimentation.

3.2.2 Selection and maintenance

The selection of the test animals was based upon weight, sex and overall health. Weight is related to age and sex, and it is a common and easy means of separating animals into test and control lots. The two groups (test and control) were arranged so that they had statistically similar mean weights and standard deviations. The size of the breeding stock was determined by the number of subjects required. If the breeding stock was too small then the size deviation was too large.

Upon weaning, the animals were housed in group cages and moved along a series of shelves so that the youngest rats were on the right while the oldest rats were on the left. Two days before the initiation of a trial, the oldest animals of the appropriate sex were removed and weighed. Animals that had a preexisting health condition i.e. skin

disease or eye infection, were culled from the experimental population. The selected rats were then housed in individual metal cages which had separate water and feed containers. The animal rooms were maintained under standard conditions (T = 27°C, 12 h darkness, 12 h light and R.H. 50-55%). Before the feeding trial began the animals were feed Ralston Purina rat chow (Chicago, IL.) which is formulated for laboratory rats.

3.3 Tumours

3.3.1 Cell line

Morris hepatoma #7777 tumours are part of a large series of over fifty transplantable rat hepatomas (Looney et al. 1978). The 7777 line was originally induced in 1954 using the chemical carcinogen N-2-fluorenylphthalmic acid (FPA) (Francavilla et al. 1984). The carcinogen was fed in a diet which contained commercial casein, edible grade skimmilk powder, whole wheat meal, brewers dehydrated yeast, whole liver powder, sodium chloride, ferric citrate, cod liver oil and corn oil (Morris and Slaughter 1978). Female Buffalo rats were used in the original experiment (Francavilla et al. 1984). The resultant FPA induced liver tumours were transferred subcutaneously to both male and female rats.

Morris hepatoma #7777 is a well differentiated trabecullar carcinoma (Francavilla et al. 1978). It is a fast growing "minimal"

deviation tumour. Since it is a minimal deviation tumour, successive transfers can be made without changing the physiological and biochemical characteristics of the line. Weight loss which is the result of protein wasting is one of the first indications of tumour take. The subcutaneous tumour nodules become apparent upon visual inspection near day 14 while death due to the neoplasm does not occur until approximately day 28. Experiments were terminated on day 21 as tumour proliferation not survival was under investigation.

3.3.2 Maintenance and preparation

The tumours were maintained in vivo throughout the experiment. Tumour cells were subcutaneously transferred to new hosts every 21 days in order to keep the tumour line active. Animals that had been culled from the breeding stock due to age or temperament were used as carriers. Cervical dislocation is the preferred method of termination when the tumours are used for transfer. Gas, solvent vapours or chemicals are not advised because biochemical changes may result thus changing the nature of the tumour. Table 2 gives a summary of the equipment and supplies needed for transfer.

The transfers were made according to the guidelines set out by the DCT Tumour Repository (NCI Frederick Cancer Research Facility, Frederick, Maryland). The rats were sacrificed by means of cervical dislocation and the tumours were excised aseptically and as rapidly as possible. The first incision was made down the spinal column from the

Table 2. Tumour Transfer Supplies[1].

Item	Quantity
Steel bar (0.5 m long, 2 cm d)	1
Tissue sieves (#03 and #100)	1 ea.
Phosphate buffered saline (pH 7.2)	50 mL
Surgical scissors	2
Tweezers	3
Haemostat	2
Glass petri dishes	2
Scalpel	1
Blades (#10)	3
Tuberculin Syringe	
50 mL	2
3 mL	*
Needles (22 gauge)	*

[1]All of the supplies were either purchased presterilized or are autoclaved at 121°C for 15 min.

*Varied with the number of experimental animals.

neck to the base of the tail. Haemostats were applied to the skin flaps and rolled to expose the tumours. The outermost pink coloured region of the tumour was removed for mincing using a scalpel and tweezers. The tumour pieces were placed in a sterile petri dish and minced using surgical scissors. Phosphate buffered saline (pH 7.2) was added to the preparation to make a suspension. The suspension was then passed through #03 and #100 tissue sieves to remove the connective tissue. The sieves were not used in the first few transfers (non-experimental) but the preparation was too viscous and hard to deliver in accurate quantities.

The phosphate buffered saline (PBS) tumour cell mix was loaded into 3 mL syringes. Originally, a 3-way surgical valve was used to aid in the injection of the tumour material. A gas tight 100 microliter syringe was attached to one outlet, a 22 gauge needle to the second and to the third, the aforementioned 3 mL syringe (tumour preparation). Using this system, 5 microliter of the suspension was injected subcutaneously into the right and left dorsal region of the rat. It was found that the delivery of such small quantities was difficult and led to inconsistant results. The procedure was therefore changed and enough PBS was added so that 0.5 mL of solution was introduced per injection. With the larger injection volume, the use of a three way surgical valve was not required.

3.3.3 Transfer

Tumour inoculation had to be done as quickly as possible. Twenty

minutes was considered the maximum time that the suspension could remain in vitro. The control and test groups for each experimental diet had to be injected with tumour preparation from the same source. Different syringes were used to inject each side of the rat in an attempt to minimize between animal variability. If the injection was made incorrectly i.e. intramuscular instead of subcutaneous, the animal was withdrawn from the experiment and destroyed. This was a rare occurrence.

