The effect of probiotic VSL#3 and lifestyle intervention in obese children with non-alcoholic fatty liver disease (NAFLD)

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Declaration

I hereby declare that the thesis entitled, **"THE EFFECT OF PROBIOTIC VSL#3 AND LIFESTYLE INTERVENTION IN OBESE CHILDREN WITH NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)"** submitted to Lovely Professional university for the award of Degree of Doctor of Philosophy (Ph.D.) in Nutrition & Dietetics is a bonafide research work and all ideas and references have been duly acknowledged. No part of this dissertation has been submitted for any other degree.

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CERTIFICATE

This is to certify that dissertation work entitled, **"THE EFFECT OF PROBIOTIC VSL#3 AND LIFESTYLE INTERVENTION IN OBESE CHILDREN WITH NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)"** submitted to Lovely Professional university for the award of Degree of Doctor of Philosophy (Ph.D.) in Nutrition & Dietetics is a bonafide research work carried out by Pooja Goyal, under my Supervision and that no part of this dissertation has been submitted for any other degree.

This assistance and help received during the course of investigation has been fully acknowledged.

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Terminologies

NAFLD	:	non-alcoholic fatty liver disease
NASH	:	non-alcoholic steatohepatitis
USG	:	ultrasonography
PAMP	:	pathogen-associated molecular pattern
DAMP	:	danger-associated molecular pattern
LPS	:	lipolysaccharide
TLR	:	toll like receptors
TNF-α	:	tumor necrosis factor alpha
ALT	:	alanine aminotranferase
AST	:	aspartate aminotransferase
GGT	:	gamma-glutamyl transferase
LDL-c	:	low density lipoprotein cholesterol
HDL-c	:	high density lipoprotein cholesterol
TG	:	triglyceride
CRP	:	c - reactive protein
FBG	:	fasting blood glucose
SCFA	:	short chain fatty acid
GLP-1	:	glucagon like peptide-1
RCT	:	randomized control trial
CTRI	:	clinical trials registry- India
CLD	:	chronic liver disease
WHO	:	world health organization
Cm	:	centimeter
Mm	:	millimeter
G	:	gram
Kcal	:	kilocalorie
CHO	:	carbohydrate
WT	:	weight
HT	:	height

Kg	:	kilogram
Mg	:	milligram
Ml	:	milliliter
Ng	:	nanogram
%	:	percentage
WC	:	waist circumference
MAC	:	mid arm circumference
TSF	:	triceps skinfold thickness
BMI	:	body mass index
BMR	:	basal metabolic rate
i.e	:	that is
Fig	:	figure
et al.	:	and others
±SD	:	standard deviation
g/d	:	gram/day
kcal/d	:	kilocalorie/day

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ABSTRACT

Background: Non-alcoholic fatty liver disease is one of the most upcoming causes of chronic liver disease in pediatric population in developing countries. Poor lifestyle and faulty dietary habits are an epidemic of obesity.

Aims & Objective: To evaluate the potential of probiotic VSL#3(De Simone Formulation) and Lifestyle modification in obese pediatrics with Non-alcoholic fatty liver disease (NAFLD). **Material & Methods**: we conducted clinical trial on 106 obese children in between age group of 5 to18 years and divided them in to four groups; group 1.VSL#3 plus lifestyle intervention (n=26), 2.VSL#3 (n=27), 3. Lifestyle intervention (diet+ physical activity) (n=26) and 4. Placebo (n=27) received interventions for four months. To identify NAFLD anthropometric, biochemical measurements and ultrasonography were carried out. The anthropometric measurements: Body mass index (BMI), mid arm circumference (MAC), waist circumference (WC) and triceps skinfold thickness (TSF) was done. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), low-density lipoprotein cholesterol(LDL-c), high-density lipoprotein cholesterol (HDL-c), triglyceride (TG), cholesterol, fasting blood glucose(FBG),C-reactive protein (CRP), uric acid, leptin and ghrelin were measured along with their dietary intake at baseline and post-trial. **Results**: NAFLD prevalence rate was 66.2% in obese children. TG, BMI, ALT and soft drinks were most significant predictors of NAFLD among obese children. Different intervention groups had significant weight loss, also reduction in BMI, MAC, TSF and WC ($p<0.001$) rather than placebo. VSL#3 plus lifestyle intervention significantly the most pronounced therapy for reducing fatty liver grades and serum AST, ALT, GGT, LDL-c, CRP, cholesterol, TG, uric acid, FBG and increase in HDL-c and also had most significant beneficial impact on leptin and ghrelin hormones ($p<0.001$) as compared to single therapy of VSL#3 and lifestyle intervention alone. **Conclusion**: Probiotic VSL#3 and Lifestyle intervention both are effective for managing the NAFLD but these interventions work independently for changing in most of the variables. In our study, combined therapy of Probiotic VSL#3 plus lifestyle intervention is promising therapeutic treatment for management of NAFLD in Indian obese children.

