



PAPER

Inhibition of systemic TNF- α cytotoxicity in cancer patients by D-peptidoglycan

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The current study was designed to investigate direct inhibitory effects of N-acetyl-glucosaminyl-muramyldipeptide (GMDP) over the cytotoxic nature of TNF- α . A lactate dehydrogenase (LDH) assay of the inhibition of TNF- α cytotoxicity was done *in vitro* on the following cell lines: A549 (human lung carcinoma cells), A431 (human breast cancer cells) and L929 (mouse breast cancer cells). In a double-blind placebo-controlled trial, cancer patients with an elevated activity of all five LDH isoenzymes were randomized to receive either a GMDP solution or a placebo; 63 patients were evaluated every third day for the mean daily number of episodes of nausea or vomiting, changes in clinical status, cell blood count and blood chemistry. A 95% inhibition of LDH release was noticed on A549 cells. Other cell lines were less sensitive to GMDP, with an observed 72% dose-dependent reduction in LDH activity. *In vivo*, LDH activity was decreased by 41% (+/-4%) (mean +/-SD) in all 21 subjects who were given 0.5-1.0 mg of GMDP daily. A lowering of LDH activity by 73.4% (+/-4%) was observed in 23 patients who received GMDP at a dosage of 1.5 mg/kg daily. Correspondingly, a 10% (+/-2%) increase in LDH activity was noticed in 19 patients who were given a placebo ($P < 0.01$). During the follow-up period, the overall clinical condition of all patients treated with GMDP was improved. No side effects were observed. In nine patients who experienced nausea from tumor toxicity before treatment, the symptom subsided. In parallel, an extremely beneficial effect on lipids metabolism was noticed in all patients with elevated cholesterol and triglyceride levels. A dietary supplementation of GMDP has been shown to reduce systemic TNF- α cytotoxicity during tumor shock.

Keywords: TNF- α ; LDH; N-acetyl-glucosaminyl-muramyldipeptide (GMDP); tumor shock; GGTP

Introduction

Tumor necrosis factor-alpha (TNF- α) was originally discovered to be a protein that produced necrotizing effects in certain types of transplantable mouse tumors.¹ This factor is considered to be a main mediator of the tumor toxicity, causing cachexia in cancer patients. In the terminal stages of cancer this peptide

induces the clinical syndrome known as tumor shock. It is a deadly complication where literally all cells, tissues and organs of the human body experience the permanent cytotoxic effects of TNF- α as it is reflected in the elevation of all five lactate dehydrogenase (LDH) isoenzymes.

Subsequent studies have shown that the spectrum of biological activities for TNF- α is not limited to cytotoxic effects, but rather it exerts a pleiotropic effect in a wide variety of mammalian cell types, including adult cardiac myocytes,² human placentas,³ dermal fibroblasts,⁴ and pleural mesothelial cells.⁵ In these cells, TNF- α stimulates collagenase and prostaglandin E2

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production. This cytokine also exerts multiple effects on lipoprotein lipase *in vivo*.⁶

In low concentrations (10^{-10} M), TNF- α is thought to act primarily as a paracrine or autocrine regulator of leukocytes and endothelial cells, serving to regulate inflammatory responses to microbes and facilitating tissue repair. In higher concentrations (10^{-8} M), TNF- α production exceeds the number of TNF receptors in a given tissue, resulting in excess TNF- α circulating in the bloodstream. There it acts as an endocrine hormone that can lead to cachexia,⁷ potentially lethal microvascular coagulation, metabolic acidosis, hemoconcentration, and hypotension.⁸

Recent clinical observations indicate that tumor necrosis factor- α is elaborated in patients with advanced heart failure,⁹ myosin-induced myocarditis,¹⁰ reperfusion injury and cardiac allograft rejection.¹¹

Thus, a successful search for the inhibitor of TNF- α signaling pathways would solve the problem of finding a universal inhibitor for the pathophysiological mechanism of diseases.