3.4 Experimental protocol

The National Cancer Institute (U.S.A.) protocol for screening chemical carcinogens and experimental animal carcinomas was used (Anonymous 1962). The animals were injected with tumour cells on day 0 and the feeding trial began on day 1. The experiment ceased on day 21 at which time the animals were sacrificed. Only those animals to be used for tumour transfer were killed by means of cervical dislocation. Nondonors were terminated using CO_2 gas. The weights of the animals and the tumours (2 per animal) were recorded.

Consumption of feed was monitored using a weigh back system. The dosage of microorganisms was calculated by multiplying the number of bacteria per gram by the weight of feed consumed. The level of bacteria per gram was determined by carrying out standard plate counts on RM agar. Animal weights were also taken every 3 days.

3.5 Skim milk based diets

3.5.1 Source, reconstitution and sterilization of skim milk powder

High heat treated skim milk powder was obtained from Alpha (Red Deer, Alberta). It was determined that the powder could be reconstituted to 30% total solids (w/w) and sterilized isothermally (93.3°C for 30 min) on two succesive days without encountering coagulation problems. Originally, reconstituted low heat treated SMP was examined however, coagulation occurred with sterilization when more than 10% total solids was used. A high solid content was required to increase the efficiency of subsequent freeze drying. Approximately 2.5 L of reconstituted skim milk was required per test animal to provide for their consumption over the 21-day test period.

3.5.2 Preparation of diets

A 1% inoculum of overnight RM culture was added to the sterile SM and incubated at 37°C until the stationary phase of growth was reached. The fermented SM was frozen and freeze dried. Freeze drying took between 3 and 4 days depending on the load in the dryer. If longer than 2 days was required, defrosting of the unit was necessary. A shelf temperature of 26°C was utilized.

The dried product was ground using a large mortar and pestle. Initially, an industrial feed grinder was utilized, however, this caused too much waste. The fermented, dried and ground skim milk was

then stored at -17°C. Lipid (vegetable oil) was added to the powder just prior to feeding to balance the ration. Control diets were prepared in the same manner except the fermentation stage was eliminated.

3.5.3 Mixed strain fermented diet

The mixed strain diet was formulated using an equal ratio mixture of the powder obtained from the individual fermentations. The microorganisms were grown separately to ensure good growth of all of the bacteria. The ration was completed by supplementation with lipid.

3.5.4 Sterility tests and cell counts

The "sterilized" milk was allowed to stand at room temperature overnight to ensure that no spore formers had survived. Spread and streakplates were made to further insure sterility. RM agar incubated at 37°C and APT agar (Difco) incubated at 30°C were used.

Total plate counts were done using RM agar prior to and post freeze drying. It was determined that there were 10^9 to 10^{10} colony forming units (CFU) per gram (depending on the strain). Freeze drying did not significantly effect the number of CFU per gram.

3.6 Fermentation products

The organic acid profile of the lipid free diet was examined using a slightly modified method of that used by Tetlow and Hoover (1988). The powder was reconstituted to 10% total solids (w/w) in distilled water. Five mL was placed in a polypropylene centrifuge tube and spun at 12,000 x g for 15 min. The supernatant was filtered through a 0.2 micrometer Nylon filter and held at -17°C until analyzed with high pressure liquid chromatography (HPLC). The samples had to be diluted 10 fold due to their high organic acid concentrations. The standards used were propionic acid (900 ppm), citric acid (300 ppm), lactic acid (400 ppm), acetic acid (700 ppm) and succinic acid (600 ppm). Standards were prepared using the above method (Tetlow and Hoover 1988) except dilution was not required.

A Shimadzu LC-6A series HPLC fitted with a BioRad Aminex Ion Exclusion HPX-87-H^+ column and UV detector was used to analyze the organic acid composition of the diets. The injection volume was 5 microliters and the flow rate of the isocratic mobile phase (0.01 N H_2SO_4) was 0.5 mL/min. The attenuation was set at 5 with a chart speed of 5 mm/min.

3.7 Casein and egg white protein diets

3.7.1 Source of proteins

The protein carriers used were egg white protein (EWP) and casein. The food grade EWP was obtained from Export Packers Co. Ltd. (Edmonton, Alberta) and the vitamin free casein from ICN Nutritional Biochemicals (Cleveland, Ohio). The total protein contents of the EWP and casein determined using the macrokjeldahl method were 85% and 91%, respectively. Sterilization prior to the addition of the bacteria was not considered necessary because the proteins were not components of the growth media.

3.7.2 Source of supplementary diet

A protein depletion diet was supplied by ICN Nutritional Biochemicals (Cleveland, Ohio). The diet contained all of the growth requirements for a rat except protein. The powdered formulation made up approximately 70% of the complete ration.

3.7.3 Formulation of the complete diets

The lactobacilli were grown in RM broth at 37°C and added to the protein just prior to freeze drying. Lactobacillus casei 03 cells were isolated from the broth by centrifugation (14,000 x g for 20 min at 4°C). The bacteria were then resuspended in sterile water for

ease of handling and added to the proteins which had been dispersed in water (33% total solids). One liter of cell (1 x 10^9 cells/mL) was added per Kg of protein. The preparation was frozen and freeze dried (shelf temperature 26°C).

The dried protein-cell mixtures were ground using a mortar and pestle and stored at -17°C until needed. The protein depletion diet was supplemented with the mix to make up a feed which contained 26% protein. The number of lactobacilli per gram in the complete ration was determined.

3.8 Nonfermented skim milk diet

Skim milk powder was used as the carrier. The powder was reconstituted to 30% total solids and the bacteria were added just prior to freeze drying (no fermentation or sterilization took place). The powder was prepared for test by supplementation with lipid.