CHAPTER -1

INTRODUCTION

Obesity is one of the major disorder and threat that increasing worldwide. According to latest Nutrition Examination and National Health Survey, the prevalence of obesity is 17% in children and adolescents from 2-18 years age in United States (Ogden *et al*., 2012).Currently, World Health Organization (WHO) globally estimated that more than 340 million children and adolescents age group ranging from 5-19 years are overweight or obese. Overweight or obesity is most prevalent in north Indian children according to epidemiology survey conducted in different states of India and found 19% combined prevalence of overweight or obesity (Fernandez *et al*., 2015 and Bozic *et al*., 2013). The prevalence of overweight in children is ranging from 4% to 22% in India (Ranjani *et al*., 2016 and Song *et al*., 2017). Obesity is associated with major complications involving all major organ system. Recent studies suggested that the prevalence of non-alcoholic fatty liver disease (NAFLD) is progressing in obese children (Fraser *et al*., 2007 and Mager *et al*., 2006).

Non-Alcoholic fatty liver is macro vesicular fat accumulation in more than 5-10% of hepatocytes encompasses liver disease such as steatosis, steatohepatitis, fibrosis and even cirrhosis and without alcohol intake; (<20g/day) (Oldfield *et al*., 2014). (Parry *et al*., 2012) concluded in his study that prevalence of fatty liver disease in obese children aged 4-18years was 61%. Another study of (Sartorio *et al*., 2007) found NAFLD was diagnosed in 44% of obese children. Obesity is consistent with NAFLD progression: NASH (non- alcoholic steatohepatitis) in adults as well as in children also if it is not treated timely. The increased prevalence of childhood obesity and NAFLD has emerged as astonishing risk factor of liver disease among children and adolescents in industrialized countries (Day, 2011 and Vajro *et al*., 2012). NAFLD is the most common cause of chronic liver disease (CLD) in general 3-11% of pediatric population in developing countries (Schwimmer *et al*., 2006).

Faulty dietary habits and poor lifestyle in these children are the major risk factors associated with obesity and NAFLD (Ojeda-Rodriguez *et al*., 2018 and Katzmarzyk *et al*., 2016). It is approximately 20-40% of NAFLD patients develop NASH and also estimated that 10-31% NAFLD patients progresses to cirrhosis after ten years

(Verduci *et al*., 2015). According to authors, NAFLD is main cause of cryptogenic cirrhosis and insulin resistance (IR) is salient attribute of NAFLD including "X" syndrome (Verduci *et al*., 2015). Pediatric NAFLD has been associated with extrahepatic involvement such as development of cardiovascular disease(CVD), Type-2 diabetes, low bone mineral density that is particularly public health agitate and threat to society (Hadizadeh *et al*., 2017). ASL, ALT levels reflect hepatocellular damage and may be increased two or three times over their normal limits. In contrast to NASH, ALT may be higher than AST. Some patients have normal AST or ALT while diagnose of simple steatosis is possible by USG. It has been found in 60% of patients with fatty liver had within normal limits of ALT in large group studies (Hadizadeh *et al*., 2017) while another studies suggested that AST and ALT levels are two to three times elevated in NASH/NAFLD. The aminotransferase levels fall over the time when hepatic inflammation attenuates. BMI could be a major predictor of obesity and NAFLD in previous studies. Leptin level is also helpful for distinguishing the pathogen severity in hepatic steatosis (Boyraz *et al*., 2013 and Neuman *et al*., 2014). Some adipokines such as leptin, adiponectin, tumor necrosis factor-alpha (TNF-α) secretion function might also disturb and consistent with regulation of body weight (Boyraz *et al*., 2013; Neuman *et al*., 2014 and Pirvulescu *et al*., 2012). Obese and diabetes person has more resistin levels with increased adipose tissues. It can also raise the bad cholesterol in hepatocytes and unable the liver to remove LDL from body. Higher triglyceride levels are also risk factor of NAFLD/NASH. It is the predictor of cardiovascular disease and may be of diabetes in further (Page *et al*., 2013; Yang *et al*., 2002; Polyzos *et al*., 2011 and Younossi *et al*., 2011).

It has been reported that diet of children is rich in high fructose corn syrup in beverages like soft drinks and high intake of fatty foods that increase fat accumulation in hepatocytes also increase in triglycerides and lipid peroxidation (Zelber-Sagi et al., 2011 and Pereira *et al*., 2017). Higher consumption of junk food increases the advance glycation end products, produce oxidative stress and nitric oxide synthesis inhibition (Elliott *et al*., 2002 and Abid *et al*., 2009). In Punjab central adiposity and higher body mass index levels are widespread in adults and also in children. The reason behind in that is more adoption of western diets among these children. Their eating pattern is poor. Somehow parents are also responsible for inculcating these habits in them initially. They consume more saturate fat, meat, poultry, cholesterol

rich foods and have lower intake of nutrient enriched foods like fruits, vegetables, milk, curd, omega-3 enriched nuts or seeds that contains also vital nutrients especially vitamin E (Abid *et al*., 2009).

Both environmental and genetic risk factors may impart in pathogenesis of NAFLD (Alisi *et al*., 2012). Diets rich in fat and fructose distorts the intestinal barrier function, increase intestinal absorbency "leaky gut" to gut-obtained products and set free of Pathogen-associated molecular patterns (PAMPs) and Danger-associated molecular patterns (DAMPs) that reach in liver cells promotes firstly fat accumulation in liver cells and secondly cellular hardening and lastly liver damage (Lin *et al*., 2014 and Marzuillo *et al*., 2014). Gut microbiota and genetic factors may play a major role in liver injury.

