CHAPTER 4

Molecular Fingerprinting of the feacal microbiota in relation to high fructose induced metabolic disorder.

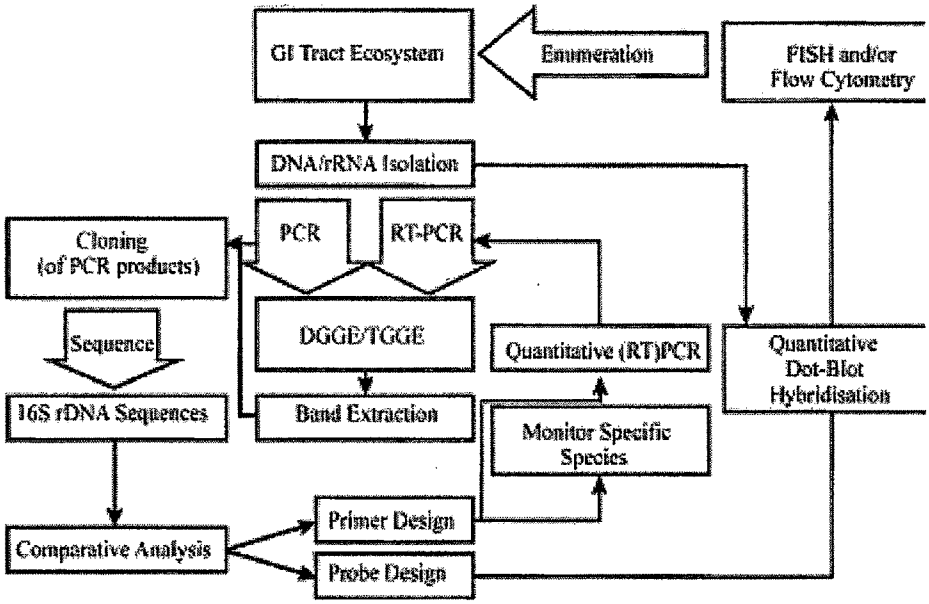
4.1 Introduction

The mammalian body harbours large microbial populations on the skin, in the oral cavity and in the genital and GI tracts. The number of bacteria is thought to exceed the number of mammalian cells by as much as a factor of 10, and a majority of bacteria are present in the GI tracts (Backhed *et al.*, 2005). Our knowledge and understanding about gene and disease is now focused towards microbial community that reside in human gut (Yang *et al.*, 2009; Benson *et al.*, 2010). The microbial community reside in human gut manipulates gut physiology and thereby influences the health. The change of human gut microbial community is associated with obesity, diabetes, hypertension, and gastrointestinal disorder. Therefore, gut bacteria has become very significant areas of research especially for dietary related metabolic disorders. The environment in various sections of the GI tracts is constantly changing; this is reflected by the variations in the composition and population of bacterial community in various sections of the human and infants' GI tracts, and faeces (Gibson *et al.* 1998; Mueller *et al.*, 2006; Vaishampayan *et al.*, 2010). More than 90% of bacteria present in gut are unculturable. A cultural technique only contributes 10% of entire gut microbiota (Sghir *et al.*, 1999). Cultural approach contributes significantly in understanding the microbial diversity but is limited by lack of precision, low level of reproducibility and labour intensive and thus limiting the effectiveness for analysing the large number of community sample for diversity study. *The development of molecular techniques to investigate microbial diversity has provided the microbiologist with a vast array of new techniques to investigate human intestinal microflora. Various molecular approaches are available to analyse the microbial diversity.* (Vaughan *et al.*, 2000; Keeley et. al., 2009) **(Fig. 4.1; Table 4.1).**

Table 4.1: Potential use and drawbacks of methods for analysis of complex microbial communities (Vaughan *et al.*, 2000; Keeley *et al.*, 2009).

Method	Uses	Drawbacks
Culturing	Isolation; "The ideal"	Not representative; slow
16S rDNA sequencing	Identification	Large scale cloning is laborious
DGGE/TGGE	Rapid comparative analysis; identification by band extraction; detection of specific groups	Semi-quantitative
T-RFLP	T-RFLP Rapid comparative analysis; very sensitive; potential for high throughput	Semi-quantitative; identification only possible with clone library
SSCP	Rapid comparative analysis	Semi-quantitative; identification only possible with clone library
FISH	Detection; enumeration; comparative analysis possible with automation	Requires probe design; laborious without automation
Dot-blot hybridisation	Detection; estimates relative abundance	Requires probe design; laborious
FISH/Flow cytometry	Enumeration; potential for high throughput	Under development

Fig. 4.1: Flow chart of molecular approaches used to analyse the human intestinal microbial community (Vaughan *et al.*, 2000).