3.9 Statistical analysis

The statistical significance of the tumour data was determined by Student's t-test. The tumour weights for the test and control groups were also used to calculate an Inhibition Ratio using the following equation: I.R.% = 1-(mean tumour weight of the test gro[illegible] mean weight of the control group) x 100. The possible correlation between initial animal weight, consumption, dosage, final animal weight and tumour

proliferation was analyzed using linear regression.

4. RESULTS

4.1 Initial screening

4.1.1 Male Buffalo rats

The effect of oral administration of Lactobacillus casei 03, 49, 28, and 44 and Lactobacillus bulgaricus 47 and 35 on the relative inhibition of Morris hepatoma #7777 tumours in male Buffalo rats is indicated in Table 3. The data was collected using a skim milk based diet. The highest inhibition ratio was observed with L. casei 03 and the lowest with L. casei 28. In the case of strain 28, a negative I.R.% was noted which means the tumour weights for the test group were larger than those of the control. Feeding L. casei strain 49 resulted in an inhibition ratio 13 percentage points lower than that observed with strain 03 (i.e. 44.73%). The equal ratio diet and strain 44 both resulted in inhibition which was approximately half of that observed with strain 03. In terms of the equal ratio mixture a value of 21.16 was obtained in male rats, which is approximately 10 percentage points lower than the mean of the other 4 single strain trials. Using this as an indication (with the results obtained for females) the L. bulgaricus strains were not examined further.

The P values were calculated using the student's t-test and were considered significant at levels which were ≤ 0.05. Although none of the trials were significant based on the above criteria, L. casei 03,

Table 3. The Effect of Selected Lactic Acid Bacteria on the Inhibition of Morris Hepatoma #7777 Tumours in Male Buffalo Rats.

Sample	Mean[1]	SEM[2]	P[3]	I.R.%[4]
Control	10.35	2.6	-	-
L. casei 03	4.38	1.1	0.059	57.68
L. casei subsp. rhamnosus 49	5.72	2.0	0.18	44.73
L. casei 28	11.06	2.1	0.84	-6.86
L. casei 44	7.34	2.2	0.40	29.08
Equal Ratio Mixture of: L. casei 03,49,28,44 L. bulgaricus 47,35	8.15	2.1	0.52	21.26

[1]Mean weight of tumour (grams) - Number of test animals per group = ten.

[2]Standard error of the mean.

[3]Probability was determined using the Student t-test.

[4]I.R.% = Inhibition ratio = [1-(mean tumour wt. for test group/mean tumour wt for control)]100.

at P = 0.06 proved to be the most promising. The P values approach 0.05 when either the control tumours are significantly larger than the test tumours (i.e. the two groups are considered to be from different populations) or vice versa. It is only the former case i.e. control tumours larger than the test tumours, which was under investigation in this study.

The standard error of the means (SEM) with respect to tumour weights varied between 2.6 and 1.1 grams (Table 3). Table 4 shows the inherent animal variability with respect to tumour proliferation; which is characteristic in solid transplantable tumour models. As can also be seen from the data in Table 4, the experimetal groups had similiar mean body weights. The standard deviation in body weight at the beginning of the trial was 34 g for rats fed L. casei 03 and the control and 33 g for those fed L. casei 49. The mean weights for the three groups at the end of the 21 day trial were all approximately 260 g. The mean body weight loss in both the control and test groups during the trial was approximately 30 g. This was expected due to the nature of the tumour i.e. Morris hepatoma #7777 causes protein wasting. Table 4 does not include all of the data for the male screening population, however, it is respresentative of the type of variation incurred.

In Table 5, the regression coefficients using initial body weights, final body weights, consumption and dosage against tumour weights are given. The highest, although insignificant correlations

Table 4. Representative Body Weight and Tumour Weight Variability in Male Buffalo Rats.

Animal	Test Group	Initial Body Wt.	Mean Wt.	S.D.*	Final Body Wt.	Mean Wt.	S.D.*	Total Tumour Wt.
1	Control	369	300	34.32	293	260	22.18	10.3
2		230			280			21.6
3		14			285			2.2
4		14			257			5.9
5		303			258			12.3
6		288			253			21.2
7		288			232			21.1
8		275			268			1.4
9		265			254			3.2
10		251			223			4.1
1	L. casei 03	362	298	34.13	250	263	21.72	4.3
2		323			256			6.0
3		320			270			12.0
4		309			308			7.7
5		302			280			3.3
6		295			279			+
7		287			259			4.5
8		273			238			2.8
9		270			251			3.0
10		238			238			+
1	L. casei 49	343	296	32.74	311	261	31.[illegible]	1.5
2		336			278			14.7
3		317			295			1.1
4		308			278			+
5		305			261			5.9
6		295			236			5.2
7		276			245			3.1
8		275			267			3.7
9		269			232			2.3
10		236			211			19.6

Note: All weights are in grams.

*Standard deviation from mean body weight.

+Tumour nodule was present (successful transfer but the tumours were less than 0.1 g.

Table 5. The Relationship Between Initial and Final Body Weight, Consumption, Dosage and Tumour Proliferation in Male Buffalo Rats as Expressed by Coefficients of Regression.

Trial		Initial Wt. vs Tumour Wt.[1]	Final Wt. vs Tumour Wt.[1]	Consumption vs Tumour Wt.[2]	Dosage vs Tumour Wt.[3]
Control	R^2*	0.070	0.008	0.176	-
L. casei 03	R^2*	0.283	0.149	0.530	0.525
L. casei 49	R^2*	0.118	0.239	0.420	0.014
L. casei 44	R^2*	0.202	0.107	0.001	0.548
L. casei 28	R^2*	0.002	0.063	0.051	0.001
L. casei 03,49,28,44 L. bulgaricus 47,35	R^2*	0.016	0.405	0.079	0.087

Note: There were ten animals per trial (eight Degrees of Freedom).