Recently, new evidence from probiotics studies found gut microbiota alteration increased the progression of NAFLD through targeting gut-liver axis (Ma *et al*., 2013). The first prevention and treatment of NAFLD in children could be lifestyle interventions such as change in dietary habits and physical activity such as moderate exercise and aerobics (30-45minutes) per day. It can alter the composition of gut-microbiota that is essential for maintenance of gut integrity and preserve a healthy gut liver axis (Ma *et al*., 2013 and Compare *et al*., 2012). Some evidence from both animal models and human studies has shown that the reduction of dietary fructose and supplementation with vitamin E, omega-3 fatty acids and probiotics improved fatty liver. (Aller *et al*., 2011) reported that patients with NAFLD improved liver enzymes after three months treatment with *Lactobacillus bulgaricus* and *Streptococcus thermophilus.* Most recently, (Alisi *et al*., 2014) attempted trial of VSL#3 Vs Placebo in obese children with NAFLD and found that four month supplementation with VSL#3 significantly managed fatty liver and also significantly reduced body mass index from 27.1 to 24.9 kg/m^2 and 8.1% weight reduction. A children pilot study suggested that Probiotic as supplemental beneficial therapy in managing pediatric NAFLD but larger randomized placebo trials in children with NAFLD are needed (Vajro *et al*., 2011 and Teixeira *et al*., 2012).

Lifestyle interventional approach is also effective tool for preventing NAFLD and first line defense treatment in obesity (Nobili et al., 2008). Indeed, unhealthy diet is more calories dense rather than essential nutrients. Healthy diet plus physical activity may improve simple hepatic steatosis. In children multi-target therapeutic approach could be successful by combining two treatments lifestyle interventional approach with probiotic VSL#3 for treating the advanced stage of NAFLD and also prevent insulin resistance.

Therefore in the present study, we planned first randomized controlled trial (RCT) to see the effect of VSL#3 and lifestyle intervention in obese children with NAFLD in planned area of Punjab, India and dividing in to four groups: **1.**VSL#3 plus lifestyle intervention, **2.**VSL#3, **3.** Lifestyle intervention and **4.**Placebo. The study results showed the most effective therapy for managing NAFLD and obesity hormones in pediatrics.

This study was attempted with following ensured objectives:

1. To assess nutritional status of obese children with fatty liver.
2. To study the efficacy of Probiotic VSL#3 supplementation Vs Placebo in obese children with fatty liver.
3. To study the role of intensive lifestyle intervention such as diet and physical activity in obese children with fatty liver.
4. To study the hunger hormones: Ghrelin and Leptin before and after intervention

Hypothesis

Null Hypothesis (Ho): Both Probiotic VSL#3 and Placebo are equally effective for managing the NAFLD in obese children.

Alternative Hypothesis (Ha): Both Probiotic VSL#3 and Placebo are not equally effective for managing the NAFLD in obese children

Null Hypothesis (Ho): Both Probiotic VSL#3 and lifestyle intervention are equally effective for managing the NAFLD in obese children.

Alternative Hypothesis (Ha): Both Probiotic VSL#3 and lifestyle intervention are not equally effective for managing the NAFLD in obese children

CHAPTER -2

REVIEW OF LITERATURE

The review of literature helps to familiarize the investigator with the work that has been done in the area of one's interest and also the study. The related to study literature have presented and discussed under following headings:

