DOI: 10.36347/sjams.2017.v05i02.058

Scholars Journal of Applied Medical Sciences (SJAMS) **ISSN 2320-6691 (Online)**
Sch. J. App. Med. Sci., 2017; 5(2D):594-599 **ISSN 2347-954X (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com

Original Research Article

Immunomodulatory effect of dietary intervention of probiotic *Lactobacillus helveticus* MTCC 5463 in geriatric volunteers of Gujarat, India

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Abstract: Aging is associated with impaired immunity. Geriatric population requires benefits of immunomodulatory capabilities of Probiotic strains as envisaged to improve quality of life. The aim of this study was to investigate the immunomodulatory effect of probiotic strain *Lactobacillus helveticus* MTCC 5463 in geriatric volunteers of Gujarat, India. In a randomized placebo controlled cross-over trial, a total of 58 subjects randomized into two groups, were given during the intervention period either the fermented drink containing MTCC 5463 (test group) and a fermented drink but without probiotic strain (placebo group). The volunteers consumed 200 ml of the product once a day for 30 days. Serum samples were collected from both the groups before and after the interventions and tested for Tumor Necrosis Factor-alpha (TNF-α), Interleukin-2 (IL-2) and Interferon-gamma (IFN-γ) for cell-mediated immunity by microwell-ELISA and IgG and IgM for humoral immunity by nephelometric tests. In placebo group, 2.9% of the subjects showed beneficiary effect on TNF-α levels; while among probiotic group, 27.2% had such benefit (p = 0.011). In placebo group, beneficiary effect for IL- 2 was observed in 3.77% of the subjects; while, such beneficiary effect was observed in 20% subjects among probiotic group (p = 0.016). There were three cases showing a beneficiary effect on IFN–γ among probiotic group; but there was no such case among placebo group for the comparison. There were no cases with beneficiary effects observed on IgG or IgM levels among the either group. The present study clearly showed a significant immunomodulatory effect on the TNF- α and IL-2 levels for the benefit of geriatric volunteers among probiotic group. There was however no significant beneficiary effect found on IFN–γ, IgG or IgM levels.
Keywords: Geriatric, immunomodulatory, probiotics

INTRODUCTION:

Probiotics are defined as live microorganisms that when being administered in appropriate dose, they confer a benefit of health to the receiver." [1]. Probiotics have established their efficacy as dietary factors which can regulate gastrointestinal functions and thereby imparting health benefits to consumers [2, 3]. Some of the health benefits which have been claimed for probiotics include the following: improvement of the normal micro flora, prevention of infectious diseases and food allergies, reduction of serum cholesterol, anti-carcinogenic activity, stabilization of the gut mucosal barrier, immune adjuvant properties, alleviation of intestinal bowel disease symptoms, and improvement in the digestion of lactose in intolerant hosts [1]. In addition to conferring health benefits to host; it has also been reported to provide benefits by modulating immune functions [2, 3]. These species are

able to survive in the human gastrointestinal (GI) tract and influence the host enteric microbiota, which subsequently modulate host immune-physiologic responses. In addition, numerous *in vitro*, animal model, and human studies also suggest that many of these same species are capable of producing a significant impact upon the host immune system as well. Many characterized probiotic effects are mediated through immune regulation, particularly through perturbations in the balance of pro-inflammatory and anti-inflammatory cytokines. Previous data has suggested that careful attention should be utilized when selecting strains to be used for probiotic therapy, as differences do exist in the immunomodulatory capacity of bacterial strains [4].

A progressive increase in the proportion and number of persons >60 year old has led to heightened attention to their physiologic and health needs, as well as to increased costs to the health care system [5]. Elderly people are especially prone to infection, as many physiological and immune responses as well as organ functions decline with age. [6]. The senescence of the immune system especially affects cell-mediated immunity with a decrease in lymphocyte proliferation capacity and Interleukin-2 (IL-2) production. However, antibody titres including mucosal IgA concentration following immunization are decreased as well. A decrease has also been observed in the ratio of mature to immature T lymphocytes and an increase in pro-inflammatory cytokine [7]. Subclinical inflammation is a frequent finding in the aging population that can be easily overlooked. The on-going inflammatory status is sustained by the abnormal production of pro-inflammatory cytokines, in particular Interleukin-6 (IL-6) and tumor necrosis factor (TNF). Elevated cytokines are known to be risk factors for age-associated pathology and mortality.