[1]Initial and Final Animal Body Weight in grams, Tumour weight in grams.

[2]Total grams of diet consumed over the 21 day test period.

[3]Total number of Lactic Acid Bacteria consumed over the test period.

*Coefficient of regression was calculated using simple linear regression.

were observed with L. casei 03 when consumption and dosage were used as the x variables i.e. 0.530 and 0.525 respectively and with L. casei 44 when dosage was the independent variable i.e. 0.548. When intial or final body weights were used to calculate the coefficient of regression, R^2 values approached 0 except in the case of the equal ratio diet where a value of 0.405 was observed.

The coefficients of regression were calculated to determine if tumour variability was due to factors such as body weight, consumption and dosage of the microorganisms. It appears from the above results that this is not the case.

4.1.2 Female Buffalo rats

The inhibition ratios observed when female Buffalo rats were fed various strains of L. casei and L. bulgaricus in skim milk based diets are shown in Table 6. The consumption of diets which contained L. casei 03, L. casei 28 and the equal ratio mixture comprised of L. casei 03, 49, 28, 44 and L. bulgaricus 35, 47 resulted in negative levels of inhibition. Positive results were noted with 2 strains i.e. L. casei subspec. rhamnosus 49 (6.49%) and L. casei 44 (2.24%). Although the inhibition ratios are positive, the P values indicate that the above test groups are from the same population as the control, and have no significant differences.

The control and test groups which led to negative inhibition ratios had tumour weight SEM values which ranged between 1.4 and 1.7

Table 6. The Effect of Selected Lactic Acid Bacteria on the Inhibition of Morris Hepatoma #7777 Tumours in Female Buffalo Rats

Sample	Mean[1]	SEM[2]	P[3]	I.R.%[4]
Control	12.01	1.4	-	-
L. casei 03	13.60	1.6	0.47	-13.23
L. casei subsp. rhamnosus 49	11.23	2.2	0.76	[illegible].49
L. casei 28	13.12	1.3	0.56	[illegible].24
L. casei 44	11.74	[illegible].6	0 93	2.24
Equal Ratio Mixture of: L. casei 03,49,28,44 L. bulgaricus 47,35	15.08	1.7	0.18	-25.56

[1]Mean tumour weight (grams) - Number of test animals per group = ten.

[2]Standard error of the mean (grams).

[3]Probability was determined using the Student t-test.

[4]I.R.% = Inhibition Ratio = [1-(mean tumour weight for the test group/ mean tumour weight for the control)]100.

g. L. casei 49 and 44 had slightly higher variation i.e. 2.2 g and 2.6 g, respectively. The mean tumour weights were between 11.23 g and 15.08 g with the control group being 12.01 g. Data for the experimental variability in terms of tumour size and body weights are listed in Table 7. The between animal variability for tumour size was large i.e. 5.1 to 19.6 g in the control, 7.2 to 21.2 g for L. casei 03 and <0.1 to 19.0 g for L. casei 49. The average body weight of the female animals in each experimental population was 195 g. The standard deviation in body weight for the 3 groups ranged between 15 and 17.5 g. At the termination of the experiment the standard deviations (in body weight) were still within the above mentioned range. As with the data for the male Buffalo rats (the data shown in Table 4), Table 7 is representative rather than all inclusive. The overall variability in tumour weight SEM values, body weight (initial and final), etc. for the female and male test groups is similiar therefore, the lack of significance in the female trial cannot be attributed to higher experimental variability.

The R^2 values in Table 8 were calculated using initial body weight, final body weight, consumption and dosage as the X variables and tumour weight as the Y variable. The highest correlation coefficients were observed when initial and final body weights were used for the control (0.603 and 0.812, respectively) and when consumption and dosage were the independent variables for L. casei 03 (0.736 and 0.748 respectively). Values between 0.415 and 0.513 were obtained using data from strain 44 and 28. The remainder of the R^2

Table 7. Representative Body Weight and Tumour Weight Variability in Female Buffalo Rats.

Animal	Test Group	Initial Body Wt.	Mean Wt.	S.D.*	Final Body Wt.	Mean Wt.	S.D.*	Total Tumour Wt.
1	Control	221	196	15.10	194	170	15.05	5.1
2		211			181			9.7
3		208			188			10.0
4		200			181			10.9
5		196			166			9.2
6		194			185			10.4
7		190			138			19.6
8		185			167			14.2
9		176			160			13.2
10		174			154			17.8
1	*L. casei* 03	227	195	17.58	192	177	14.35	7.2
2		211			193			14.8
3		206			186			21.2
4		200			175			12.9
5		195			173			7.2
6		194			193			7.5
7		189			163			11.3
8		185			183			18.4
9		181			161			17.0
10		163			154			18.5
1	*L. casei* 49	215	195	16.75	189	179	15.26	16.8
2		213			195			19.0
3		206			185			0.7
4		205			198			+
5		199			188			16.5
6		194			172			16.7
7		188			180			5.6
8		187			166			13.8
9		180			170			11.4
10		160			148			11.7

Note: All weights are in grams.

*Standard deviation from mean body weight.

+Tumour nodule was present (successful transfer) but the weight of the tumour was less than 0.1 grams.