Lately, a few human inhibitors have been isolated from body fluids.^{12,13} One of them shows an immunological cross-reactivity with TNF receptors.¹⁴

The first demonstration of the presence of N-acetylmuramyl-D-peptide (MDP) in human urine was reported by Krueger.¹⁵ Numerous reports testify to the fact that muramyl peptides induce different mediators of the immune response: TNF- α released to macrophages, interleukin-1 (IL-1) and Ia-antigen in both immunocompetent cells and in brain astrocytes.¹⁶ Similar to some vitamins, MDP is utilized but not synthesized by the host organism, acting as a regulator of various physiological systems. It has been suggested that muramyl peptides enter the organism through the adsorption of the degradation products of normal colibacilli.

Subsequently, glucosaminylmuramyl dipeptide (GMDP) was identified in human milk and amniotic fluid.¹⁷ It has also been suggested that GMDP enters the human body after the degradation of a probiotic part of the microflora.¹⁸ Previously, this compound was isolated during an analysis of the anti-tumor drug blastolysine which is a lysozyme cell wall hydrolysate of *Lactobacillus Bulgaricus*.¹⁹ GMDP has been extensively studied in animals, demonstrating adjuvant activity, antitumor activity, low pyrogenicity and a hypnogenic effect. It causes tumor necrosis by stimulating TNF- α mediated cytotoxicity.²⁰ This muramyl dipeptide also has shown a reducing effect on lipopolysaccharide (LPS), TNF- α and MDP, resulting in the prevention of

the toxic action of LPS during septic shock.²¹ Later, GMDP proved to be a most efficient stimulator of CD8 mediated cytotoxicity.²² However, the inhibition of TNF- α cytotoxicity *in vitro* has never been determined. Furthermore, the potential efficacy of GMDP for the prevention of TNF- α systemic cytotoxicity has not been assessed in controlled clinical trials. We have done placebo clinical trials evaluating the feasibility of treating TNF- α cytotoxicity in tumor shock, a condition where it is of vital importance to treat negative systemic apoptosis by protecting the whole body from TNF- α toxicity.

Patients and methods

Patients

All patients aged between 13 and 58 years who were admitted to the Institute from 19 October 1996 to 24 January 1997 were evaluated for inclusion in this study. Patients with at least one LDH isoenzyme falling within a normal reference range were excluded. Informed consent was obtained from all patients; 63 patients in the terminal stages of various cancers were selected for this clinical trial. The study group consisted of 41 females and 22 males between 14 and 57 years of age.

Malignancies consisted of the following: (17) brain tumors, (27) patients with breast carcinoma and massive metastasis to the liver or brain, (2) stomach cancers with liver metastasis, (1) hepatoma with lung metastasis, (3) melanomas with liver metastasis, (10) lung carcinomas with brain or liver metastasis and (3) colon cancers with liver metastasis.

All patients had received conventional treatment with chemotherapy and radiation. All of them suffered from cachexia and their blood tests revealed a 3–10 fold increase in LDH activity for all five LDH isoenzymes. The activity of liver enzymes was also increased; nine patients had experienced vomiting or nausea.

Methods

Study D-peptidoglycan N-acetyl-glucosaminylmuramyl-dipeptide was prepared and supplied in powdered form by The Institute of Infectious Gynecology, Conroe, Texas. This semisynthetic D-peptidoglycan is identical to the wall fragment of *Lactobacilli Bulgaricus*. A control powder was composed of vegetable oil, corn syrup solids and lactose.

Quality control of the GMDP (checking its identity and purity) was undertaken at The Institute by high performance liquid chromatography HPLC by comparison with reference samples (Sigma Corp., St Louis,

MO, USA). Each powder was diluted with 10 ml sterile distilled water. Daily GMDP dosages ranged from 0.5–1.5 mg/kg of the body weight.

LDH assay of the inhibition of TNF- α cytotoxicity in vitro The following cell lines were used for this assay: A549 (human lung carcinoma cells), A431 (human breast cancer cells) and L929 (mouse breast cancer cells).

All cells are available commercially from American Tissue Culture Collection ATCC. They were maintained in a Daldec Modified Eagle DME medium supplemented with 10% FCS and glutamine stored in a CO₂ incubator.

Lactate dehydrogenase (LDH) assay Cells were seeded into 35 mm Petri dishes and grown to a 70% confluency. They were then treated with human recombinant TNF- α (gift from Cetus Co. Emeryville, CA, USA) in a concentration indicated in the figures.

In cases where the cells were not spontaneously sensitive to TNF- α induced cytolysis, cycloheximide (CHX), a powerful blocker of the protein synthesis which sensitizes the cells to TNF- α action, was used in concentration 25 μ g/ml.