The foods containing probiotics and prebiotics are termed as synbiotic foods and are important part of functional foods. Functional foods such as probiotic products may have a particular application in this high-risk group, especially in terms of protection against entero- and urogenital pathogens, and perhaps also in the prevention of several age-related diseases [8]. Symbiotic foods have been found useful in various categories such as pregnant women and early infancy Children and healthy adults [9-11] the same has however not been well studied in elderly persons. The effects of two different probiotics in healthy adults have been evaluated, but these studies did not have a placebo group [12, 13]. In the analyses of the profiles of cytokines induced by some lactic acid bacteria, it has been observed that the most remarkable effect was the increase in the tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) and in the regulatory cytokine IL-10 for all the probiotic strains assayed. However, the induction of TNF-α by the probiotic bacteria would be necessary to initiate the cross talk between the immune cells associated with the lamina propria and the intestinal epithelial cells. IFN-γ would also play a physiological role and it has been demonstrated that this cytokine is necessary for the maturation of some immune cells, such as dendritic cells, and also controls their cellular proliferation at the intestinal level [1]. Several studies report that ingestion of probiotic bacteria or fermented milk products triggers spontaneous and enhance mitogen-induced production of IFN-γ and IL-2 by blood leucocytes. However; a single study reports increased levels of IFN-γ in serum after intake of yoghurt supplemented with lactobacilli and streptococci, whereas other studies are negative for IFN-γ and IL-2 [3]. In one study, the diet of a group of healthy elderly subjects was supplemented with *L. casei* for three weeks, and reported that the duration of ‘‘winter infections’’ (gastrointestinal or respiratory) was decreased by 20% compared with a control group [14].

Nutritional factors play important role not only in delaying normal ageing but also reducing the onset of age related diseases. The dietary intervention containing probiotics and prebiotics may provide ideal way to improve the age related complications but this aspect has not been adequately studied. In the present study, we have investigated the immunomodulatory effect of the probiotic strain *Lactobacillus helveticus* MTCC 5463 in healthy elderly geriatric volunteers of Gujarat, India; with specific objective of measuring its effect on the serum level of Tumor Necrosis Factor-alpha (TNF-α), Interleukin-2 (IL-2), Interferon-gamma (IFN–γ) as indicators of cell mediated immunity and IgG & IgM as indicators for humoral immunity.

MATERIAL AND METHODS:

The study was conducted between March 2012 and October 2014.

Origin and maintenance of bacterial strains

The indigenous probiotic strain *L. helveticus* MTCC 5463 and starter culture *Streptococcus thermophilus* MTCC 5460 [14-16] were maintained by the Department of Dairy Microbiology, Anand Agricultural University, India at −80°C as 15% glycerol stocks and were routinely cultured in de Man, Rogosa, Sharpe (MRS) and M 17 medium, respectively (Hi Media India Ltd., India).

Product preparation

The test product was a fermented probiotic drink (*Lassi*) with double toned milk fermented with culture containing *S. thermophilus* MTCC 5460 and *L. helveticus* MTCC 5463. The cultures were added at 0.1% each and incubated aerobically till an acidity of 0.8–0.9% lactic acid was obtained. Both the test and

placebo products contained sugar and prebiotic honey in a standardized ratio. The control product was made in a similar manner without the addition of MTCC 5463. The shelf life of the fermented drink was 28 days at 4°C, corresponding to the lower level (10^9 CFU/ml) of strain MTCC 5463.

Subjects

All subjects included in the study were between 64-74 years of age healthy volunteers, not suffering from any chronic illness. During pre-test counselling, they were explained about the study design and written consents were taken. Exclusion criteria included lactose intolerance, recent antibiotic treatment, frequent gastrointestinal disorders, or metabolic diseases. Participants included in the trial had no known allergies or intolerance to dairy foods. No antibiotics or laxatives were taken 2 months before or during the study.

Study Design and Intervention

The study was a randomized placebo controlled cross-over trial. Volunteers enrolled were randomly divided into two groups, Probiotic & placebo. During feeding period, 200 ml of product containing the test strain to one group and a similar product but without the test strains as placebo were fed regularly at the time of breakfast in morning for 4 weeks. Subjects of each group were given a washout period of 4 weeks before they were crossed over and included to the other group. The study protocol was approved by the Human Research Ethics Committee of H. M. Patel Center for Medical Care & Education, Karamsad (Gujarat). The trial was registered at CTRI on 22-11-2012, CTRI/2012/11/003136.

Study Product

A honey based product (in form of "Lassi") containing at least $1x10^8$ per ml of viable *Lactobacillus helveticus* MTCC 5463 was selected for feeding trial. A total $2x10^{10}$ cells were consumed in 200 ml per day by each subject for 4 weeks.