Table 8. The Relationship Between Initial and Final Body Weights, Consumption, Dosage and Tumour Proliferation in Female Buffalo Rats as Expressed by Coefficients of Regression.

Trial		Initial Wt. vs Tumour Wt.+	Final Wt. vs Tumour Wt.+	Consumption vs Tumour Wt.++	Dosage vs Tumour Wt.+++
Control	R^2*	0.603	0.812	0.021	-
L. casei 03	R^2*	0.169	0.022	0.084	0.080
L. casei 49	R^2*	0.001	0.022	0.090	0.071
L. casei 44	R^2*	0.021	0.172	0.482	0.512
L. casei 28	R^2*	0.416	091	0.736	0.748
L. casei 03,49,44,28 L. bulgaricus 47,35	R^2*	0.00?	0.224	0.015	0.024

Note: There were ten animals per trial (eight Degrees of Freedom).

+Initial and final animal body weight and tumour weight (grams).

++Total grams of diet consumed over the 21 day test period.

+++Total number of Lactic Acid Bacteria consumed over the test period.

*Coefficient of Regression was calculated using simple linear regression.

values approached 0. As with the male Buffalo rats, the R^2 values were not significant and therefore do not explain the variability in tumour weight between animals. If, however, the coefficients of regression were significant, future trials would have to be designed to accomodate the dependency.

4.1.3 Confirmation

The data in Table 3 for L. casei strain 03 led to a confirmatory study (Table 9). An inhibition ratio of 58% at a P value of 0.049 was obtained when male Buffalo rats were fed the fermented diet. In Table 10 the regression information for initial and final body weight versus tumour weight is given, however, the consumption and dosage data was unavailable. The data was missing because the animals kept overturning their feed dishes and it was therefore impossible to monitor their consumption. The R^2 values with respect to body weight were all low i.e. less than 0.320. This data confirms that there is no significant dependence between body weight and tumour proliferation.

4.2 Protein carriers

Food grade casein and egg white proteins were used to carry L. casei strain 03 in a protein depletion diet (Table 11). When casein was used an I.R.% of 26.10 was obtained. The inhibtion was significant at a P value of 0.22; according to the test criteria, the

Table 9. The Effect of Perorally Administered Skim Milk Fermented With Lactobacillus casei on the Inhibition of Morris Hepatoma #7777 Tumours in Male Buffalo Rats

Sample	Mean[1]	SEM[2]	P[3]	I.R.%[4]
Control	4.63	1.1	-	-
Test (L. casei 03)	1.94	0.45	0.049	58.10

[1]Mean tumour weight (grams) - Number of test animals per group = ten.

[2]Standard error of the mean.

[3]Probability was determined using the Student t-test.

[4]I.R.% = Inhibition Ration = [1-(mean tumour weight of the test group/mean tumour weight of the control)]100.

Table 10. The Relationship Between Initial and Final Body Weights and Tumour Proliferation in Buffalo Rats Receiving a Fermented Diet as Expressed by Regression Coefficients.

Trial		Initial Wt. vs Tumour Wt*	Final Wt. vs Tumour Wt.*	Consumption vs Tumour Wt.	Dosage vs Tumour Wt.
Control	R^2 **	0.233	0.298	ND[1]	ND[1]
L. casei 03	R^2 **	0.320	0.229	ND[1]	ND[1]

Note: There were 10 animals per test (Eight degrees of Freedom)

*Initial and final animal weights in grams, tumour weight (grams).

**Coeffients of Regression were caluculated using simple linear regression.

[1]ND - No data due to problems with the animals i.e. kept over turning their feed dishes.

Table 11. The Effect of *L. casei* strain 03 on the Inhibition of Morris Hepatoma #7777 Tumours in Male Buffalo Rats When Casein and Egg White Proteins Were Used as the Dietary Carriers.

Sample	Mean+	SEM++	P*	I.R.%**
Casein Control	7.46	1.2	-	-
Casein/*L. casei* 03 Test	5.51	1.0	0.22	26.10
Egg White Protein Control	6.73	1.3	-	-
Egg White Protein/ *L. casei* 03 Test	7.44	1.3	0.70	-10.58

+Mean tumour weight (grams) - Number of test animals per group = ten.

++Standard error of the mean (grams)

*Probability was determined using the student t-test.

++I.R.% = Inhibition Ratio = [1-(mean tumour weight for test group/ mean tumour weight for control)]100.

control and test group are considered to be of the same population. An inhibition of -10.58 was observed using the egg white carrier. The P value was 0.70 which means the test and control populations are not significantly different i.e. the test diet had no effect on tumour growth. In the above trials, tumour weight SEM values were quite similiar i.e. 1.0 to 1.2 g (casein test and control, respectively) and 1.3 g for both the egg white control and test groups.

As with the previous data in Tables 5, 8 and 10, no significant correlation between initial body weight, final body weight, consumption or dosage and tumour weight exists (Table 12). The highest R^2 value seen in Table 12 is for the control (0.641) which was obtained by using final body weight as the X variable. This was predictable, as the tumour causes protein wasting and weight loss. Even though no significant correlations were noted in the initial screening, it was still necessary to analyze these data because the diets were changed i.e. the diet itself may have had an effect.