2.1. Non-alcoholic fatty liver disease (NAFLD) widespread in obese children

2.2. Causative factors for developing obesity and NAFLD in children

2.1.1. Manifested role of fat and fructose in developing NAFLD

2.3. Concerned parameters to diagnose fatty liver disease

2.4. Function of gut microbiota for managing NAFLD

2.5. Function of probiotics for management of NAFLD

2.6. Function of Lifestyle intervention for managing NAFLD

2.7. Obesity hormones; leptin and ghrelin

2.1. Non-alcoholic fatty liver disease (NAFLD) widespread in obese children

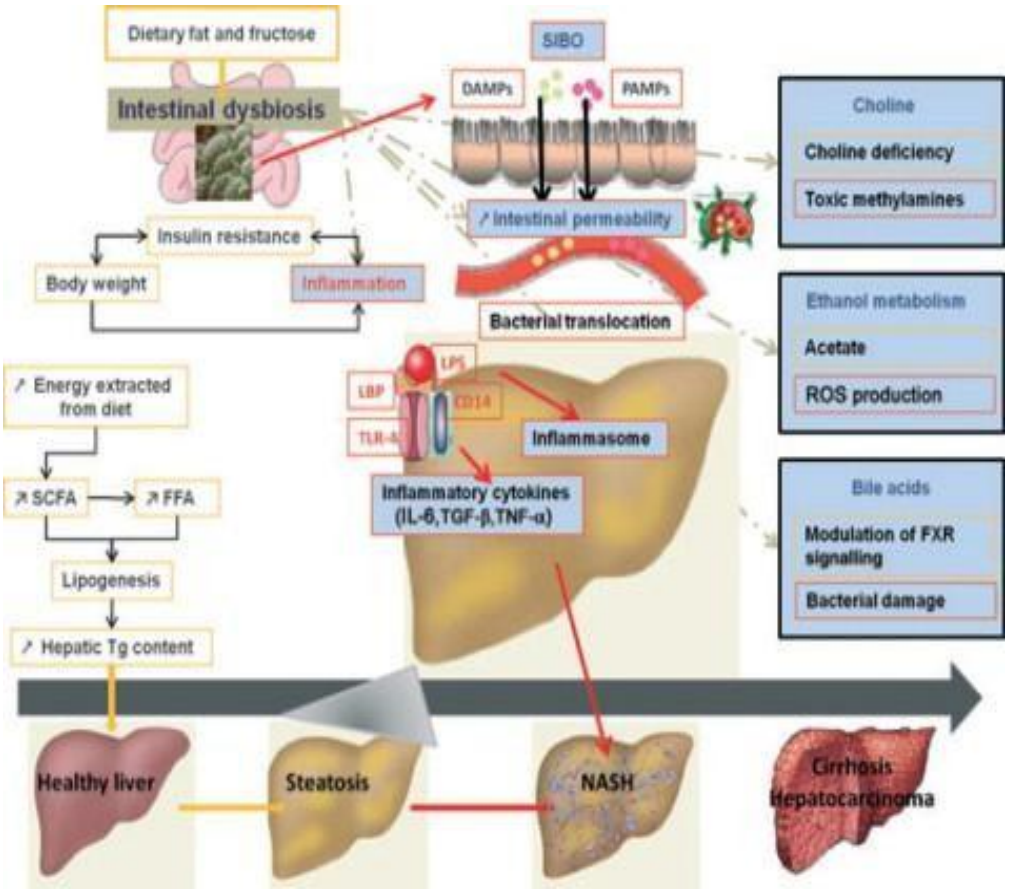
Obesity is most widespread in Punjab, India and increasing various metabolic disorder in adults and children in all over worldwide (Lobstein *et al*., 2004 and Fan *et al*., 2017). NAFLD and diabetes are common salient features of obesity (Dehghan *et al*., 2005). NAFLD is the greatest recurrent root of chronic liver disease in developing countries and also unknown cause of cryptogenic cirrhosis in obese children (Nobili *et al*., 2015). Obese person has 30-80% chance of fatty liver (hepatic steatosis), 30-40% of simple steatosis progresses to NASH and then 6-10% may suffer from full blown cryptogenic cirrhosis after ten years of NASH without treatment (Mathur *et al*., 2007). NAFLD is also found in non-obese children that may be a genetic susceptibility of single nucleotide polymorphism, (SNP) that regulate fat metabolism and oxidative stress in body. Most significance gene PNPLA3 (patatin-like phospholipase domain-containing 3 gene) also called adiponutrin, is require for breakdown of triacylglycerol in adipose cells (Romeo *et al*., 2008).

2.2. Causative factors for developing obesity and NAFLD in children

The causative factor of obesity with NAFLD is high fructose corn syrup intake and fatty food overconsumption (Nseir *et al*., 2010 and Marchesini *et al*., 2008). Diet of children is poor and unhealthy including lack of essential nutrients in the diet which results lower consumption of omega-3, vitamin E, B complex vitamins, fiber and fish. HFCS is highly consumed in the form of beverages i.e. soft drinks, canned juices. It contains 53-55% fructose and 42% glucose (Ouyang *et al*., 2008 and Jin *et al*., 2012). It is highly sweet toxin that increases denovo-lipogenesis more than 5 folds in obese person than in healthy individual. Excessive soft drink consumption commonly seen in these patients cause insulin resistance and increased in hepatic triglycerides (Jin *et al*., 2012 and Pereira *et al*., 2017). Individual who consumed more than 1 soft drink (1 glass) daily had higher widespread of metabolic syndrome (MS) (El-Koofy *et al*., 2012). In one study MS found mostly in 41% of NAFLD and 20% in control group. Another study suggested 50- 60% of these children also suffered from pre-diabetes (Havel *et al*., 2005). All disorders are associated with unusual dietary pattern of obese children and also faulty dietary practices of their parents. In another study high fructose (3.5g/kg/day) increased advance glycation end products related to various degenerative diseases and caused ATP depletion in liver cells without action of

enzyme (Vos *et al*., 2013). A 20 ounce (600 ml) of soft drink contains 32.6g of fructose that expected to increase post prandial blood glucose. A normal healthy person can absorb 25g fructose in small intestine through GLUT 5 transporters (Vos *et al*., 2013). Unusual fermentation of fructose increased by gut microbiome and further release of endotoxin, bacterial translocation, increased intestinal porosity leads to inflammation and bacterial over growth "leaky gut". Moreover, increase in ATP and AMP further converted to uric acid including imbalance of ketohexokinase activity by fructose ingestion and impaired nitric oxide synthesis (Vos *et al*., 2013). It has commonly seen in some patients that they have high uric acid because of sensitivity of fructose sugar and may develop hyperuricemia after ingestion of fructose containing drinks (Dhingra *et al*., 2007 and Vartanian *et al*., 2007). High fructose diet develops fat in liver cells and modulates the gut microbiota composition. Uric acid breakdown product of purine increases in cells and overturn in circulatory system and more ATP depletion occurs in this condition (Israel *et al*., 1983). So, uric acid is also concerned indicator of NAFLD and diabetes among people who consume excess amount of fructose drinks (Reiser *et al*., 1989). It is rapidly absorbed in small intestine and increases the post prandial blood sugar levels. High fructose diet induced lipogenesis and synthesis of triglycerides including higher level of non-esterified fatty acids (NEFA) (Reiser *et al*., 1989 and Sartorio *et al*., 2007). Recovery of these patients may be delayed because of excessive ATP depletion and more fat accumulation in liver cells. Abdominal fat and more visceral fat (high waist circumference) have commonly seen in these patients because of disturbed metabolic pathways.