Metagenomic approach is a comprehensive study of nucleotide sequence, structure, regulation, and function, providing a picture of the dynamics of complex microbial communities (Tringe *et al*, 2005). This approach can identify the diversity, but not the relative numbers, of each species residing in that particular environment. Metagenomic approaches will provide a wealth of sequence information from single ecosystems, but they are not suitable for high-throughput monitoring. Denaturing gradient gel electrophoresis (DGGE) and related fingerprinting techniques have proven their power in comparing and monitoring ecosystems at the 16S rRNA gene or functional gene level (Ercolini, 2004; Licht *et al.*, 2006). DGGE and Temperature gradient gel electrophoresis (TGGE) are gel-electrophoretic separation procedures for double stranded DNA's of equal size but with different base-pair composition or sequence (Muyzer and Smalla, 1998). In principle, the methods are sensitive enough to separate DNA's on the basis of single point mutations (Sheffield *et al.*, 1989).

The composition of the diet influences the bacterial flora of the GIT. Diet and frequency of feeding influences the distribution of bacteria within the GIT of both man and animals (Sekirov *et al.*, 2010). Amylase resistant starch (ARS) that is low digestibility starches alters the intestinal microflora of rats (Martínez-Puig *et al.*, 2007). The mucosa associated flora of the large intestine is important in determining mucosal function. Microbial population on the mucosal surface depend on the prebiotic carbohydrates oligofructose and inulin (Langlands *et al.*, 2004). The dietary analysis method provides a new tool for establishing relationships between diet and disease and indicates a potentially therapeutic diet for various disease i.e. UC (ulcerative colitis), diarrhoea, obesity and many gastrointestinal disorder (Elizabeth *et al*, 2005).

Dietary sugar such as fructose and sucrose is readily absorbed and rapidly metabolized by human liver. In 1977, average intake of fructose sugar in united nation was amount approximately 37 grams per day, largely from fresh fruits (Park and Yatley, 1993). Westernization of diet has resulted in significant increase in added fructose, leading to typical daily consumptions amounting to $\geq$ 100 grams of fructose per day (Basciano *et al.*, 2005). The exposure of the liver to such large quantities of fructose leads to rapid stimulation of lipogenesis and triglycerides (TG) accumulation (**Fig 4.2**), which in turn contributes to reduced insulin sensitivity and hepatic insulin resistance/glucose intolerance. Intake of nutritive sweetener above 25% of total energy consumed will cause hypertriglycridemia and gastrointestinal symptoms (Tappy and Kim, 2010). The long term effect of high fructose diet can include changes in digestion, absorption, apetite and hepatic metabolism leading to development of various metabolic disorders such as diabetes, obesity and cardiovascular disease.