4.3 Supplemented skim milk diet

In the following experiment L. casei strain 03 was added to skim milk just prior to freeze drying; no fermentation took place. In this case, the I.R.% obtained for L. casei 03 was negative i.e. -25.75 (Table 13). On average the test tumours were 1.0 g larger than the control tumours. The SEM values were 1.3 for the control and 2.0 for

Table 12. The Relationship Between Initial and Final Body Weights Consumption, Dosage and Tumour Proliferation when L. casei strain 03 is Fed to Male Buffalo Rats in Egg White Protein and Casein Carriers.

Trial		Initial Wt. vs Tumour Wt.+	Final Wt. vs Tumour Wt.+	Consumption vs Tumour Wt.++	Dosage vs Tumour Wt.*
Control	R^2***	0.043	0.641	0.088	-
Egg White Carrier/ L. casei 03**	R^2***	0.670	0.006	0.062	0.482
Control	R^2***	0.002	0.207	0.561	-
Casein Carrier /L. casei 03**	R^2***	0.047	0.323	0.256	0.268

Note: There were seventeen animals per trial (fifteen Degrees of Freedom).

+Initial and final animal weights (grams), tumour weight in grams.

++Total grams of diet consumed over the 21 day test.

*Total number of L. casei strain 03 consumed over the 21 day period.

**Egg white protein and casein were used to carry the L. casei 03. in a protein depletion diet.

***Coefficients of Regression were calculated using simple linear regression.

Table 13. Effect of a Skim Milk Based Diet Supplemented with L. casei strain 03 on the Growth of Morris Hepatoma #7777 Tumours in Male Buffalo Rats.

Sample	Mean[1]	SEM[2]	P[3]	I.R.%[4]
Control	4.00	1.3	-	-
Nonfermented L. casei 03 Skim Milk Test	5.03	2.0	0.67	-25.75

[1]Mean tumour weight (grams) - Number of test animals per group = ten.

[2]Standard error of the mean.

[3]Probability was determined using the Student t-test.

[4]I.R.% = Inhibition ration = [1-(mean tumour wt. for the test group/ mean tumour wt. for the control)]100.

the test.

The regression coefficients for the nonfermented skim milk experiment were much the same as those observed in the previous trials (Table 14). The largest R^2 value was 0.301, all of the other data points were less than 0.263.

4.4 Organic acid production

An example of the type of organic acid profile which is obtained by analyzing fermented milk samples using high pressure liquid chromatography is shown in Figure 1. In Table 15, the results from the analyses of the fermented milk diets containing L. casei strains 03, 49, 28, 44 and L. bulgaricus strains 47, 35 and RM broth from L. casei strain 03 are given. Two of the peaks in the profiles were identified as lactic acid and citric acid, the remaining peaks did not correspond to the standards used. Further investigation was not done because the purpose was to observe rather than quantify the differences between strains.

The acid which was in highest concentration was lactic acid with the exception of L. bulgaricus strain 47. This was expected as heterofermentative lactic acid bacteria are defined as producing at least 50% lactate (Kandler 1983). The results for broth and fermented milk obtained through L. casei strain 03 fermentations are the most interesting. It can be seen in Table 15 that lactate concentration is

Table 14. Effect of Lactobacillus casei strain 03 Nonfermented Skim Milk Test Ration of the Proliferation of Morris Hepatoma #7777 Tumours in Male Buffalo Rats.

Trial		Initial Wt. vs Tumour Wt.+	Final Wt. vs Tumour Wt.+	Consumption vs Tumour Wt.++	Dosage vs Tumour Wt.*
Control	R^2**	0.301	0.136	0.263	-
Non-F[illegible]ted L. ca[illegible] Test [illegible]tion	R^2**	0.143	0.034	0.040	0.039

Note: There were ten animals per test group (eight Degrees of Freedom).

+Initial and final animal weights (grams), tumour weights in grams.

++Total grams of diet consumed over the 21 day test.

*Total number of L. casei strain 03 consumed over the trial.