At present, the mechanism responsible for the action of cycloheximide (CHX) is unknown. It does not affect TNFR1 and TNFR2. Rather, CHX seems to block the synthesis of proteolytic enzymes, resulting in the destruction of compounds used in the TNF death pathway, thus protecting molecules sensitive to proteolytic degradation. This then leads to an accumulation of proteins and cells otherwise totally resistant to TNF acquired sensitivity.

Sixteen hours after the treatment, 20 μ l samples of the cultured supernatants were removed and assayed for LDH release by the Cytotox 96 assay (Promega Biotech, Madison, WI, USA) in accordance with the manufacturer's instructions. This methodology is based on the fact that when cells are dying they release LDH. These cells are given a substrate which will produce a color reaction, and in presence of LDH the reaction visibly changes color.

All samples were assayed in triplicate on an EL340 Microplate reader (Bio Tech Instruments Inc. Winooski, VT, USA) at 490 nm wavelength. An average of the three readings was plotted on the graphs (Figure 1, Figure 2 and Figure 3).

In vivo LDH assay Study protocols and informed consent procedures were approved by the Review Board of The Institute of Infectious Gynecology.

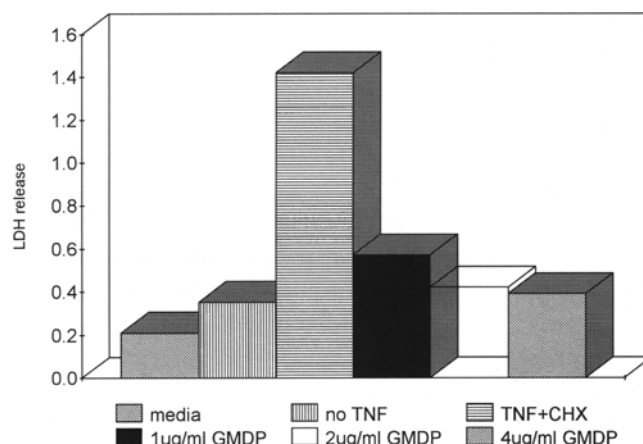


Figure 1 LDH assay in A549 cells (human lung cancer), 100 U/ml TNF+ cyclotex.

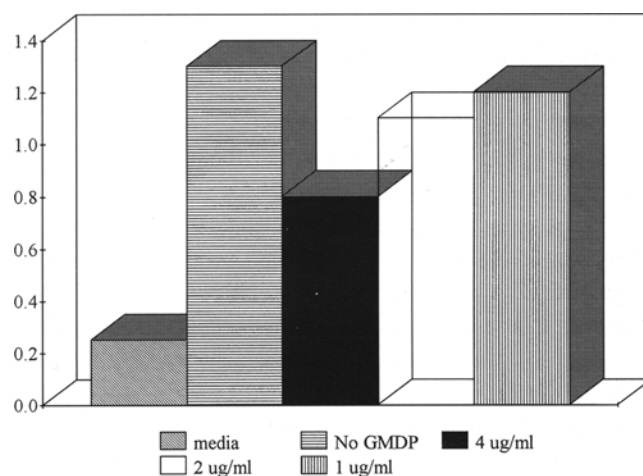


Figure 2 LDH assay in L929 cells (mouse mammary cancer), 500/ml rh TNF, 16h treatment, no CHX.

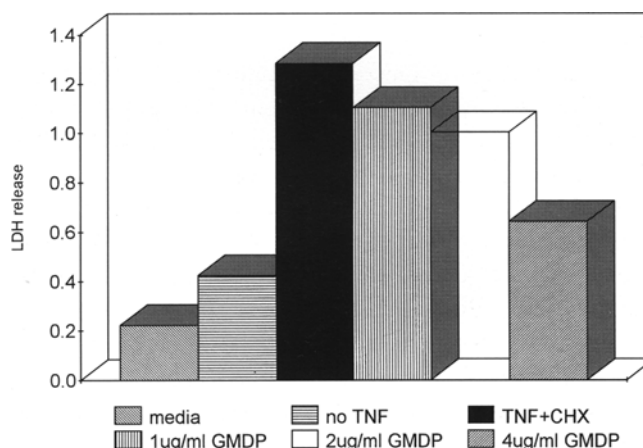


Figure 3 LDH assay in A431 cells (human lung cancer), 100 U/ml TNF+ CHX.