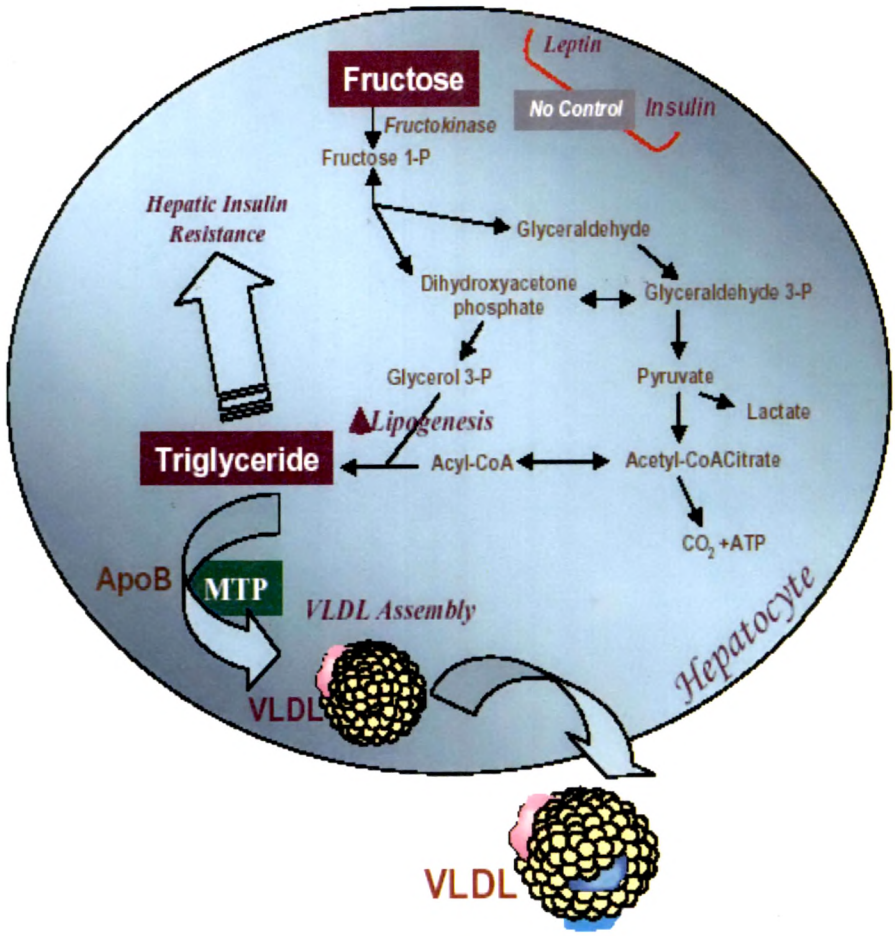


Fig. 4.2: Hepatic fructose metabolism: A highly lipogenic pathway (Basciano *et al.*, 2005).

In this chapter the relationship between different dietary sugar and intestinal microbial community profiles was investigate by using DGGE.

4.2 Materials and Methods

4.2.1 Animals and treatment:

Male Charles Foster rats (body weight 200-250 g) were used for the study. They were allowed *ad libitum* access to water and food. The animals were cared in accordance with principles and guidelines of the Institutional Animal Ethics Committee (IAEC), Department of Biochemistry, M. S. University of Baroda,Gujarat, India. The experimental protocol was approved by the IAEC.

4.2.2 Experimental groups:

The animals were divided into four groups of six rats each. Group 1, control animals (control), received the control diet containing 61 per cent starch and tap water *ad libitum* [61% starch, 20% casein, 0.7% methionine, 5% groundnut oil, 9.7% wheat bran and 3.5% salt mixture (The mineral mix in a kg contained $MgSO_4$ $7H_2O$-30.5 g; NaCl-65.2 g; KCl-105.7 g; KH_2PO_4-200.2 g; $MgCO_3$ g, -3.65 g Mg $(OH)_2$. $3H_2O$-38.8 g; $FeC_6H_5O_7.5H_2O$-40.0 g; $CaCO_3$- 512.4 g; KI-0.8 g; NaF-0.9 g; $CuSO_4.5H_2O$-1.4 g; $MnSO_4$-0.4 and $CONH_3$ -0.05 g) and water *ad libitum*, 0.2 ml of vitamin mixture (The vitamin mix in a kg contained vitamin A concentrate I.P., 2500 I.U.; vitamin D3 cholecalciferol, 200 I.U.; thiamine hydrochloride, 0.5 mg; riboflavin, 0.5 mg; pyridoxin, 0.5 mg; sodium pantothenate, 1.5 mg; nicotinamide, 5 mg; ascorbic acid, 25 mg multivitamin tablets, Piramal healthcare Ltd., Mumbai, India) was added per kg feed. Group 2, fructose-fed animals (Fructose), received the fructose enriched diet, which was similar in composition to the control diet (except for starch which was replaced by 61% fructose). Group 3, Control animals treated with streptomycin, received the starch diet and were allowed to drink 5gm/ litre streptomycin solution 15 days interval and other days allowed *ad libitum* access to water. Group 4, fructose-fed animals treated with streptomycin, received the fructose fed diet and were allowed to drink 5gm/ litre streptomycin solution 15 days interval and other days allowed *ad libitum* access to water. The diets were prepared fresh everyday based on the method of Cohen *et al.*, (1977). The animals were maintained in their respective groups for 90 days. Body weight changes were measured initially and in the end of treatment. At the end of the experimental period oral glucose tolerance test was carried after 12 h fasting (blood samples were collected by

retro-orbital plexus) and blood samples (separated plasma) were used for biochemical estimations.

4.2.3 Genomic DNA isolation from feacal samples.

Starch powder

TE buffer

10% sodium dodecyl sulphate

20 mg /ml Proteinase K

5 M NaCl

C TAB / NaCl solution

24:1 chloroform / isoamyl alcohol

25:24:1 phenol/chloroform

Isopropanol

The genomic DNA isolation from feacal sample of rats was done by using standard method of Zhang *et al.*, (2006), with slight modification. Shortly, 1-1.5 g of freshly collected faeces of rat was dissolved by vigorous vortexing in 0.85% saline. Pellets were collected by centrifugation at 5000g. The pellets were washed with TE buffer, resuspended in 3ml TNE reagent and incubated at 55°C for 1-2 h after addition of 50 µl of proteinase-K. Supernatant was collected in fresh eppendorf containing 2 g starch powder, gently mixed by vortexing and incubated for 1 min at room temperature. 100 µl of 5M NaCl and 180 µl CTAB /NaCl solution were added to the supernatant collected after centrifugation and incubated for 10 min at 65°C. The mixture was extracted twice with equal volume of phenol / chloroform / isoamyl alcohol followed by once with Chloroform/isoamyl alcohol. Finally the supernatant was collected in a fresh tube and added 0.6 vol of isopropanol, mixed gently and incubated at room temperature for 1 h. Precipitate was collected by centrifugation followed by 70% ethanol wash. After complete removal of ethanol by air drying, the pellet was finally dissolved in 25 µl 10:1 TE buffer.

