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Original Article

Community Medicine Section

Effect of Probiotic Dietary Intervention on Calcium and Haematological Parameters in Geriatrics

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ABSTRACT

Introduction: Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Sufficient calcium intake has been reported to support bone growth and prevent bone loss during the ageing process.

Aim: To determine the effect of *Lactobacillus helveticus* MTCC 5463 probiotic dietary intervention on serum calcium & haematological parameters in geriatric population.

Materials and Methods: Healthy volunteers with age ranging from 64-74 years were recruited from the nearby residential areas in and around Anand, Gujarat. Study duration was from 2012 to 2015. Of the 112 subjects initially enrolled in the trial, 36 withdrew before the intervention because of not matching with criterias. Of the 76 participants, 5 subjects (4%) under test group and 12 subjects (11%) under placebo left the study. We had 59 subjects who successfully completed a double blind cross over trial. Probiotic fermented milk products (in form of "Lassi") was prepared by supplementing toned milk with honey and fermenting with probiotic *Lactobacillus helveticus* MTCC 5463 and *Streptococcus thermophilus* MTCC 5460. The final product had at least 10^8 CFU/ml of viable *Lactobacillus helveticus* MTCC 5463 at the time of feeding. During feeding period, 200 ml of fermented product containing the test strain to one group and a similar product but without the test strain 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 was approved by institutional ethics committee.

Results: The socio-demographic and clinical profiles were similar at baseline. The mean (SD) calcium level improved significantly in test {9.36 (0.45) vs 8.45 (0.61), p<0.001}. No significant effect was observed with respect to haemoglobin & haematological parameters.

Conclusion: The well-documented probiotic *Lactobacillus helveticus* MTCC 5463 confirmed increase in serum calcium level but no effect on haematological parameters when administered to geriatrics.

Keywords: Feeding trials, Healthy volunteers, *Lactobacillus helveticus* MTCC 5463, Lassi

INTRODUCTION

There has been a steady rise in the share of elderly population (aged 60 years or above) in the total population over the decades. As against 5.6% in 1961, the proportion went up to 7.4% in 2001. According to official population projections, the number of elderly persons will rise to approximately 140 million by 2021 [1]. As the global population grows older, maintaining the health of elderly individuals proves to be increasingly more important and as the medical expenditure is highest during the later years of life, the ability to maintain the health of elderly individuals becomes critical for economic reasons. Geriatric group presents a paradoxical nutrition-related challenge. The physiological changes that occur with ageing should also be considered [2]. Calcium balance varies with different age groups. It is positive during growing age, zero in full grown adult and changes into negative balance with ageing consequently turning into loss of bone mass in geriatrics. Cacium plays a vital role in functions within the cells in all living organism. During ageing process, it has been reported that adequate calcium intake maintain the bone growth and avert bone loss. There have been growing evidence reporting that inadequate calcium intake may put in calcium loss because of decreased mechanical loading of the skeleton because of ageing [3].

Gut microbiot a can play an important role in improving the nutritional status in emaciated geriatrics. Modification of microbiota through increasing the variety and inclusion of probiotic in diet can be easy and a reasonable way to support beneficial microbial metabolism in geriatics [2].

As per the currently adopted definition by Food and Agriculture Organization and World Health Organization (2002), probiotics are, live microorganisms which when administered in adequate amounts confer a health benefit on the host. Few experimental studies have shown that consumption of probiotics in animal models has resulted in maintaining the serum calcium in the normal range. Sayed reported that goat's kids diet supplemented with probiotics lead to significant increase the Haemoglobin(Hb) concentration, Packed Cell Volume (PCV), erythrocyte count, and serum total protein [4]. Previous studies on the effect of probiotics on serum calcium and haematological parameters have been mostly performed in animal models.

Numerous human studies are being done on evaluation of the potential of probiotics by modulating the gut microbiota composition in improving the health of geriatics in the last decade [5-7]. There is very limited data available in this regard in humans, particularly in geriatrics. There are no comprehensive precise reports available on the effects of probiotics on haematological parameters and serum calcium level in geriatric population.

AIM

This study was undertaken to investigate the effect of dietary supplementation of probiotic *Lactobacillus helveticus* MTCC 5463 on calcium and haematological parameters and in geriatrics.