Informed consent was obtained from all patients. They were either assigned a GMDP treatment or a placebo by means of a block randomization procedure. Attendant physicians and nurses were unaware of which compound was being administered. Every third day the following data was recorded for each patient: cell blood count, blood chemistry, mean daily number of episodes of nausea or vomiting and changes in clinical status.

Statistical analysis was done with the Epi-Info Statistical Package (Center for Disease Control and Prevention, Atlanta, GA, USA), with two-way comparisons by the Fisher exact test (two tailed).

Results

Figure 1, Figure 2 and Figure 3 demonstrate GMDP's inhibitory effect on LDH release. One can see dose dependence on all cell lines. Mouse breast cell carcinoma proved to be less sensitive to the inhibitory action of GMDP.

GMDP was given to 21 patients orally at a dosage of 0.5–1 mg/kg daily in a water solution for 14 days; 23 patients were administered GMDP at dosage of 1.5 mg/kg daily; 19 patients received a placebo.

At enrollment there were no significant differences between the two groups with respect to age, height, weight or the severity of diagnosed tumor shock. Results were expressed as percentage of change in LDH activity from pretreatment values. Figure 4 shows the mean (SD) in LDH activity. In all control patients,

LDH activity increased approximately 10% (2) during this observation period. On the other hand, all treated patients exhibited persistent 41% decrease in levels of LDH activity (6) in a dosage of 0.5–1 mg/kg, and 73.4% (4) in a dosage of 1.5 mg/kg, with the lowest level achieved on the day 14. These changes were significantly different from those in the control patients ($P < 0.01$).

During the follow-up period, the overall clinical condition of all patients treated with GMDP improved. Nobody experienced any side effects. Nausea subsided in nine patients all of whom had this symptom of tumor toxicity before treatment. In two patients with high levels of free iron, the feeding of GMDP resulted in full normalization of this test. An augmenting effect on low platelet count by more than 200% was observed in three patients who had platelet counts below 30 000/ml. In parallel, a beneficial effect on gamma glutamyl transpeptidase GGTP activity was observed. Figure 5 demonstrates that GMDP decreased GGTP activity by 34% (6) in all seven patients who had elevated levels of this enzyme ($P = 0.025$, Fisher's exact test). Lowering effects on alkaline phosphatase was also noticed in three of six patients with previously elevated levels of this liver enzyme. Reductions in bilirubin concentrations by 19% (3) were observed in all six patients from elevated levels ($P = 0.015$ exact Fisher's test).

In the previous examples, 11 treated patients and seven control patients also had elevated triglycerides and cholesterol levels 2–10 times above the normal reference range. Results were expressed as a percentage of change in both triglycerides and cholesterol levels from pretreatment values.

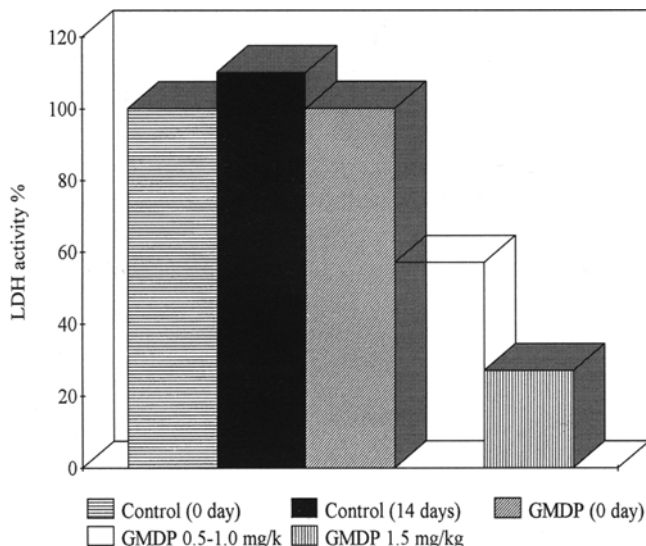


Figure 4 Reduction of the LDH activity of cancer patients after administration of GMDP in dosages of 0.5–1.0 mg/kg and 1.5 mg/kg.