4.2.4 PCR amplification of V3 region of 16S rDNA from *feacal* matter.

The variable V3 region of 16S rDNA gene was amplified by PCR using the following primers complementary to conserved regions of the 16S rRNA genes correspond to positions
341 and 534 in *E. coli* (Muyzer *et al.*, 1993). Forward primer contains 40 nucleotide GC-rich sequence which acts as a clamp.

Forward Primer: 5' CCTACGGGAGGCAGCAG 3'
Reverse Primer: 5' ATTACCGCGGCTGCTGG 3'
Forward primer GC-rich sequence:
5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG 3'

A combination of Forward primer GC-rich sequence and reverse primer was used to amplify the 16S rDNA regions from DNA obtained from *feacal* samples.PCR cycling conditions used to amplify the 16S rDNA gene fragment were 96° C for 5 min, followed by 10 cycles of 94° C for 1 min, 61° C (reduced by 0.5° C each cycle) for a 45 s and 72° C for 1 min; 25 cycles of 94° C for 1.0 min, 55° C for 45 s and 72° C for 1.0 min; and a final extension at 72° C for 10 min.

4.2.5 Denaturing gradient gel electrophoresis (DGGE)

The Dcode universal mutation detection system (Bio-Rad Laboratories, Richmond, Calif.) was used for a DGGE analysis of the PCR products obtained faecal microbial community DNA. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (10 % [wt/vol] acrylamide-bisacrylamide [37.5:1]) by using denaturant gradient from 40 to 60% (100% denaturant was 7 M urea plus 40% [wt/vol] formamide increasing in the direction of electrophoresis, were used. The gels were subjected to a constant 80 V for 12 h at 60°C, and after electrophoresis the gels were stained by silver staining method.

4.2.6 Silver staining

The silver staining was done by the standard method as described in Sambrook *et al* (2001).

4.2.7 Biochemical analyses in serum samples

Oral glucose tolerance test, total cholesterol, triglycerides, HDL and LDL cholesterol concentrations were measured.

4.2.8. Statistical analysis

Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean ± SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software,San Diego, California, USA.

4.3 Results

4.3.1 Microbiota of the rat faecal matter fed with starch and fructose in diet.

PCR amplifications of 16S rRNA gene fragments were obtained from total DNA extracted from faecal samples using specific primers as mentioned in material and method (4.2.4) (**Fig. 4.3**).