2.2.1. Manifested role of fat and fructose in developing NAFLD



High fructose diet changes the microbial function and loosens the tight junctions with passage of freely endotoxin in to the portal system; this mechanism involves the increased porosity of intestine and caused hepatic inflammation and insulin sensitivity with activation of TLR- 4. When TLR-4 acts on stellate cells and examined the liver cellular hardening and if it acts on kupffer cells then fosters inflammation of liver. Moreover, intestinal dysbiosis invites other degenerated diseases. Steatosis may developed by higher amount of NEFA returning from adipose tissue where fat stored (Neuman *et al*., 2014). Various studies demonstrated that soft drinks, sweeteners i.e aspartame and caramel are the major risk factors for developing obesity with NAFLD and also insulin resistance (Kelley *et al*., 2004; Hadizadeh et al., 2017; Vlassara *et al*., 2002; McDevitt *et al*., 2001). (Abid *et al*., 2009) postulated that soft drinks consumption was the strong independent predictor of NAFLD and MS with higher CRP levels through logistic regression analysis and aldolase B enzyme deficiency common in this fatty liver progression.

2.3. Concerned parameters to diagnose fatty liver disease:

Various clinical biomarkers predict NAFLD in obese children. (Sartorio *et al*., 2007) resulted that USG, HOMA, ALT, BMI and uric acid were the independent markers of NAFLD in Italian obese children but the major markers were BMI and ALT. Another study of (Hadizadeh *et al*., 2009 and Neuman *et al*., 2014) suggested triglyceride, total cholesterol, IR, HDL-c, LDL-c commonly used for numerous decades in fatty liver disease or either in metabolic syndrome. But some new biomarkers have been emerged namely adiponectin, leptin, TNF-α, resistin, ghrelin, interleukin (IL-6) and HSCRP.

Recently, (Hadizadeh *et al*., 2009) also suggested that adiponectin generally reduced in Obesity and NAFLD and most declined in NASH. It is a protein from adipose tissue prevents excessive accumulation of fat in liver and has anti-inflammatory and antagonist to lipogenesis. Some studies recapitulated that it has been seen lower in moderate steatosis and fibrosis (Esteghamati *et al*., 2010 and Armutcu *et al*., 2013)

Leptin is ob gene extract and secreted by white adipose tissue and also some secreted by stellate cells of liver. It is kind of peptide hormone that controls the appetite and maintain the energy levels and also prevents weight gain. Some studies postulated that leptin marker is strong predictor of fibrosis and liver inflammation (Silha *et al*., 2003

and Grigorescu *et al*., 2012). On the flip side, leptin could not distinguish the stages of fibrosis. (Fitzpatrick *et al*., 2010) concluded that leptin and CK-18 M30 was strong estimator for diagnosing the severity of NAFLD/NASH. Some studies demonstrated that Cytokeratin-18 during apoptosis process secretes more in blood stream and its higher levels defined hepatic fibrosis. These tests were also sensitive parameters for diagnosis fibrosis. They also found 13% of children with fatty infiltration had acanthosis nigricans and 49% had enlarged spleen (Yilmaz *et al*. 2009 and Ulukaya *et al*., 2007). Another study (Kwok *et al*., 2014) concluded that CK-18 M30 was sensitive parameter for diagnosing NASH. Moreover, another studies showed that higher level of leptin was connected with cardiovascular disease (CVD) and insulin resistance (Ikejima *et al*., 2001 and Aleffi *et al*., 2005). (Marchesini *et al*., 2003) concluded in his study that lower concentration of ghrelin levels were associated with insulin resistance in NAFLD patients because of its orexigenic nature.

Another pro-inflammatory marker, TNF-α is a strong predictor of liver disease. It may increase during endogenously higher level of triglyceride and cholesterol (Lydatakis *et al*., 2006). It has been reported that improvement in TNF-α prevents the insulin resistance and hepatic steatosis (Tacer *et al*., 2007). A study conducted in fatty liver obese adults found serum TNF-α was increased and lower in control group (Jamali *et al*., 2016). Previous studies demonstrated that some patients with NAFLD suffered from iron homeostasis including higher level of cytokines because TNF-α may play a role in regulating the iron overload in the liver (Aigner *et al*., 2008). These inflammation leads to dysfunction of mitochondria and higher the reactive oxygen species. Moreover, mitochondrial disruption and cell damage are strong estimators of risks and also replacing the liver biopsy test specially in children because of invasive tool and difficult for taking consent for this diagnostic procedure (Armutcu, *et al*., 2013; Silha *et al*., 2003 and Trujillo *et al*. 2005).