**Coefficients of Regression calculated using simple linear regression.

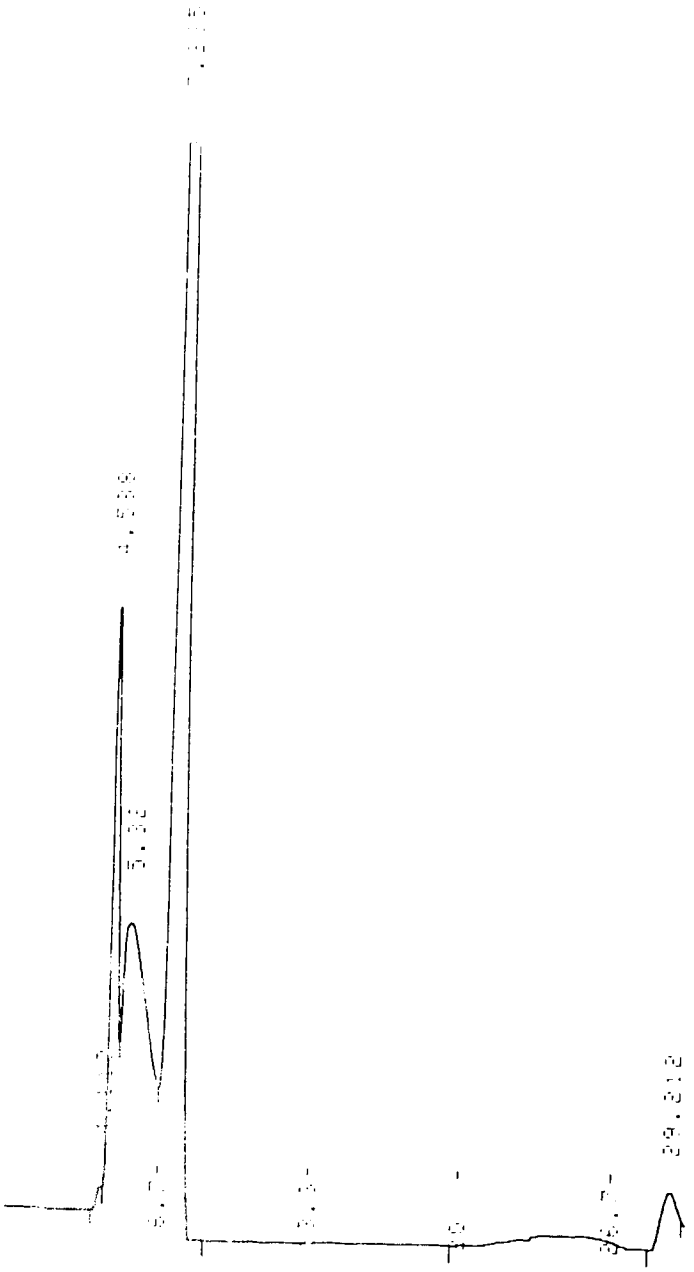

Figure 1. Organic acid profile of skim milk fermented with L. casei strain 49.

Table 15. Concentration of Organic Acids in Milk and RM Broth Fermented with Various Strains of Lactobacilli.

Sample	Acid	Retention Time (min.)	Concentration % of Total	Concentration mg/mL
Milk/	Citric	4.6	44	1.3
L. bulgaricus 47	Unknown 1	5.2	4	*
	Lactic	7.4	46	0.7
	Unknown 2	29.3	5	*
Milk/	Unknown 1	4.0	<1	*
L. bulgaricus 35	Citric	4.6	11	2.4
	Unknown 2	5.1	6	*
	Unknown 3	5.6	20	*
	Lactic	7.2	62	6.7
	Unknown 4	29.2	1	*
Milk/*L. casei* 49	Unknown 1	4.1	1	*
	Citric	4.6	12	1.3
	Unknown 2	5.3	24	*
	Lactic	7.2	62	3.6
	Unknown 3	29.2	2	*
Milk/*L. casei* 28	Unknown 1	4.1	<1	*
	Citric	4.6	14	1.2
	Unknown 2	5.1	20	*
	Lactic	7.2	62	2.8
	Unknown 3	29.1	3	*
Milk/*L. casei* 44	Citric	4.6	11	1.0
	Unknown 1	5.2	23	*
	Lactic	7.3	64	3.0
	Unknown 2	29.2	2	*
Milk/*L. casei* 03	Citric	4.7	20	1.6
	Unknown 1	5.2	28	*
	Lactic	7.3	52	2.1
	Unknown 2	29.2	<1	*
RM broth/	Citric	4.5	13	3.1
L. casei 03	Lactic	7.1	86	10.5
	Unknown 1	29.3	<1	*

*Unable to calculate unknown concentrations in mg/mL as organic acids differ in their absorbance.

35% higher in the media than in the milk. The unknown which had a retention time of 5.18 (concentration was 28% of the total) in the milk was not present in the broth. It appears that the metabolic processes of this strain vary between the milk and formulated broth.

5. DISCUSSION AND CONCLUSION

5.1 Initial screening

The most promising result in the initial screening of the fermented milk diets in male and female Buffalo rats carrying Morris hepatoma #7777 tumours was obtained with L. casei strain 03. An I.R.% of 57.68 was obtained when male rats were the carriers, however, a negative ratio (-13.23) resulted when female rats were tested (Tables 3 and 4, respectively). Upon comparison of Tables 3 and 4, it was noted that the effect in male rats was between 27 and 47 percentage points higher than in female rats except for L. casei strain 28 in which case the results for both male and female rats were essentially the same. This tumour type produces an estrogen binding protein (Francavilla et al. 1984) and it may be an intrinsic factor such as this influencing the treatment.

During the initial chemical induction of various liver tumours Morris and Slaughter (1978) observed a difference in effect between sexes. It is therefore not surprising that a sex dependent relationship was observed in this study. The sex of the test animal is often specified in the literature with males being most commonly used. It is however, not practical to assume that only male animals should be used, as effect also varies with tumour type. Screening both sexes against a particular treatment when setting up an experimental animal model is therefore advisable.

From the data in Tables 3 and 4 it can be seen that there was a sex and strain dependent effect when Buffalo rats inoculated with Morris hepatom #7777 tumours were used as the experimental model. Shimizu et al. (1987) reported data which screened 13 species (24 strains) of lactobacilli against Meth A solid and ascites tumours in BALB/c mice. An inhibition ratio of 87.6% was obtained for Yakult Institute of Tokyo strain 901. It is not appropriate to directly compare our results with those reported by Shimizu et al. (1987) because of differences in species of animal, tumour type and mode of administration. Even though an I.R.% of only 57.58 was obtained with L. casei strain 03, the overall effectiveness, when used in the model outlined by Shimizu et al. (1987) may be greater because: 1) a direct mode of administration was utilized i.e. intraperitoneal injection; and 2) the tumour may be more susceptible to treatment.

Shimizu et al. (1987) screened 24 strains of lactobacilli because of the well documented strain differences. There are also differences in the susceptibility of different tumours to treatment with bacteria. Shahani et al. (1983) selected and used an Ehrlich ascites tumour in their research because it is very responsive to immunoadjuvant treatment. Solid tumours such as Morris hepatoma #7777 do not respond to immune activation to the same degree as ascites tumours. There are also differences in the effects reported within solid tumours. Tumours are a very heterogenous population and response prediction is therefore impossible.