MATERIALS AND METHODS

Healthy volunteers with age ranging from 64-74 years were recruited from the nearby residential areas in and around Anand

city. Study duration was from 2012 to 2015. Total 112 geriatric participants were recruited by contacting them through various senior citizen forums, clubs and personal communication. The inclusion criteria included the wish to participate, no known health problem requiring drug therapy, no known allergies or intolerance to dairy foods. No antibiotics or laxatives were taken 2 months before or during the study. Exclusion criteria included lactose intolerance, recent antibiotic treatment, frequent gastrointestinal disorders, or metabolic diseases. During pre-test counselling, they were explained about the study design and asked to sign the consent form before recruitment.

The cultures, *Streptococcus thermophilus* MTCC 5460 and probiotic strain *Lactobacillus helveticus* MTCC 5463 were obtained from the culture collection of Dairy Microbiology Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India. Both the cultures were maintained by propagating in sterilized reconstituted skim milk (10% total solids) and stored at 5±2ºC. *Lactobacillus helveticus* MTCC 5463(earlier known as *L. acidophilus* V3) has been proven for its probiotic properties [8-11] and its whole genome is sequenced [12]. *Streptococcus thermophilus* MTCC 5460 is a curd isolate, fully characterized and studied extensively for its application in fermented milks preparation such as curd, lassi and yoghurt either alone or in combination with *lactobacilli*.

Probiotic fermented milk product (in form of "Lassi") was prepared by supplementing toned milk with honey and fermenting with probiotic *Lactobacillus helveticus* MTCC 5463 and *Streptococcus thermophilus* MTCC 5460. Standard procedure for probiotic lassi making with minor modifications of the procedure adopted by Patidar and Prajapati (1998) was used [13]. Fresh toned milk having minimum 3.0% fat and 8.5% Solids Not Fat (SNF) was used for preparation of probiotic lassi. Pre-warmed (45ºC) toned milk was supplemented with Honey 5% and sugar 5%. All the rate of additions were based on works carried out previously in the department. After proper mixing, a heat treatment of 90ºC for 5 minute was given to the mixture followed by cooling to 40 ± 2ºC. Active cultures of *S. thermophilus* MTCC 5460 and *Lactobacillus helveticus* MTCC 5463 were inoculated 1% v/v each and stirred well.

Lassi samples for microbial analysis were prepared by aseptically transferring 11g of sample to 99ml sterile phosphate buffer to obtain 1:10 dilution. Subsequently, 1 ml of the said dilution was used for making further dilutions in 9 ml phosphate buffer. Suitable dilutions were prepared and poured in a set of sterile petri dishes in duplicates. Microbial counts were determined as per standard procedures. *Lactobacilli* count on MRS agar (De Man et al., 1960) and *Streptococci* count on M17 agar [14]. The media and chemicals were purchased from Hi Media, Mumbai, India. All chemicals used in this study were of analytical grade.

The inoculated milk was incubated at 37 ± 2ºC till the acidity reached to 0.65 % lactic acid (approximately 6 hour). The set curd was immediately transferred to refrigerator to arrest further acid production. After allowing it to cool to 10ºC, the curd was broken by mechanical stirrer to get probiotic lassi. The product was then filled in polyethylene cups with lids and stored at refrigeration temperature (5±2ºC) for shelf-life study. The final product had at least 10^8 CFU/ml of viable *Lactobacillus helveticus* MTCC 5463 at the time of feeding. A similar product without probiotic *Lactobacillus helveticus* MTCC 5463 served as control/placebo.