IL-6 is inflammatory cytokine that maintains the immune function and responses. It is produced by cells of adipose tissue and reason behind that it is responsible for increasing CRP levels and also gives specific response to lipolysaccharide (PAMP) a microbial product. Some studies showed correlation of IL-6 with TNF-α, insulin resistance and fatty liver (Khera *et al*., 2009; Hui *et al*., 2004 and Tilg, 2010). (Zamora *et al*., 2007) showed higher level of IL-6 in sleep apnea obstructive individuals with higher fatty liver grade.

C-reactive protein is an independent marker of hepatic steatosis and liver inflammation. In addition to this HSCRP predicts the lower degree of inflammation and also the higher degree of hepatic fibrosis. It is also predictor of future heart disease. Some studies recapitulated that HSCRP was the best predictor for differentiating in liver inflammation especially fibrosis (Siebler *et al*., 2008; Kogiso *et al*., 2009; Kerner *et al*., 2005 and Yoneda *et al*., 2007). Another study of (Uchihara *et al*., 2006) also concluded that HSCRP level was higher in moderate and severe grades of NASH as compared to lower grade of NASH. C-reactive protein is created in liver and also produced in adipose tissue. Some inflammatory cytokines have been seen interlinked with higher level of HSCRP. In a nut shell, HSCRP is most considering predictor of steatohepatitis during screening of NAFLD in obese person and also future index of CVD (Zimmermann *et al*., 2011; Park *et al.*, 2004 and Anty *et al*., 2006).

2.4. Function of gut microbiota for managing NAFLD

Gut microbiota has important role in digestion, absorption and also ferment the unusual substrate, preserving immune function and help in the synthesis of various enzymes, vitamins especially K and H (Guarner and Malagelada, 2003). Fiber is nondigestible carbohydrate that fermented by colon bacteria itself and production of short chain fatty acids (SCFA) acetate, propionate and butyrate (Usrell *et al*., 2013 and Parekh *et al*., 2014). These metabolic products are main source of energy for the host by free fatty acid receptors ($FFAR_2$) and ($FFAR_3$) that to stabilize intestinal hormones. Butyrate production prevents obesity and IR also suppressed food intake. Butyrate has major role for maintaining the mucosal barrier function (David *et al*., 2014 and Benson *et al*. 2010).

Colon cells metabolized 89% butyrate and it has beneficial effects in the gut integrity and motility. Specially butyrate has antipathogenic activity and prevents bacterial over growth during inflammation and in hepatic damage as well (Schwiertz *et al*., 2010 and Fernades *et al*., 2014). It is a major metabolic product for colon cells in humans and has various functions. It has been defined that SCFA consist 6-16% of total requirement of calories for humans (Bedford and Gong, 2018 and Bergman, 1990). (O'Kefee *et al*., 2011) suggested that fiber diet and its supplement improved the gut condition of critically ill patients and also reduction of diarrhea by improving the absorption of electrolytes through butyrate producing beneficial bacteria. In

NAFLD condition it has been observed that there is an inappropriate oxidation of butyrate and impaired cellular homeostasis. Probiotics in combination of fiber diet attenuated inflammation and increased butyrate producing bacteria in the colon and benefit the health of patient. It has anti-inflammation property to reduce inflammatory cytokines (Williams *et al*., 2003; Mathew *et al*., 2014 and Jahns *et al*., 2015).

SCFA has important role in maintaining gut integrity (Lupton, 2004; Cani *et al*., 2008 and Wang *et al*., 2012) to regulate the tight junctions and enhance barrier functions through rise in zonula occuldens-1(ZO-1) and it can backward the nature of ZO-1 and declined the bacterial translocation (Liu *et al*., 2014). Researchers are thriving to explore the role SCFA in gut permeability. SCFA has also crucial role in maintenance of glucose and propionate play a glycogenic role in liver while butyrate and acetate may play a role for lipids metabolism. Furthermore, studies suggested rise in propionate flow in the liver reduced the hepatic triglycerides and maintained the blood glucose levels (den Besten *et al*., 2015). Higher acetate has antagonist role for glucose maintenance (Layden *et al*., 2012).

SCFA also has effective role in metabolism of lipids. Recently, one study showed propionate is effective to decline the belly fat and hepatic fat (Nishina *et al*., 1990 and Demigne *et al*., 1995). However, acetate in the liver has role in fresh lipogenesis and synthesis of cholesterol and may be impeded by propionate (Hong *et al*., 2005). Higher acetate also raised the level of leptin in adipose cells that maintains the satiety and has anorexic effects (Kimura *et al*., 2013; Sun *et al*., 2011; Zaibi *et al*., 2010 and Halaas *et al*., 1995). Some nerve reflexes may be modulated by SCFA. In short studies showed that fermented fibers had effective role in appetite modulator (Archer *et al*., 2004 and Parnell *et al*., 2009). Butyrate and propionate basically regulated appetite through releasing the gut hormones PYY and GLP-1 from intestinal cells. More studies are required to elucidate the importance of these SCFA for managing appetite (Chambers *et al*., 2014).

