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Original Article

INFLATED LEVELS OF SCFA, *BIFIDOBACTERIA* AND *LACTOBACILLUS* IMPROVES THE STATUS OF PRE HYPERTENSION AND TYPE 2 DIABETES MELLITUS IN SUBJECTS RESIDING IN NORTH EAST INDIA–A RANDOMIZED CONTROL TRIAL WITH SYNBIOTIC SUPPLEMENTATION

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ABSTRACT

Objective: The study was undertaken to determine the effect of synbiotic supplementation on glycemia, gut health and Short chain fatty acid (SCFA) levels in pre hypertensive type 2 diabetic adults.

Methods: 60 pre hypertensive adults with type II NIDDM aged 35-55yrs were purposively selected from Sun Valley Hospital, form north east state of India, and based on the informed consent given by the subjects they were randomly divided into Control (N=10) and Experimental groups (N=25). The experimental group was supplemented with 1 gm of freeze dried synbiotic product (2 species of *Lactobacillus, Bifidobacterium* each, one species of *Streptococcus*, one species of yeast along with 300 mg Fructo oligosaccharide) daily to be taken along with meals, for 45 days. Glycemic parameters were determined using an enzymatic method. DNA was isolated from the stool samples and was quantified for determination of LAB, Bifidobacteria and Enteric pathogens using PCR techniques. SCFA was determined using Gas Chromatography Mass Spectrophotometer (GCMS).

Results: Intervention with synbiotic supplementation resulted in a significant reduction in FBS, PP_2BS, HbA1c, by 3.3%, 6.7%, 14%, respectively along with a significant increment in butyrate (547.4%) and propionate (310%) levels. Gut health of the subjects improved significantly as indicated by increased colonization of Bifidobacteria (131.6%) and LAB (32.6%) and a significant reduction in enteric pathogens (44.6%).

Conclusion: Daily intake of 1 g freeze dried synbiotic product is an attractive therapy to improve glycemia, gut health which may be implicated by increased production of SCFA.

Keywords: Type 2 Diabetes Mellitus, Pre-hypertension, Synbiotic, Gut health, Glycemia, SCFA.

INTRODUCTION

India leads the world with the largest number of diabetic subjects earning the dubious distinction of being termed the "diabetes capital of the world". Type 2 diabetes mellitus along with hypertension epidemic in India is a result of societal influences and changing lifestyles. In the recent times, science based strategies have been established to prevent CVDs using functional foods. Amongst these the less explored foods include the probiotics, prebiotics and the synbiotics. Few scattered studies are available which have shown synbiotics can serve a great potential as agents to improve or maintain a balanced intestinal microflora to enhance health and well-being. They can be incorporated into many foodstuffs which may influence the functioning of gut microbiota and thereby improve glycemic index, SCFA levels and thus help to assist diabetic and hypertension control. Therefore, the present study was undertaken "To study the inflated levels of SCFA, *Bifidobacteria* and *Lactobacillus* improves the status of pre hypertension and type 2 diabetes mellitus in subjects residing in North east India with synbiotic supplementation.

MATERIALS AND METHODS

60 pre hypertensive adults with type II NIDDM aged 35-55yrs were purposively selected from Sun Valley Hospital, form north east state of India, and based on the informed consent given by the subjects they were randomly divided into Control (N=10) and Experimental groups(N=25). The experimental group was supplemented with 1 gm of freeze dried synbiotic product (2 species of *Lactobacillus, Bifidobacterium* each, one species of *Streptococcus*, one species of yeast along with 300 mg Fructo oligosaccharide) daily to be taken along with meals, for 45 days. Glycemic parameters were determined using an enzymatic method. DNA was isolated from the stool samples and was quantified for determination of LAB, Bifidobacteria and Enteric pathogens using PCR techniques. SCFA was determined using Gas Chromatography Mass Spectro Photometer (GCMS).

Biochemical evaluation and assay methods

After the overnight fast, venous blood sample was collected in clean, sterilized vacuum containers and allowed to stand at room temperature for 15 minutes. For determination of postprandial blood glucose, blood was collected after 2 hours of a meal. Serum was immediately separated and stored at-80 ° C until analysis. The blood was then analyzed for Glucose and Total Lipid Profile using standardized kits.