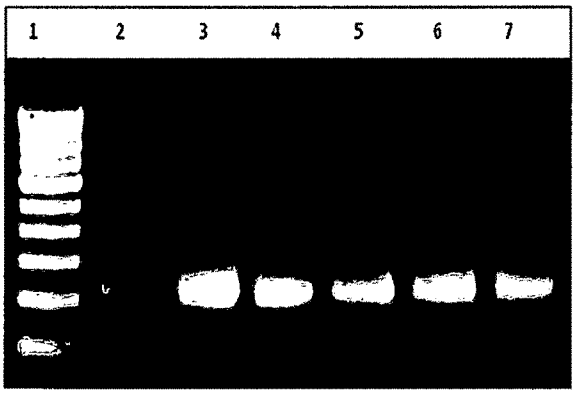
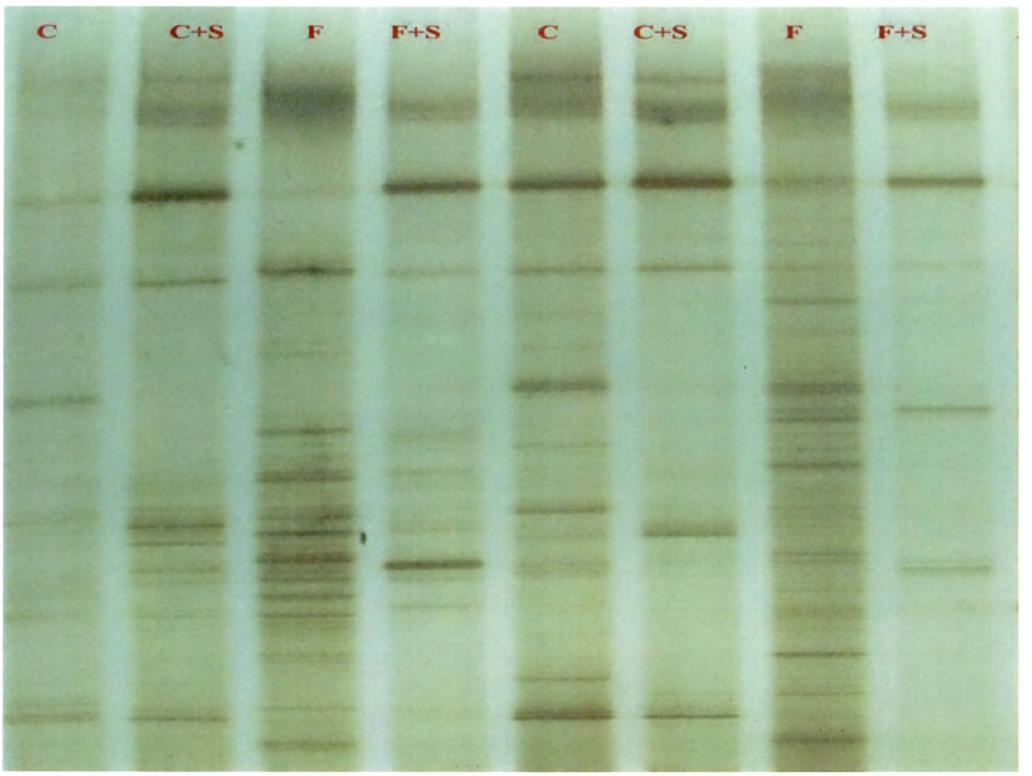


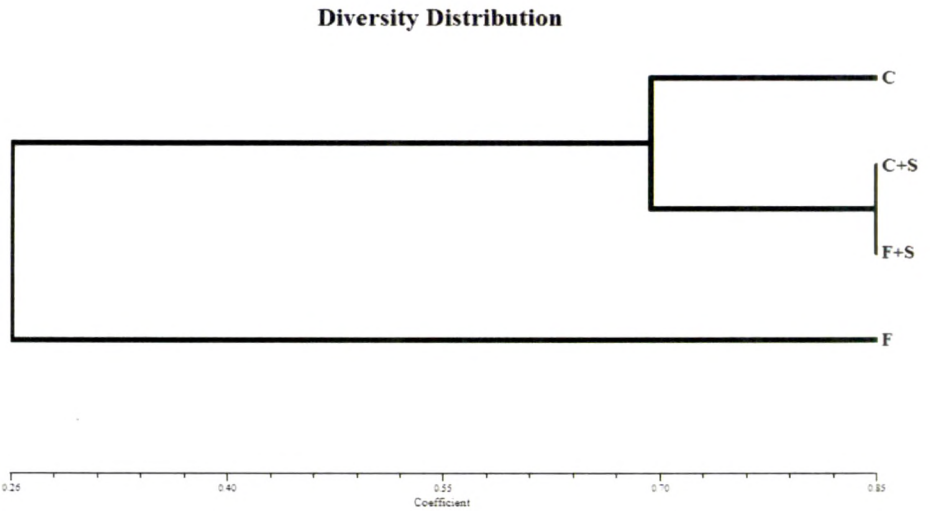
Fig. 4.3: 16S rRNA gene PCR of starch and fructose treated feacal microbial population. (2% agarose). Lane 1: 100 bp Ladder, Lane 2, 3, 4, 5, 6 and 7 PCR from feacal matter of different dietary group.

DGGE profiles revealed that faecal samples from animals belonging to the same dietary groups showed similar banding pattern but differed from other dietary groups (**Fig. 4.4**). The microbial profiles of rats fed on starch and fructose were entirely different but the profile of streptomycin treated groups was similar. Changes in the carbohydrate composition (starch fed and fructose fed rats) influenced the phylogenetic composition of the GI tract microbiota

Fig. 4.4: DGGE profile and dendrogram of faecal genomic DNA samples from rats receiving starch and fructose -base diet.