When interpreting data from experimental trials, general anticarcinogenic claims cannot be made. The observations only hold true for the model studied; in our case, L. casei strains administered orally to Buffalo rats carrying Morris hepatoma #7777 subcutaneous tumours. However, general findings such as: 1) the immune system is stimulated upon administration of some gram positive bacteria (Zbar et al. 1970); 2) the effect varies with strain (Perdigon et al. 1986; Shimizu et al. 1987); 3) macrophages isolated from challenged hosts are cytotoxic (Freedman et al. 1980); 4) in vitro assays with tumour cells are unsuccessful (Kato et al. 1983); 5) antimacrophage agents abolish established effects (Goguel et al. 1982), etc. may be used to speculate on the mechanisms involved. Based on the antitumour and immunoadjuvant work involving gram positive bacteria, it has been hypothesized that immune activation rather than direct cytotoxicity is responsible for the antitumour activity.

Namba et al. (1981), Nishimura et al. (1985) and Roitt et al. (1985) have all taken the generalization further by indicating that immunity derived from gram positive bacteria is possibly due to peptidoglycan structure. Our work has not evolved to the point of examiniation of the immune system or active component, however, our research does indicate that L. casei strain 03 does show promise (Table 3 and 9).

Other variables which we examined were initial body weight, final body weight, consumption and dosage of microorganisms. Simple linear

regressions using these factors as the independent variables and tumour weight as the dependent variable were done (Tables 5 and 8). This attempt at explaining the variability in tumour size between animals was unsuccessful as no correlation was found. In the literature, inhibition ratios have been used to represent the data concerning the effect of a treatment modality on the proliferation of solid tumours (Matsuzaki et al. 1987; Shimizu et al. 1987). When such an equation is used tumour variability is only indirectly evident, i.e. seemingly high inhibition ratios are shown to be insignificant. Based on this and the guidelines set out by the National Cancer Institute (U.S.A.) we considered that the variability in this study was within the accepted range.

5.2 Protein carriers

Both Perdigon et al. (1986) and Shahani et al. (1983) noted that it was necessary to have milk as the carrier when gram positive bacteria were administered orally. Shahani et al. (1983) proposed that casein may a pl y a protective role. Because of this we examined casein as a potential oral carrier for *L. casei* strain 03. An inhibition ratio which was only half of that obtained when milk was both the growth medium and carrier was observed. This difference in the results could be due to: 1) other milk components besides casein are required; 2) the casein was not as protective because of a looser association between the casein and the bacteria; 3) milk fermentation is required in order to obtain a full effect because metabolic

products are also involved; and 4) fermentation in milk enhances the resistance of the bacteria directly e.g. possible changes in the peptidoglycan thickness. Although point 3 is a possibility it is unlikely based on the literature.

To determine whether the effect is due to fermentation itself or some other factor(s) in the milk it would be necessary to grow the bacteria in milk and then separate them from the milk. This could be facilitated by use of a buffered milk which would prevent acid coagulation. The bacteria could then be added to skim milk and to casein.

Egg white protein was also examined as a possible carrier. The purpose of this was to compare the effectiveness of a nonmilk protein with casein in terms of effectiveness. Factors in the egg white such as lysozyme were not considered problematic as Namba et al. (1981) found an increased in vitro response when lysozyme was used. The justification for this observation was that lysozyme may cause the release of absorbable peptidoglycan fragments thus causing an immune response.

A negative inhibition was obtained when egg white protein was the carrier which could indicate: 1) that fermentation in milk is a requirement; 2) casein is at least in part responsible for an effect; and 3) unpredicted inhibitory factors were present in the egg white. The use of egg white as a carrier suited our purposes, however, future

investigation using this carrier would be unproductive.

5.3 Nonfermented skim milk carrier

In this case, L. casei strain 03 was grown in RM media and then transferred to skim milk just prior to freeze drying. This experiment was done to investigate our assumption that other factors besides casein play a protective role. In this experiment a negative inhibition ratio of the same order as that found with egg white carrier was observed. This may indicate that: 1) casein is the primary protectant and when diluted out with other milk components in an unfermented medium it is less effective; 2) Acid coagulation positively affects the functionality of the system; 3) fermentation in the milk is a requirement; or 4) a combination of the above. The profiles comparing the organic acid concentrations in fermented milk and broth tends to support point 3. As it currently stands, the role of milk is undefined and the experiment using a buffered milk medium (outlined above) would be the logical next step.

5.4 Conclusion

Tumours are a very heterogenous population an no specific solid tumour or group of solid tumours has provided all the necessary information for the effective treatment of cancer. The Morris group of hepatomas represents a diverse group of solid tumours through which tumour agents such as Lactobacillus casei, can be studied. The

use of a chemical carcinogen directly is not practical as it is both time consuming and unpredictable. Transplantable tumours such as Morris hepatoma #7777 are nearly 100% transferrable and have characteristic growth kinetics (Looney et al. 1978). Treatment modalities are therefore easier to assess in a tumour transplant animal model system.

Based on this study it can be concluded that L. casei strain 03 has an effect on the proliferation of Morris hepatoma #7777 tumours in male Buffalo rats when administered orally in a fermented skim milk vector. Due to the varied: 1) susceptibility of different animal species and strains to treatment modalities; 2) effectiveness of different routes of administration; and 3) morphological, physiological and biochemical characteristics of tumours, it is impossible to make any broader statements.

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