Fasting blood glucose (FBS) and postprandial blood glucose (PP2BS)

FBS was estimated using enzymatic reference method with hexokinase (Reinauer *et al.* 2002)[1]. Hexokinase (HK) catalyzes the phosphorylation of glucose by ATP to form glucose 6 phosphates and ADP. To follow the reaction, a second enzyme, glucose 6 phosphate de hydrogenase (G6PDH) is used to catalyze oxidation of glucose 6 phosphates by NAD^+ to form NADH. The concentration of the NADH formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at 340 nm.

$$\text{D-Glucose + ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate+ADPD}$$

$$\text{Glucose- 6 phosphate + NADH} \xrightarrow{\text{G6DH}} \text{D-6-phsphogluconate +NADH}$$

Glycated Hemoglobin (HbA_{1c})

HbA_{1c} was quantified assayed using IFFC and FDA approved automated dedicated high performance liquid chromatography (HPLC) method (IFFC 2002). The principle involved ion exchange of HPLC. The samples are automatically diluted on the d-10, injected into the analytical flow path, and applied to the analytical cartridge. The d-10 delivered a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobin is separated based

on their ionic interactions, then pass through the flow cell of the filter photometer, where change in the absorbance at 415 nm is measured. The d-10 software performs reduction of raw data collected from each analysis; two level calibrations are used for quantization of HbA_{1c} values. A sample report and a chromatogram are generated for each sample.

The A_{1C} area is calculated using an Exponentially Modified Gaussian (EMG) algorithm that excludes the labile A_{1C} and carba mylated peak areas from the A_{1C} peak area. HbA_{1c} covers all fractions; this includes labile $HbA_{1a,}$ HbA_{1b} and $HbA_{1c.}$ The former two fractions are labile and hence do not represent the stable or long term change. HbA_{1c} represents the true long term glycemic control.

Table 1: Cut offs for HbA_{1c}

Classification	HbA_{1c} value
Good	≤6
Borderline	7-8
Poor	>8

American diabetes association 2007 standards

Determination of the gut microbiota

The gut microbiota was determined with respect to the microorganisms-*Lactic acid bacteria*, *Bifidobacterium* and Enteric pathogen.

The steps involved in the determination of the fecal flora were:

Collection of fecal sample and isolation of DNA for enumeration of micro-organisms

The stool samples from the subjects were collected in an air tight sterile containers kept with dry ices and stored at-80˚C until analysis.

Extraction of DNA using QIAagen kit

180-220 mg of stool was weighed in 2 ml tube and place in 2 ml tube (placed in ice) for thawing. Thereafter, 1.4 µl of ASL buffer was added to the stool sample and vortexed for 1 minute until thoroughly homogenized. The suspension was heated for 5 min at 70˚C (95˚C for gram positive bacteria) and vortexed for 15 second and centrifuged the sample at 14,000rpm for 1 minute to pellet stool sample. 1.2 ml of the supernatant was pipetted into a new 2 ml micro centrifuge tube discard pellet. 1 inhibit Ex tablet was added to each sample and vortexed for 1 minute to allow inhibitors to absorb to the inhibitor Ex motrix. The sample was centrifuged at 14000rpm for 3 minutes to pellet inhibitors bound to inhibitors Ex. Supernatant was taken into a new tube and centrifuge at 14000rpm for 3 minutes. 15 µl protinase K was taken into a new tube and added 400 µl of supernatant to it. 400 µl of AL buffer was added and vortexed 15 sec. 400 µl of ethanol was added to the lysate and mix by vortexing. Complete lysate was added from previously step to the QIAamp column and centrifuged at 14000rpm for 1 minute place the QTAamp spin column in a new 2 ml collection tube and the filtrate was discarded. 500 µl of AW1 buffer was added to the spin column and centrifuge at 14000rpm for 1 minute. The QIAamp column was placed in a new 2 ml collection tubes and the filtrate was discarded. 500 µl AW1 buffer were added to the QIAamp spin column and centrifuged 3 minute and the filtrate was discarded. The QIAamp spin column was transferred into a new tube and 100 µl AE buffer was added on to the QIAamp membrane. Incubation was done at room temperature for 1 minute centrifuged at 14000rpm for 1 minute and the DNA was eluted.