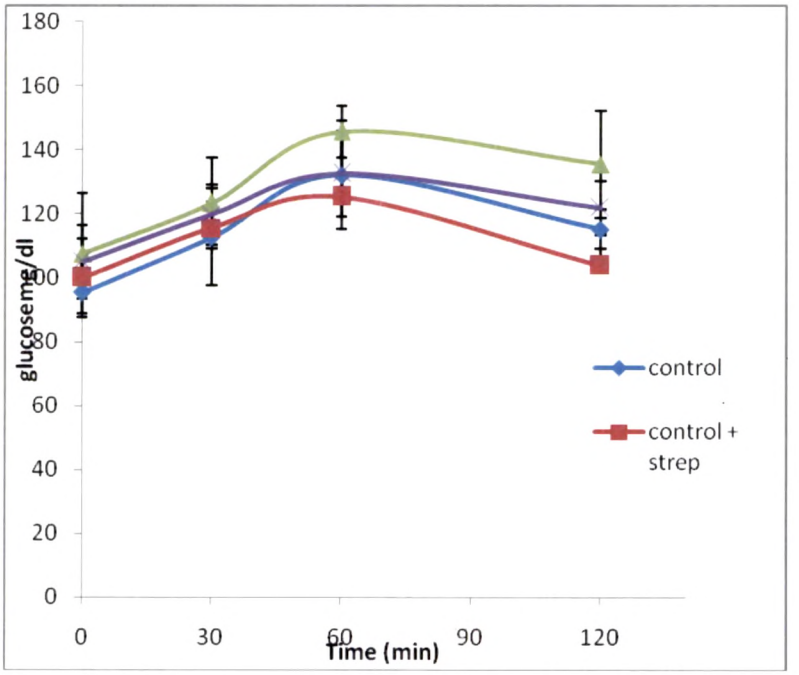
C= Control, C+S= Control+ Streptomycin, F= Fructose, F+S= Fructose + Streptomycin



4.3.2 Oral glucose tolerance

After 70 days on the 61% (w/w) sucrose rich dietary group, the rats developed severe glucose intolerance (**Fig. 4.5**). Compared to control there is significant increase in plasma glucose level in fructose (61%) fed groups.

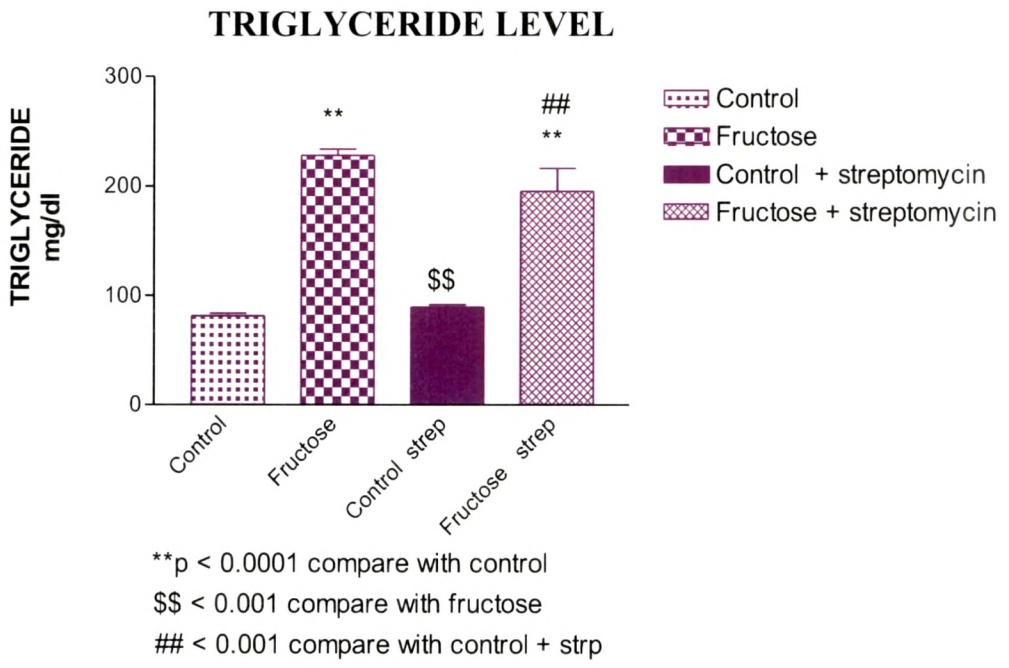
Fig. 4.5: Oral glucose tolerance tests in plasma samples of different groups of rat fed with Starch and Fructose.