Blood samples

Blood samples were collected at 0, 4, 8 and 12 weeks before and after each intervention. TNF-α and IFN–γ were determined by ELISA method (Immunotech, France), IL-2 by ELISA method (Thermo scientific, USA); while IgG and IgM by nephelometric method (Spin react, Spain). These assays were carried out according to the manufacturer's instructions. Analytical sensitivity was 5 pg/ml, 0.08 IU/ml & <6 pg/ml for TNF-α, IFN-γ & IL-2 respectively. Complete blood count (CBC) was carried out using a semi-automated blood cell counter (SYSMEX KX 21) and ESR was carried out manually. All the tests were done at the Central Diagnostic Laboratory, Shree Krishna Hospital and Pramukhswami Medical College Karamsad, Anand, Gujarat, an NABL accredited laboratory.

Defining beneficiary effects

Beneficiary effects of Probiotic intervention on each parameter were defined. For TNF-α, a moderate increase of TNF-α level from normal value to maximum three times than the upper limit of normal range; as well as, a decrease from abnormally high value (more than ten times higher than the upper limit of the normal range) to at least half of the baseline abnormal level was considered as beneficiary. For IL-2, IFN–γ, IgG & IgM; a moderate increase of levels from sub-optimal or normal level to at least double of the upper limit of normal range was considered beneficiary.

RESULTS:

Initially, 112 subjects were enrolled in the trial, 36 had to withdraw because of antibiotic consumption. With 18 dropped out during the intervention period; 58 subjects completed the study, and hence included in final analysis. The baseline values of IL-2 in all the subjects under the present study; who were essentially healthy subjects of geriatric age group, were slightly higher than that found in various studies carried out elsewhere [17]. It was therefore decided to derive a region and the age-group-specific local range of normal value of IL-2. Base line values of such subjects, whose all other haematological and immunological parameters were within normal range, were taken into consideration for the same. Baseline values, as observed between 5^{th} and 95^{th} percentile cut-offs, 13.32 to 53.34 pg/ml, was considered as normal range for the present study. In each of the probiotic and placebo group, subjects with normal or abnormal baseline value were observed for significant benefit after intervention as defined for each parameter and % correction was calculated as shown in table 1.

As shown in table 1, significant beneficiary effect was seen among probiotic group on TNF-α (p = 0.011) and IL-2 ($p = 0.016$) levels. There were as such three such cases with sub-optimal level of IFN–γ among probiotic group and all of them showed a beneficiary effect. It would however not be possible to consider probiotic- intervention responsible for this change as there was no such case among placebo group for the comparison. Only 3 cases in placebo group showed improvement in IgG levels while no cases in intervention group showed any beneficial effect but the difference was not statistically significant (p=0.24 [Fisher's Exact Test]).There were no such cases with beneficiary effects observed on IgM levels among the placebo group or Probiotic group.

Table 1: Effect of Probiotic administration on the Immunological parameters of geriatric population (n=58)

Immunological parameters	Placebo group			Probiotic group			*P value*
	Subjects with normal or abnormal value(as defined for each parameter) before dietary supplement (a)	Subjects with Significant benefit observed (b)	% Correction (b/a X100)	Subjects with normal or abnormal value (as defined) before dietary supplement (c)	Subjects with Significant benefit observed (d)	% Correction (d/c X100)	
TNF-α	34	01	2.9%	44	12	27.2%	0.011
IL-2	53	02	3.77%	55	11	20%	0.016
IFN–γ	00	00	00	03	03	100%	NA
IgG	58	03	5.1%	58	00	00	0.24
IgM	58	00	00	58	00	00	NA

DISCUSSION:

The potential health benefits of consuming Probiotic products have been well established. In addition to its beneficial role in the diseases such as cancer, various infections, GI disorders, asthma, etc.; its effect on immune system has recently been studied in the population of various age-groups [7]. Elderly people represent a significant social issue requires immediate attention. The problem is compounded by increased health costs. People of old age encounter deterioration in functions of various systems such including digestive, hormonal and diminished immunity. Aging has also been associated with increased circulating levels of TNF-α. Moreover, older age has been associated with a concomitant increase of TNF-α, IL-6, acute phase proteins, suggesting an interrelated activation of the inflammatory cascade. Elevated cytokines are known to be risk factors for age-associated pathology and mortality. However, it is still a matter of debate whether this mild activation of pro-inflammatory mediators is the result of on-going sub clinical disease or is an integral part of the aging program itself [13].