RNase treatment protocol

The precipitated was re suspended and dried genomic DNA in 500 µl of 1×TE buffer. 15 µl of 1µg/µl RNase was added and incubated in a 65˚C water bath for 10 minutes. 0.4 volume of 7.5M ammonium acetate was added to precipitate the RNase A, the tube was flicked several times to mix. Let stand the sample for 30 minute at 4˚C, spin at 13,000rpm for 30 minute, at 4˚ C and carefully removed supernatant to the new tube and the supernatant was saved. DNA was precipitated with 1 ml of cold 100% ethanol, mix gently for 1 minute and let it stand at -20˚C for 30 minute. Spin at 13,000rpm for 20 minute at 4˚C dicant and discarded the ethanol and the sample washed with 70% ethanol. 500 µl of 70% ethanol was added and centrifuged it for 10 minute. Pellet was removed and 500 µl of 70% ethanol was added in the same test tube. Again the pellet was removed and dried it in laminar to get the DNA. All sample were put in the refrigerator at -20˚C for 48 hours.

Gel formation procedure

Stock solution of TAE buffer is 50X TAE and working concentration is 1X. 1lt. of the total solution was transferred to the electrophoresis tank. Pipette out 1 ml of 50X TAE buffer to 100 ml of the above solution. 0.8g of agarose was added with 1 µl of Ethidium bromide and dissolved and was poured to the casting tray and was allowed to cool. 1 µl of the DNA samples are loaded into the wells of an agarose gel using a using a pipette. After 30 minutes DNA formation is visualized using bioDoc-It™ Imaging system.

The Thermo Scientific Nano Drop™ 1000 Spectrophotometer measures 1 ul samples with high accuracy and reproducibility. The full spectrum (220 nm-750 nm) spectrophotometer utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place. A 1 ul sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1 mm and 0.2 mm paths. The instrument is controlled by PC based software, and the data is logged in an archive file on the PC. With the sampling arm open, pipette the sample onto the lower measurement pedestal. Close the sampling arm and initiate a spectral measurement using the operating software on the PC. The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement made. When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. And calculate RNase impurity in the ratio of 260/280.

A critical factor in PCR is the selection of appropriate primers for maximal efficiency and specificity. Primer specificity is affected by a number of factors, including sequence, primer location, and the PCR system used. General primer-design rules for PCR are also applicable to avoid mispriming and primer–dimer formation. Therefore, the following primers used for the study are:

Primer	Primer seq (5'-3')	Annealing temp
Lac-F	AGCAGTAGGGAATCTTCCA	55·C
Lac-R	CACCGCTACACATGGAG	
BifidoF	CTCCTGGAAACGGGTGG	60·C
Bifido-R	GGTGTTCTTCCCGATATCTACA	
Uni 331F	TCCTACGGGAGGCAGCAGT	60·C
Uni 797R	GGACTACCAGGGTATCTATCCTGTT	
Enter F	TGCCGTAACTTCGGGAGAAGGCA	60·C
Enter R	TCAAGGACCAGTGTTCAGTGTC	

Fig. 1: List of primers with sequencing

The sample runs in the Master cycler nexus gradient model PCR. The concentration of the bacteria is determined.

Procedure of PCR technique

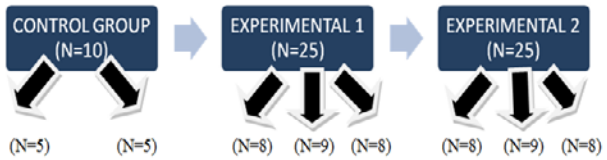
Metagenomic DNA was extracted from the stool samples using the Qiagen kit. PCR amplification of V6-V8 region of bacterial 16S rDNAwas carried out using the genus specific primer for Bifidobacterium, Lactobacillus, Enterococcus and an universal primer for overall bacteria. PCR reaction was performed in a 10 µl reaction volume in a thermal cycler (Mastercycler Nexus gradient, Eppendorf, Germany). Each PCR reaction constituted a final concentration of 1x standard Taq buffer, 1.75 mM of $MgCl_{2,}$ 200µM of dNTPs, 0.2µM of each primer, 0.5U of Taq DNA polymerase (Sigma