Some inflammatory and immune responses could modulate by SCFA. Two studies examined that butyrate impeded the histone deacetylation in mild leukemia and also obstructed the activation of Kappa b. In totality, SCFA played major role in preventing human body against the damaging effects of metabolic harmful products and also inhibit the inflammation associated with poor diet rich in fat and fructose.

(Luhr *et al*., 2002 and Maeda *et al*., 2000).

Deficiency of butyrate leads to endogenous malnourishment and extreme hunger of enterocytes induced intestinal inflammation. Choline deficiency is also common inNAFLD because of metabolic disturbance. In NAFLD, choline is converted to methylamine rather than acetylcholine and it is essential for making main phospholipids for membrane of cells and modulates the expressions of genes and lipid metabolism (Karen *et al*., 2012). Choline rich foods and supplement is required in severe form of NAFLD/NASH (Zeisel, 2006).

Excessive ethanol production and bile acid metabolically disturbed in liver disease. Normal healthy person has more butyrate production that maintains GLP-1 and PYY hormone secretion from intestine that reduce food intake, gives satiety value and prevent weight gain (Chambers *et al*., 2014).

The proportion of firmicutes and bacteriodetes are disturbed in obese person. They have more numbers of firmicutes and less number of bifidobacteria and bacteriodetes. Gut microbiota of healthy person contains 1-2kg weight of microbes i.e. 100 trillion. In obese person their ratio is imbalanced and they harvest more energy and there is no control of their hunger without burning calories (Sharma *et al*., 2013). Non digestible carbohydrates are not digested by humans itself and gut bacteria are capable to digest fiber and harvest energy for the cells that lining on the surface of the colon. Liver and gut microbiota are linked with one another (Kalliomaki and walker, 2005) as liver provides eventually immune response to microbes and their components also their outer and inner toxins exist in portal blood supply and protect from unusual gut-derived toxin products. It also distributes various substances to intestine through internal hepatic circulation and bile (Carmine *et al*., 2014). Treatment with probiotics is promising for management of NAFLD/NASH through targeting the gut liver-axis.

2.5. Function of probiotics for management of NAFLD

Probiotics are identified by FAO (Food agriculture organization)/WHO (world health organization) as “Live microorganisms”, when it is supplemented in defined dose that impacts positive on the health of host” and also alters the IM composition including interacts with immune response or gut epithelium.It is recent evidence from various studies that probiotics have anti-inflammatory and positive metabolic effects.

Modulation of the gut microbiota entitles a new effective therapy for NAFLD (Aller *et al*. 2011; Loguercio *et al*. 2002; Solga *et al*., 2008 and Vajro *et al*., 2011). Probiotic VSL#3(De Simone Formulation) is a mixture of eight Probiotic strains *(Bifidobacteria [B. breve, B. infantis, B. longum] Lactobacillus acidophilus, L. plantarum, L paracasei and L. delbrucki subsp. bulgaricus, Streptococcus thermophilus).*Few studies with Probiotic VSL#3 in animal or human models with NAFLD have been conducted by various researchers (Alisi *et al*., 2012; Li *et al*., 2003; Mancerlli *et al*., 2012; Mancerlli *et al*., 2011and Velayudham *et al*., 2009).

In animal models (Bathena *et al*., 2013) demonstrated that *Lactobillus fermentum* ATCC significantly reduced liver fat accumulation, decreased total cholesterol, TG, uric acid and IR.

(Yadav *et al*., 2013) recently, demonstrated that VSL#3 in rats modulated the gut flora composition i.e. (decreased firmicutes and enhanced bacteriodetes and bifidobacteria) encouraged the production of SCFAs like butyrate, that has a positive beneficial effect on weight loss in obese mice and major source of energy for bacteria in the colon. Changes in the gut microbiota composition changed the peptide hormone (YY) of gut that regulates food intake, having anorexigenic effect and also decreases water or electrolytes absorption in the intestine. This hormone is directly concerned with dietary fiber that gives satiety value and improves the absorption of nutrients that prevents obesity, IR. Although, acetate is the antagonist short-chain fatty acid that are generally increased in obese rats and also increase hunger hormone for food. High fat and fructose diet is consistent with higher intestinal level of acetate cause rise in TG, cholesterol, IR. Acetate producing bacteria are responsible for increasing the circulatory levels of acetate in the intestine. It has been found commonly in obese mice because of high intake of fat and also disturb IR. In mice models various studies showed that VSL#3 and other probiotics were effective for reducing inflammation, fibrosis and declined the steatohepatitis and also protected NASH and dyslipidemia (Li *et al*., 2003; Esposito *et al*., 2009; Ma *et al*., 2008;Velaudham *et al*., 2009 and Menacarelli *et al*., 2012).

In human, one study used Probiotic (bioflora: 4 tablets/day) in 10 adult NAFD patients for 2 month resulted in significantly decreased ALT and GGT (Marzuillo *et al*., 2014). (Loguercio *et al*., 2002 and Loguercio *et al*., 2005) concluded that VSL#3

contains 450 billion bacteria used in 22 patients for 120 days openly labeled significantly attenuated plasma malondialdehyde (MDA) and also 4-hydroxynonenal (results from lipid peroxidation of polyunsaturated fatty acid and marker for oxidative stress). (Vajro *et al*., 2011) performed RCT for 2 months with using Lactobacillus GG: 12 billion /day in 10 pediatric obese controls and 10 obese patients resulted in significantly reduced aminotranferase and anti- peptidoglycan-polysaccharides and TNF-α.