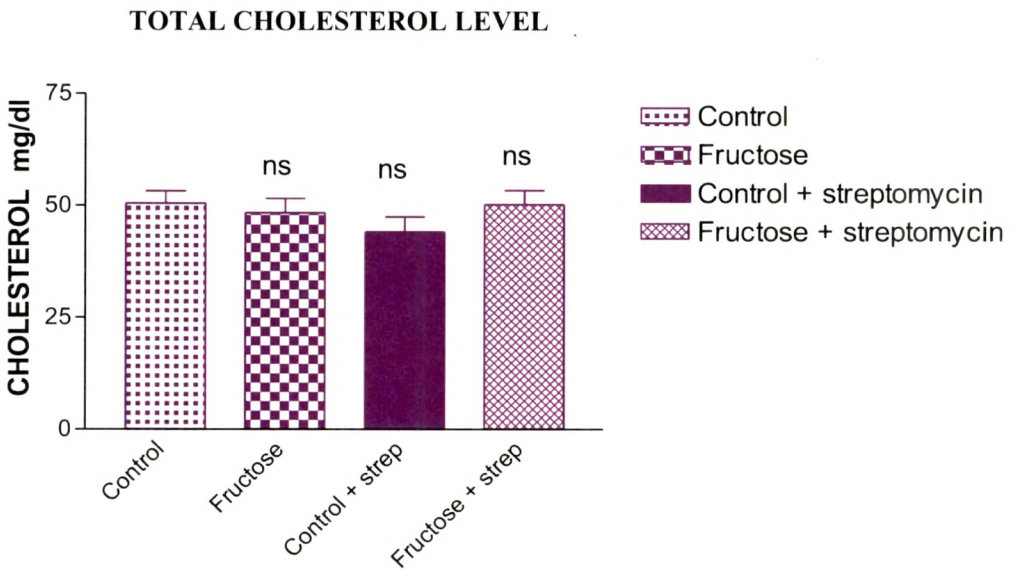


4.3.3 Serum lipid levels

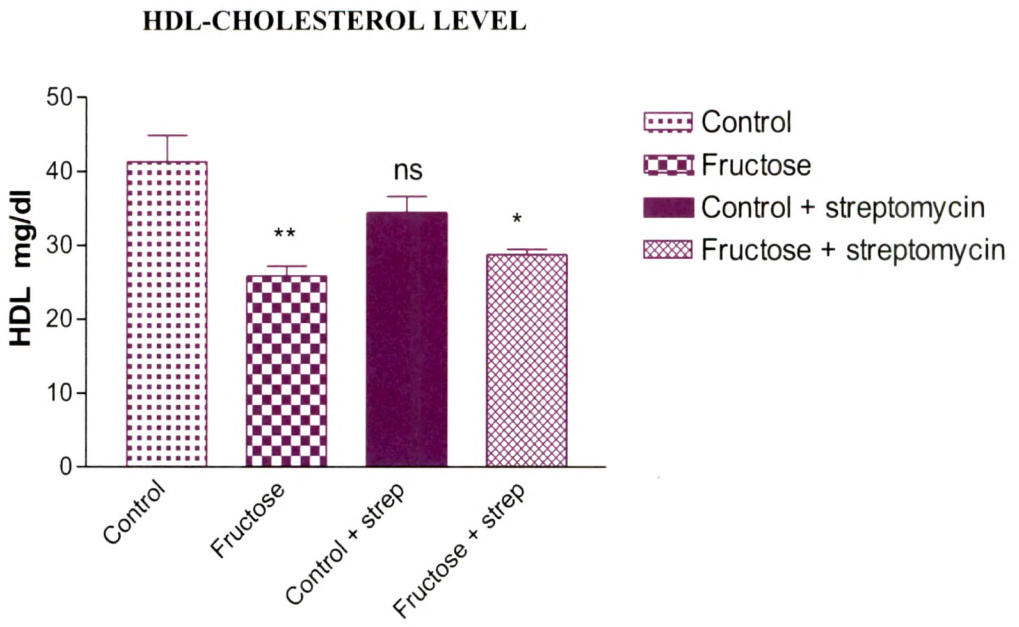
Comparison of the effect of fructose and starch supplementation on the fasting serum lipid was estimated in rats (**Fig. 4.6**). TG was significantly increased 2.8 fold in fructose rich diet compared to control. Cholesterol levels were not changed in the entire diet group. HDL-Cholesterol was reduced to 0.76 fold in fructose rich diet compared to control. Similar trends were found when compared between control and fructose treat streptomycin groups.

Fig. 4.6. Lipid profile in serum samples of different groups of rat fed with Starch and Fructose.