Aldrich, USA) and 25ng of template DNA. PCR conditions were, initial denaturation at 94℃ for 5 min. followed by 25 cycles of denaturation at 94˚C for 30 sec., annealing at for 30 sec., extension at 72˚C for 30 sec. and a final extension at 72 ˚C for 7 min. PCR products were separated in a 1.5% agarose gel along with 100 bp DNA size and 50 bp DNA mass ladder for size and mass calculation, respectively and visualized under BioDoc-It Imaging System (UVP, USA). B and quantification was performed using Image J software comparing with the mass ladder.

Determination of Short-chain fatty acid

Sample preparation

The samples were pooled by the following method



GC-MS was performed on the Shimadzu GC 2010 plus with triple quadrupole MS (TP-8030) fitted with EB-5MS column (length-30 m, thickness-0.25 µm, ID-0.25 mm). Temperature programme was started at 60 °C, held for 2 mins and then raised finally to 250 °C @ 4 °C/min at which it was held for 15 mins. Injection temperature was 250 °C and column flow rate was 1.0 ml/min with He as carrier gas. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV with an ion source temperature of 230 °C and a continuous scan from 45 to 800 m/z. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST) library.

RESULTS

As indicated in table 2, intervention with synbiotic supplementation resulted in a significant reduction in FBS, PP_2BS, HbA1c, by 3.3%, 6.7%, 14%, respectively along with a significant increment in butyrate (547.4%) and propionate (310%) levels. Gut health of the subjects improved significantly as indicated by increased colonization of Bifidobacteria (131.6%) and LAB (32.6%) and a significant reduction in enteric pathogens (44.6%).

Table 2: Impact of Synbiotic supplementation on glycemic index, gut health and SCFA

Parameters	Groups	Mean±SD	t stat	Paired two tail	% difference
	Control Pre	141.5±17.56	3.61	0.005	4.24↑
FBS	Control Post	147.5±17.55			
	Exp Pre	154.12±17.59	8.11	**2.43*****	**4.67↓**
	Exp Post	146.92±16.51			
	Control Pre	170.6±15.19	3.36	0.008	2.63↑
PP2BS	Control Post	175.1±16.73			
	Exp Pre	176.2±18.66	12.06	**1.11*****	**7.49↓**
	Exp Post	163±16.91			
	Control Pre	7.48±0.407	1	0.34	0.13↑
HbA1c	Control Post	7.47±0.402			
	Exp Pre	7.7±0.65	11.27	**4.5*****	**14.8↓**
	Exp Post	6.5±0.50			
	Control Pre	49.25±14.95	2.55NS	0.23	64.24↓
Butyrate	Control Post	17.61±2.53			
	Exp Pre	37.73±13.81	4.86	**0.03**	**547.41↑**
	Exp Post	244.27±76.16			
	Control Pre	58.36±5.27	3.2NS	0.19	41.79↓
Propionate	Control Post	33.97±5.41			
	Exp Pre	46.04±20.61	3.9	**0.05***	**310.03↑**
	Exp Post	188.77±75.04			
	Control Pre	176.85±90.68	2.54NS	0.03	23.19↓
Lactobacillus	Control Post	135±7.81			
	Exp Pre	191.55±183.7	5.36	**0.0001*****	**32.61↑**
	Exp Post	254.03±231.52			
	Control Pre	177.64±70.58	0.43NS	0.67	3.52↓
Bifidobacteria	Control Post	171.40±85.80			
	Exp Pre	131.14±56.18	3.54	**0.001*****	**131.61↑**
	Exp Post	303.75±252.64			
	Control Pre	223.82±232.72	2.42NS	0.03	5.91↑
Enteric pathogen	Control Post	356.01±263.15			
	Exp Pre	248.77±222.05	4.43	**0.0001*****	**49.6↓**
	Exp Post	125.33±130.90			

* Correlation is significant at the 0.05 level (2-tailed), *** Correlation is significant at the 0.001 level (2-tailed)

DISCUSSION

In the present study supplementation of synbiotic product (1 gm) for a period of 45 days to pre hypertensive type 2 diabetic adults brought about a significant reduction in glycemic status, fecal enteric pathogen along with a significant increment in beneficial gut microbiota and SCFA.