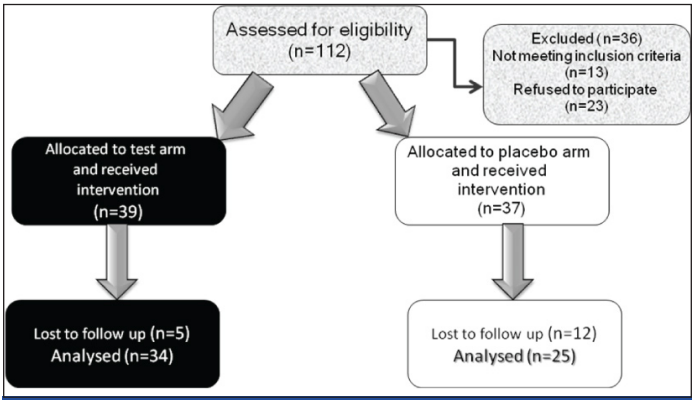
The subjects flow is given in [Table/Fig-1]. Of the 112 subjects initially enrolled in the trial, 36 withdrew before the intervention because of not meeting inclusion criteria, antibiotic consumption, physiological discomfort etc. Of the 76 subjects, 5 subjects (4%) under test group and 12 subjects (11%) under placebo left the study. At the end of the trial we had 59 subjects who successfully completed the trial. The male:female ratio was ensured as 1:1 using balanced randomization technique. The subjects were advised to maintain their lifestyle unchanged and not to use preparations made from unhygienic practices like street foods to avoid gastrointestinal disturbances. All subjects were examined by physician to check their health status and medical history. All relevant health parameters such as use of anti-microbials, history of surgery, gastrointestinal and immunological disorders and details of socioeconomic aspects were also documented. Volunteers enrolled were randomly divided into two groups, viz., probiotic and placebo.

The study was carried out as a cross over double blind randomized controlled study as shown in [Table/Fig-2]. During feeding period, 200 ml of fermented product containing the test strain to one group and a similar product but without the test strain 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 trial was divided into five consecutive periods: a pre-feeding period (2 weeks), followed by a feeding period (4 weeks), a washout period (4 weeks), a second-feeding period (4 weeks), and a final washout period (2 weeks).

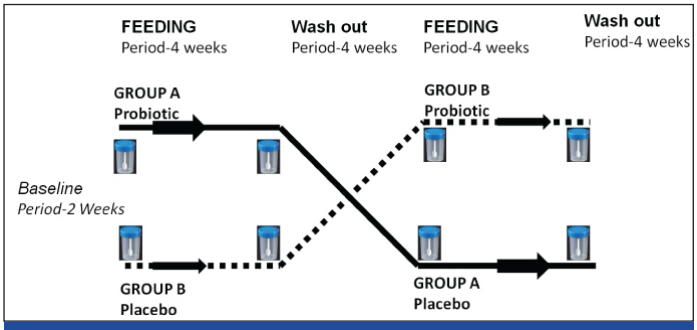
The amounts of energy nutrients were similar in both study groups. The research workers ensured that the participants consumed the product in their presence and maintained a diary with information on their diet and symptoms, if any.

The follow up of the subjects was done during and after the study to identify compliance problems. The subjects were instructed to keep themselves away from unhygienic foods; to consume lassi as per schedule & time; to stay away from infection; avoid to take medications which affect gut bacteria or lead side effects. Encourgement was given to subjects for discusssing thier queries or concerns regarding feeding trials or any other related issues.

This study was conducted in collaboration between two insititues, namely Anand Agricultural University (AAU) & HM Patel Centre for Medical Care and Education, Karamsad, Gujarat. It is approved by Human and Research Ethical committee of HM Patel Centre for Medical Care and Education (HMPCMCE:HREC/FCT/41/01) and Anand Agricultural University (AAU), Anand (AAU/DR/RES/DM/IEC/659/2011). The trial was registered at ICMR Clinical Trial



[Table/Fig-1]: Enrolment and randomization of participants.



[Table/Fig-2]: Study design and sample collection.

Particulars (n=76)		Mean	SD
Age		68.93	4.100
Number of children		2.8182	1.20140
		Frequency	Percent
Gender	Female	38	50.0
	Male	38	50.0
	Total	76	100.0
Marital status	Married	56	76.7
	Widow	12	16.4
	Widower	5	6.8
	Total	73	100.0
	Missing	3	
	Total	76	
Education	Diploma	2	3.0
	Graduate	12	18.2
	Higher Secondary	4	6.1
	Primary	24	36.4
	Secondary	15	22.7
	Uneducated	9	13.6
	Total	66	100.0
	Missing	10	
	Total	76	

[Table/Fig-3]: Socio-Demographic characteristics of the study population.

Registry (REF/2012/10/004135). Blood samples were collected at 0, 4, 8 and 12 weeks before and after each intervention.

Complete Blood Count (CBC) was carried out using a semi-automated blood cell counter (SYSMEX KX 21). Serum calcium level was determined by Cobas integra 400 plus (Roche Diagnostics, Germany) and ESR was done manually. These assays were carried out according to the manufacturer's instructions. Blood samples were taken from each volunteer immediately before and after each study period for calcium and various haematological parameters. Investigations were carried out from all blood samples collected at Central Diagnostic Laboratory, Shri Krishna Medical College Karamsad, Anand, Gujarat; an NABL accredited laboratory.