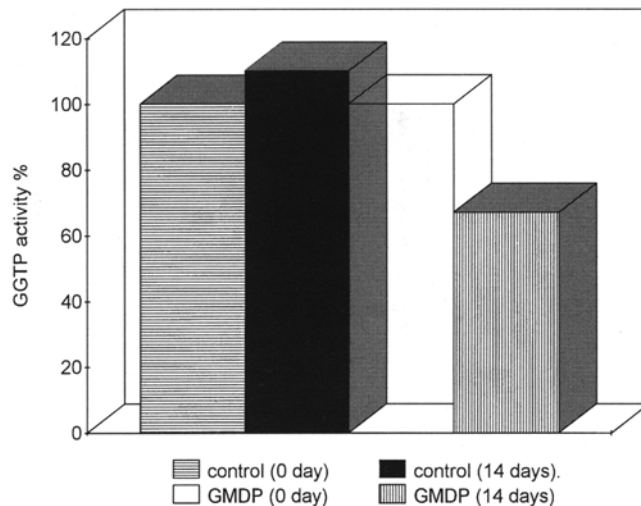


Figure 5 GGTP activity during GMDP treatment.

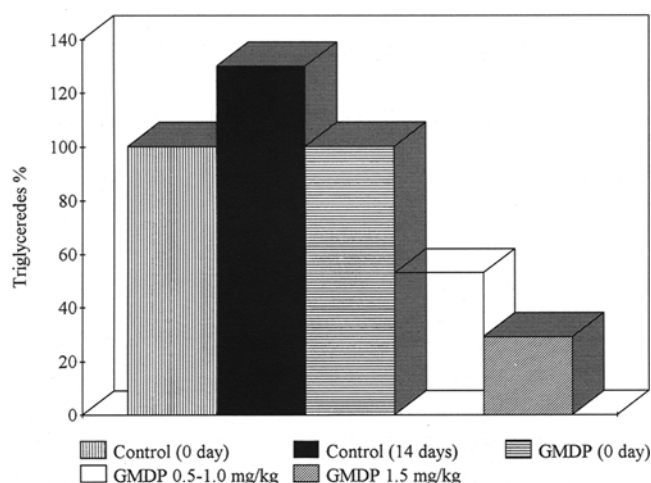


Figure 6 Reduction in triglyceride concentration in cancer patients in dosages of 0.5–1.0 mg/kg and 1.5 mg/kg.

Figure 6 shows a 71% (8) decrease in triglyceride concentrations in a dosage level of 1.5 mg/kg and a 41% (4) reduction in a dosage level of 0.5–1.0 mg/kg. In contrast, the control patients did not exhibit a significant change in blood levels of this lipid ($P > 0.5$).

Figure 7 shows a 21% (40) and 32% (5) reduction in elevated cholesterol levels in patients treated with GMDP in dosage levels of 0.5–1.0 mg/kg and 1.5 mg/kg.

Discussion

The problem of inhibiting cytokine cytotoxicity remains a real challenge in the development of a pathogenic treatment for many diseases which on the surface appear unrelated to each other. Despite common knowledge that all muramyl peptides (including

GMDP) stimulate TNF- α production by means of macrophages and other cell types, we decided to use GMDP as a blocker not only of the toxic but also of the pleiotropic effects of TNF- α .

It is worth pointing out that in cancer patients, it is necessary to maintain high levels of TNF- α along with a concurrent reduction in its systematic cytotoxicity.²³ A lowering TNF- α levels in such patients may stimulate tumor growth. Consequently, it at first may be hard to accept the fact that one compound can be used for both the stimulation of TNF- α production and the selective inhibition of its systemic cytotoxicity. Finding an apoptosis modulator which combined antitumor activity would be beneficial not only in treatments of tumor shock, but also those conditions (eg burn shock, stroke and chronic heart failure) which would be characterized by systemic apoptosis.

Yet it has been shown by us that in a variety of cell lines this negative systemic effect of the cytokines could be treated according to this newly discovered phenomenon of the inhibitory effects of GMDP over the cytotoxic nature of TNF- α . Our *in vitro* LDH assay demonstrated GMDP dose-dependent reductions of LDH release in a number of cell lines, thus preventing apoptosis. However, we did not define *in vitro* the exact molecular mechanism for this inhibition. Thus the precise levels for blockage of TNF- α killing pathways remains to be determined.