Previous research has also shown that the synbiotic supplements may lower glucose levels in human. A higher reduction in fasting blood glucose levels (38.89%) was observed in individuals who consumed the synbiotic shake [2]. A study conducted [3]. On adults supplemented with fermented Dahi fortified with probiotic bacteria plus inulin which showed a signi ficantreduction in fasting blood glucose by 7.78%.

Present study also revealed significant rise in LAB and *Bifidobacteria* by 32.6% and 131.6% respectively. Human trials conducted by Giovanni M *et al.* in 2010 elicited that oligosaccharides that are fermented by colonic microflora enhanced the growth of beneficial commensal organisms like *bifidobacteria* and *lactobacillus*. At the end of the treatment period, after eating the prebiotic (20 gm) for 90days a significant increment in the colonization of *Bifidobacteria* (10.78%) and *Lactobacillus* (30.51%) was observed [4].

Earlier studies have also shown that FOS supplementation led to increase in SCFA formation in the gut and related beneficial effects on the host metabolism like glucose tolerance [5, 6]. SCFA, particularly acetate, propionate, and butyrate, are the dominating end-products of bacteria fermentation in the large bowel and could

affect the transport processes of colonic epithelial cells, energy metabolism, growth, and cellular differentiation [7, 8].

During the fermentation process, a number of short chain fatty acids are produced which enter the portal blood stream where they are utilized by the liver. Propionate has been reported to inhibit synthesis of lipid, thereby lowering the rates of triacylglycerol secretion while butyrate is taken up by the large intestinal cells and provide a source of energy for human colon epithelial cells [9]. In the present study, a significant increase in fecal butyrate by 547% and propionate by 114% was observed in the synbiotic supplemented group. SCFAs and lactate in mice cecum were significantly increased (P<0.05) by intake of oligosaccharides [10]. The possible mechanism proposed for the reduction in the glycemic parameters when supplemented with synbiotic food could be due to the influence of SCFA produced as a result of increase colonization of Bifidobacteria and Lactobacillus which may have raised the gut incretins namely GLP-1 and GIP [11, 12].

A positive correlation was observed between the SCFA levels and enteric pathogen in the gut. The mechanism for this is not entirely understood but it may be related to acidic pH generated by the intense production of SCFA inhibiting the growth of pathogenic bacteria [13]. Butyrate and butyrate producing microbes have been associated with gastrointestinal health in human [14, 15]. In the present study a positive correlation of 0.6 has been observed between SCFA levels and *Bifidobacteria* indicating the role of *Bifidobacteria* in raising the levels of SCFA.

In our study, we have found the significant reduction in enteric pathogens post supplementation with synbiotic and this can be explained by increase in butyrate and propionate. The possible mechanism for this could be reduced local pH rendering the intestinal milieu acidic [16, 17]. This effect eliminates the pathogenic bacteria from gaining access to the gut. Another mechanism involves enterobacteriaceae binding to the glyco lipid binding receptor namely gangliotetraslyceramide (GAI), sites on the intestinal brush border. Also *Bifidobacteria* has been reported to inhibit adhesion of pathogenic strains to the GAI molecule and prevent binding of pathogens to intestinal epithelial surfaces [18, 19], reported that B. *lactis* DR10 adhere to intestinal epithelial cell and inhibits colonization by *E. Coli* O157:H7 by producing proteineous substance. This complex system of the gut when exposed to synbiotic has ultimately helped in improving the glycemic status of the subjects.

CONCLUSION

Synbiotic (1 gm) supplementation can prove to be a novel therapy for the management of pre hypertension type 2 diabetes, as it not only helps in glycemic control but also helps in increasing the colonization of beneficial gut microbiota, improving metabolic control and thereby reducing the risk of development of secondary complications of diabetes.

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