RESULTS

Socio-demographic characteristics of the study population in the current clinical trial are shown in [Table/Fig-3]. The mean age of the participants was 68.93 years. Most of the participants were married (76.7%) and either were house wives (40%), retired (22.9%), farmer (18.6%) or doing some jobs (17.1%). Very few participants (21.2%) had professional qualification.

The effect of probiotic dietary intervention on haematological and calcium parameters of the subjects with comparative evaluation of the same between probiotic and placebo groups are shown in [Table/Fig-4 & 5]. The mean (SD) calcium level improved significantly in probiotc group {8.45(0.61) vs, 9.36(0.45) p<0.001}. There was no significant difference of means for haemoglobin {12.41(1.52) vs,12.40(1.39) p=0.08} in probiotic and placebo {12.29(1.71) vs, 12.17(1.71) p=0.89} groups of volunteers before and after intervention. There was no significant difference of means for haematocrit between test as well as placebo groups of volunteers before and after intervention.

DISCUSSION

Healthy volunteers (n=76, 38 males and 38 females) with age ranging from 64-74 years recruited for feeding trial after applying selection crieterias but we had 59 subjects who successfully completed the trial at the end of study. The clinical profile revealed that the baseline values were similar for test (probiotic lassi) and placebo group (lassi without probiotic). The calcium level improved significantly in test group after feeding with probiotic lassi containing *Lactobacillus helveticus* MTCC 5463.

A study was done by Narva et al., on the effect of *Lactobacillus helveticus* fermented milk on acute changes in calcium metabolism in postmenopausal women. The effects of probiotic on bone in 20 postmenopausal women with a mean age of 65 years and mean Body Mass Index (BMI) of 26 revealed the increase in serum calcium levels [15]. Asemi and Esmaillzadeh conducted controlled clinical trial to determine the effects of daily consumption of probiotic yoghurt on serum calcium and iron levels among Iranian healthy pregnant women. Consumption of probiotic yogurt resulted in maintaining serum calcium levels compared with the conventional yogurt (p = 0.01) [16]. A study on male rats with osteoporosis, reported that *Lactobacillus helveticus* fermented milk increased bone mass density and bone mass composition which were measured by using dual-energy X-ray absorptiometry (DEXA) [17]. *Lactobacillus helveticus* from fermented milk whey (LBK-16H) confirmed an increase in bone mass density *in vitro* study, where the results of calcium accumulation measured in osteoblast cultures. It found that *Lactobacillus helveticus* (LBK-16H) can increase osteoblast activity [18]. A study done by Q. Qu et al., reported increased bone mass density by *Lactobacillus helveticus* through its effects on osteoblasts and osteoclasts, which were measured by culturing bone marrow of mice derived from osteoblast and osteoclast precursor [19].

Characteristics	Placebo group			Test group			Overall				
	N	Mean	Std. Deviation	N	Mean	Std. Deviation	N	Minimum	Maximum	Mean	Std. Deviation
Total leucocyte count (04-10x1000/ul)	69	6.61	1.68	69	6.51	1.34	138	3.80	11.80	6.56	1.51
Red blood cell count (4.5-5.9mill/cmm)	71	4.60	0.62	61	4.75	0.46	132	2.84	6.16	4.67	0.56
Haemoglobin(13-17 g/dl)	67	12.12	1.72	66	12.42	1.41	133	6.70	15.40	12.27	1.58
Haematocrit(36-53%)	61	39.48	3.08	65	39.59	3.26	126	30.00	46.40	39.54	3.16
Mean corpuscular volume (80-100 fl)	62	85.28	8.66	68	84.64	9.44	130	60.70	106.4	84.94	9.04
Mean corpuscular hb (25-35picogm)	61	27.26	3.93	70	26.67	4.65	131	15.40	36.40	26.94	4.32
Mean corpuscular Hb concen.(31-37 g%)	64	31.38	2.05	70	31.05	2.25	134	23.80	35.20	31.21	2.15
Red cell distribution width(39-46 fl)	52	45.40	2.89	70	46.94	4.41	122	39.80	64.70	46.28	3.90
Platelet count (150-410x1000/ul)	71	233.51	69.74	69	239.48	77.71	140	8.00	410.00	236.45	73.57
ESR(upto 30mm)	61	20.00	12.92	64	19.31	12.82	125	2.00	72.00	19.65	12.82
White blood cell series P	70	57.56	10.09	70	58.17	10.58	140	22.00	77.00	57.86	10.31
White blood cell series L	70	31.97	7.34	70	30.94	7.59	140	16.00	50.00	31.46	7.45
White blood cell series E	55	4.62	1.72	57	5.11	2.94	112	0.00	14.00	4.87	2.42
White blood cell series M	70	3.00	1.84	70	3.34	1.54	140	0.00	10.00	3.17	1.70
Calcium (8.6-10.2mg/dl)	71	8.64	0.76	70	9.40	0.46	141	7.06	11.37	9.02	0.73