Nutritional factors such as synbiotic foods have potential to influence body parameters which can delay the onset of age related disorders. *Lactobacillus helveticus* MTCC 5463 has been isolated and characterized indigenously. Its applications as Probiotic, including bile tolerance and cholesterol reducing properties have extensively been studied under *in vitro* and *in vivo* conditions and the hypo-cholesteromic effect of culture has been verified in human volunteers [18]. In the present randomized placebo-controlled trial, effects of daily consumption of a honey-based fermented milk-product with probiotic strain *Lactobacillus helveticus* MTCC 5463 for 4–weeks to geriatric volunteers on the serum level of several immunological parameters were studied.

In literature, there have been mixed reports on increase, decrease or no effect on these cytokines due to probiotic interventions, which appear to be strain-specific and dose-dependent as well [12]. In the present study, all the geriatric subjects included were healthy and not suffering from any acute or chronic illness at the time of inclusion. It was therefore expected that their haematological and immunological parameters were within normal range; where, appreciating any beneficiary effect of Probiotic intervention has been quite difficult. We therefore defined the beneficiary effect for each cytokine considering a significant deviation from the baseline value in each subject studied rather than calculating the mean deviation across all subjects.

This is particularly useful to appreciate effect on TNF-α level which is a pro-inflammatory cytokine, possesses both growth stimulating and growth inhibitory properties. Its low level maintains homeostasis and promotes the replacement of injured and senescent tissue by stimulating fibroblast growth. It has beneficial functions in the immune response to bacterial and certain fungal, viral, and parasitic invasions. TNF-α secreted by the macrophage causes blood clotting which serves to contain the infection. TNF-α causes necrosis of some types of tumors and promotes the growth of other types of tumor cells. High levels of TNF-α correlates with increased risk of mortality. Its normal range is 0-16 pg/ml [17]. As observed in the present study; a moderate increase has been beneficial for the host, but; as high levels of TNF-α correlate with increased risk of mortality, a significant decrease is beneficial to the host. IL-2 helps the immune system to produce T4 cells, key lymphocyte which helps both CMI and Humoral immunity. It plays a role in anti-inflammatory reactions, in hematopoiesis and in tumor surveillance and promotes the

proliferation of activated B-cells. It stimulates the synthesis of Interferon-gamma in peripheral leukocytes and also induces the secretion of IL 1, TNF-α and TNF-beta. Its normal range is 13-54 pg/ml [17]. The baseline values of IL-2 in all the subjects under the present study; who were essentially healthy subjects of geriatric age group, were slightly higher than that found in various studies carried out elsewhere. An age-group-specific local range of normal value of IL-2 was therefore derived. Base line values of such subjects, whose all other haematological and immunological parameters were within normal range, were taken into consideration for the same.

The present study, one of the very few studies carried out on geriatric population, clearly showed a significant immunomodulatory effect on the TNF-α and IL-2 levels for the benefit of the subjects among probiotic group in comparison to placebo group; which correlated well with some other studies performed *in vitro*, on animal models and in other age groups [3, 5]. IFN–γ has an antiviral and anti-parasitic activity. It inhibits the proliferation of a number of normal and transformed cells and synergizes with TNF-α and TNF-beta in inhibiting the proliferation of various cell types. Innate production of IFN–γ is critical in immunological defence against certain pathogen, such as intracellular bacteria, viruses and fungi. Normal range of IFN–γ is 0-0.89 IU/ml [17]. IgG is the most abundant immunoglobulin. IgG antibodies are predominantly involved in the secondary immune response and plays an important role in Antibody-dependent cell-mediated cytotoxicity (ADCC). IgM is involved in primary response. Normal range of IgG & IgM is 7.0-16.0 g/l and 0.3-2.4 g/l respectively. There have been some studies which have indicated effect on IFN-γ and IgG levels; no significant beneficiary effect was however found on IFN-γ, IgG or IgM levels in the present study [3]. It is further envisaged that the product needs to be investigated among patients with some kind of immunodeficiency disorders to further appreciate the immunomodulatory benefits.

CONCLUSION:

The present study clearly showed a significant immunomodulatory effect of a probiotic product containing *Lactobacillus helveticus* MTCC 5463 on the TNF-α and IL-2 levels of geriatric volunteers in comparison to that of placebo group. No significant beneficiary effect was however found on IFN-γ, IgG or IgM levels.

ACKNOWLEDGEMENT:

Authors wish to acknowledge the financial support received from Department of Biotechnology, Government of India, to this study (Project No.: No. BT/PR-14950/FNS/20/490/2010).

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