We found that the administration to the patients of a D-aminoacid containing peptidoglycan (GMDP) in large dosages (40–0 mg daily) also led to lowering of LDH activity in the blood. This lowering effect is dose dependent with maximum efficacy observed at 1.5 mg/kg daily. GMDP is a natural wall component of probiotics and the mediator of their systemic effect.¹⁷ Thus, through modulation (administering GMDP to patients) of these newly discovered effects of the probiotic microflora, we have seen an inhibition TNF- α systemic cytotoxicity. Furthermore, this lowering effect on LDH activity *in vivo* demonstrates that GMDP protects the cells against cytotoxic effects of any kind of cytokines. In this respect, we intentionally neglected the opportunity to check the level of TNF- α because it neither reflects the actual cytotoxic effects nor does it indicate effects on interleukins. Therefore, it cannot be used as a reliable assay for the monitoring of apoptosis during the GMDP treatment of the tumor shock.

It is noteworthy that this D-peptidoglycan in large dosages (1–1.5 mg/kg daily) also inhibits the pleiotropic effects of TNF- α such as those multiple effects on lipoprotein lipase. In all patients with elevated levels of

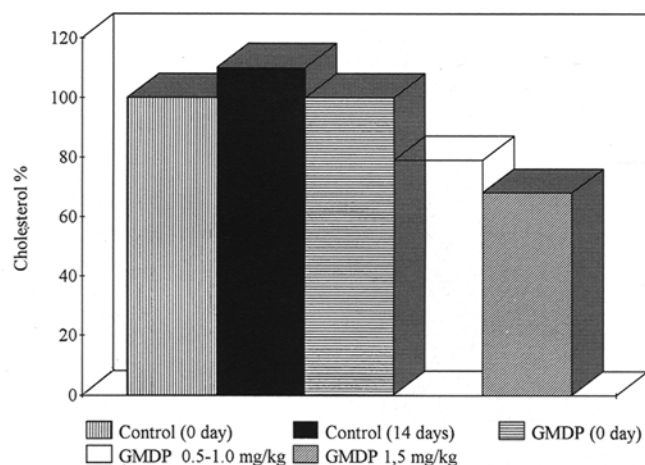


Figure 7 Lowering of cholesterol levels during tumor shock in dosages of 0.5–1.5 mg/kg.

triglycerides and cholesterol, the concentration of these lipids decreased during treatment. Larger studies should be done to ascertain this effect not only in patients with tumor shock, but also in all people with lipid metabolism disturbance.

We also found that feeding the cancer patients with GMDP led to a significantly lower activity of liver enzymes such as alkaline phosphatase and GGTP. Bilirubin levels were also lowered. In parallel, we have noticed that in patients responding well to GMDP treatment, nausea has subsided along with a concurrent improvement in appetite. Our data is consistent with the results of previous controlled studies in which cancer patients were fed a lower dosage of GMDP (1–10 mg daily) in the treatment and prevention of septic complications. In these studies, it was found that levels of bilirubin, creatinine and the activity of the liver transaminases were also similarly reduced.²⁴

In two patients with high levels of free iron in their blood, we observed a quick lowering after the first three days of GMDP feeding. This interesting finding can be explained by the well known phenomenon of neutrophil activity stimulation by GMDP.²⁵ This muramyl peptide stimulates a release from specific granules of collagenase, apolactoferrin, lysozyme, and C5 cleaving protease, binding iron useful to bacterial metabolism. However, we did not address the possible mechanism for the flourishing platelet count noticed in three patients who had severely decreased platelets before GMDP treatment.

In promoting yoghurt as being therapeutic, Mechnikov was the first to imply that ingested lactobacilli could have beneficial effect on normal microflora of the gut. Identification of GMDP (a bacterial wall fragment of a probiotic) in human milk and later in amniotic fluid has suggested a systemic effect of the probiotics in the endogenous microflora.^{17,18} We also believe that there is a strong justification for the use of this bacterial peptidoglycan not only as a health promoting product with well established immunomodulating properties, but also as an apoptosis modulating agent capable of counteracting the systemic effect of any cytokines. As a result of the evolutionary process, the systemic effects of endogenous lactobacilli appear to contribute to the beneficial effects of the probiotic microflora on homeostasis.

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