[Table/Fig-4]: Effect of intervention on haematological parameters and calcium of the subjects.

Characteristics	Probiotic group				Placebo			
	N	T0	T30	p-value	N	P0	P30	p-value
Haemoglobin (13-17 g/dl)	58	12.41±1.52	12.40±1.39	0.89	65	12.29±1.71	12.17±1.71	0.08
Haematocrit (36-53%)	57	38.89±3.45	39.57±3.13	0.00	59	39.88±3.40	39.61±3.06	0.24
Calcium (8.6-10.2mg/dl)	62	8.45±0.61	9.36±0.45	<0.001	69	9.56±0.68	8.65±0.76	<0.001

[Table/Fig-5]: Comparison of haematological parameters and calcium before and after intervention

A study by K. Sjögren et al., reported that probiotics in the gut have a significant effect on bone health by reducing intestinal inflammation and increases bone mass density [20].

Mechanism of *L. helveticus*-fermented milk could be by reducing PTH and raising calcium levels in the blood serum [15]. *Lactobacillus helveticus* produces the proline-containing peptides, isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP), which may induce a greater availability of minerals [21]. We didn't study the mechanism of effect of *Lactobacillus helveticus* in human beings in this trial. Previous studies on the effect of probiotics and bone have all confirmed that probiotics can increase BMD and BMC and help reduce osteoporosis by different ways [22].

There was no significant difference in total leukocytes count, red blood cell count, Mean Corpuscular Volume (MCV), platelet count and erythrocyte sedimentation rate in test versus placebo group. There was no significant difference found with respect to haemoglobin and haematocrit in placebo versus control group as shown in [Table/Fig-5] in result section. The mean of heamoglobin was 12.12 ±1.72 in placebo group and 12.42±1.41 in test group. A study by Pătrascanu et al., of the probiotic administration in pregnant sows found insignificant fluctuation and within the normal physiological ranges in Hb, PCV and red blood cells values [23]. A study of effects of probiotic on haematology and biochemical parameters in mice by Salahuddin et al., found that total erythrocyte count and haemoglobin concentration were significantly ($p<0.01$) higher in treated group than in controls [24]. A Study by Alkhalf et al., of effects of commercial probiotic (Bactocell®) in broiler chickens study revealed that there was no significant change for Hb and PCV concentrations among different groups at all studied times [25]. We did not find any study showing the effect of probiotic on hematological parameters in human being during literature search. Probiotics demonstrated potential effects on metabolism through different mechanisms with outstanding results in the animal model. The results of this study confirmed that geriatric populations who suffered from low calcium level are potential targets to consume probiotic for increasing calcium. Participants reported improved bowel movement & a generalized well-being and liked the taste of probiotic lassi.

LIMITATION

Geriatric volunteers were recruited at one time point for study might not represent the variation that exists in geriatrics over a period of time. Wide inter-personal variations were observed among subjects in context of sociodemographic profile which might have some impact on diet consumed by subjects and so on results. This study presents few important parameters of the study. Other parameters were also studied but are not part of this paper.

CONCLUSION

The well-documented probiotic *Lactobacillus helveticus* MTCC 5463 confirmed increase in serum calcium level which is well tolerated and liked by geriatric volunteers. All strains of *L. helveticus* do not affect calcium level. There was no effect found on haematological parameter. This study can be replicated in future among different age group individuals having clinical bone related problems which might expolore the new facet of treatment. This is the first study on Indian geriatric population which can be used as base for further research in future.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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