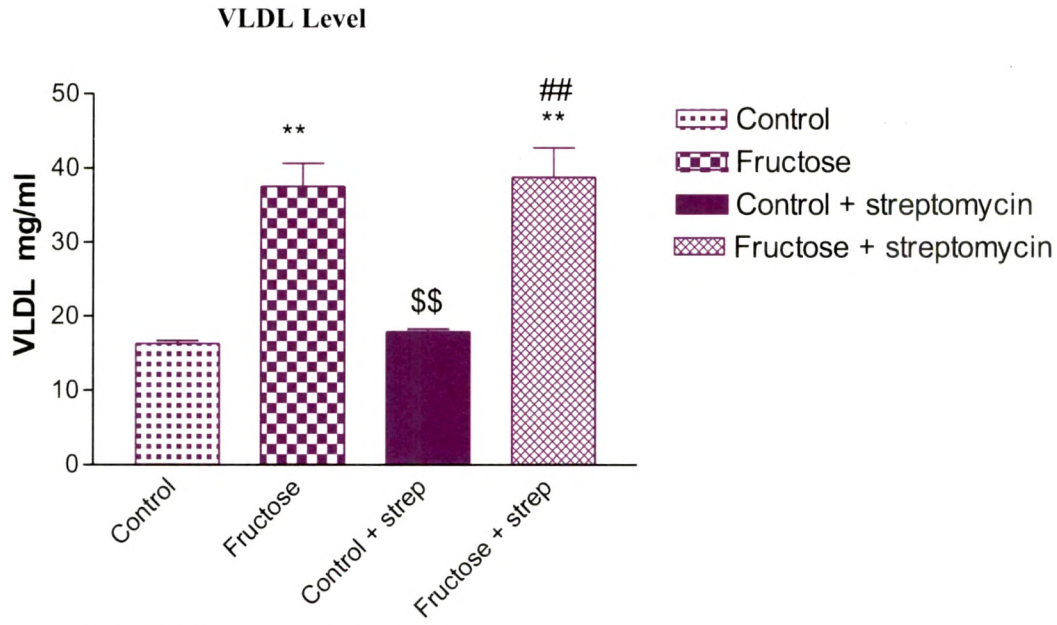




No significant change between groups , N = 6-8



**P < 0.001 & *P < 0..01 compare to control N = 6-8
other group are non significant.



$$ < 0.001 compare to fructose

< 0.001 compare to control + strp

**P < 0.001 & *P < 0.01 compare to control N= 6-8

4.4 Discussion

The worldwide increase in the incidence of metabolic syndrome correlates with marked increase in total fructose intake in the form of high-fructose corn syrup, beverage and table sugar. High fructose diet causes hypertension, insulin resistance and hyperlipidaemia (Johnson *et al.*, 2007). The complex interrelationship between dietary components, gut microbiota composition and function and the human cardiometabolic phenotype are largely lacking (Diamant *et al.*, 2011). However, recently various meta-omics-based approaches have been developed to unravelling the secrets of our gastrointestinal tract microbiota (Zoetendal *et al.*, 2008). In this study, culture-independent approach based on the 16S rDNA sequence variability was used. DGGE profile showed that changes in the dietary sugar affected the phylogenetic composition of the faecal microbiota. Even though sucrose and starch are both easily digestible, they are not expected to reach the large intestine. The DGGE band patterns obtained indicated that these carbohydrates indeed affected the composition of bacteria in the large gut. Based on similarity and cluster analysis, starch and fructose treated dietary groups had different phylogenetic composition of the faecal microbiota. Recent reports mentioned about dietary change that influenced the gut microbial diversity (Cani *et al.*, 2008; Turnbaugh *et al.*, 2009; Faith *et al.*, 2011, Wu *et al.*, 2011). On the other hand, streptomycin treated groups did not show any variation.

High fructose diet also altered the clinical parameters such as OGTT, triglycerides, VLDL, HDL-Cholesterol level (Bantle *et al.*, 2004; Stanhope *et al.*, 2009; Haung *et al.*, 2011; Hudgins *et al.*, 2011). Present study confirmed that high fructose diet led to reduction in glucose tolerance and increase in TG. High fructose consumption by rats has been shown to reduce glucose tolerance effects (Elliott *et al.*, 2002; de Moura *et al.*, 2009). High fructose diet, induce hypertriglyceridemia and other metabolic diseases (Basciano *et al.*, 2005; Tappy and Kim, 2010). 32% fructose solution in diet of rats for 6 week led to 1.85 fold increase in TG (Robin *et al.*, 1982). Rats fed with 66% of fructose in diet for two week increased the plasma TG levels increase 2 fold as compared to starch diet (Busserolles *et al.*, 2002). Fructose consumption by rats has been shown to produce elevated TG, cholesterol, and body fat (Elliott *et al.*, 2002; de Moura *et al.*, 2009; Bocarsly *et al.*, 2010).

Further research is needed to understand the regulation of microbiota during fructose diet and its relation to metabolic disease. Analysis of DGGE profile could be useful in understanding the dynamics of microbial population.