(Wong *et al*., 2013) recently, performed RCT for 6 months with using lepicol 10g/day in 10 adults non-alcoholic steatohepatitis (NASH) controls and 10 adult NASH patients resulted in significantly reduced AST and changed interahepatic triglyceride content (IHTG). (Alisi *et al*., 2014) recently, performed RCT of VSL#3 Vs placebo in obese children with histology examination in NAFLD concluded that VSL#3 improved NAFLD and glucagon-like peptide (GLP-1) significantly also decreased BMI or improved ultrasound fatty liver scores with 4-months trial.

(Malaguarnera *et al*., 2012) performed randomized control trial (RCT), concluded that bifidobacterium longum with lifestyle intervention enriched with B vitamins improved fibrosis and reduced HOMA, cholesterol, TG, AST, ALT, TNF-α. (Shavakhi *et al*., 2013) used probiotics and metformin in 36 adults with NASH found that approach was effective for reducing ALT and BMI. (Cani *et al*., 2007) suggested that gut microbiota has major role in pathogenesis of NAFLD and NASH. Most of the higher firmicutes in gut suppress various beneficial bacterial activities which cause derangements of metabolic process and increase various biomarkers i.e. lipid, liver enzymes and IR. (Xiao *et al*., 2003) concluded that fermented milk products enriched with Bifidobacterium longum were effective for reducing lipids levels in adults. Another study of (Ejtahed *et al*., 2012) resulted that probiotic yogurt enriched with Bb12 and La5 ameliorate higher blood glucose levels.

(Mouli *et al*., 2015) performed RCT in liver disease with use of VSL#3 was effective for treating hepatic encephalopathy (HE) in adults. (Dhiman *et al*., 2014) concluded that VSL#3 was potent probiotic for managing HE with reduction of hospital stay of adult patients. (Rincon *et al*., 2014) proved that oral dose of VSL#3 reduced the force of circulating disturbance in cirrhotic patients with ascites. (Lunia *et al*., 2014) inferred that probiotic therapy prevented liver damage. (Gupta *et al*., 2013) invented

that pharmacology approach VSL#3 had effective role on portal haemodynamics of cirrhotic patients. (Yang *et al*., 2003) investigated that probiotic therapy managed the NAFLD in adults. Another study of (Gupta *et al*., 2010) suggested that probiotic therapy with propranolol reduced variecal bleeding in prophylaxis. Marlicz *et al*., 2010) investigated that multistrain of probiotics were effective with decompensated and compensated cirrhosis. (Marlicz *et al*., 2009) invented in another study that short term therapy with higher quantity of VSL#3 modulated gut microbiota composition and had no effect on cytokines leads to pathogenesis of hepatic renal disease.

2.6. Function of Lifestyle intervention for managing NAFLD

It has been proved that diet of obese children with NAFLD is rich in fructose (HFCS), red meat, saturated fat and cholesterol and less consume fiber, fish, seeds, nuts enriched with vitamin E (Angelika *et al*., 2014; Gentile and Pagliassotti, 2008; Leclercq and Horsmans, 2008; Spandaro *et al*., 2008). These children consume more fructose rich processed food and beverages soft drinks: soda, coke, pepsi usually carbonated and canned juices. HFCS usually contains 53%-55% fructose and 42% glucose. Children consume soda drinks that rich in fructose and more calories. It is very difficult to lose weight and defined therapy along with lifestyle modification could give best results. Currently authors have been concentrated on antioxidants, omega-3 fatty acids, dietary fructose and pre/probiotics for improving hepatic steatosis (Le and Bortolotti, 2008 and Zivcovic *et al*., 2007). (Jin *et al*., 2014) demonstrated that reduction of dietary fructose in American adolescents with NAFLD improved cardiovascular disease risk, insulin sensitivity, and high sensitivity C - reactive protein and oxidation of low density lipoproteins.

Lifestyle intervention entitles first line treatment in children with NAFLD. (Nobili *et al*., 2008) demonstrated in RCT that 12-month lifestyle intervention with diet and increased physical activity induced average weight loss of 4.75kg and also significant improvement in liver histology and abnormal laboratory levels in pediatric NAFLD. (Koot *et al*., 2011) showed that a lifestyle intervention (dietary change, physical activity and behavioral modification) of 6 months in 144 children with NAFLD significantly ameliorate hepatic steatohepatitis and serum aminotransferases. (Groanbaek *et al*., 2012) observed that a 10 week “weight loss camp” (moderate exercise for 1 hour/day and energy restriction) in 117 obese children resulted in an average weight loss of 7.1kg and better the ultrasonography scores of liver steatosis

and reduced liver transaminases and insulin sensitivity.

(Africa *et al*., 2016) prescribed low calorie diet to all NAFLD children during entire study, each patient received diet: carbohydrate 50-60%, fat 23-30% (Saturated fat two third& unsaturated fat: one third, omega-6 or omega-3 ratio 4:1), protein 15-20% for total calories 25-30 kcal/day. In spite of that, a moderate aerobic exercise (30-45min at least 3 times a week) was also recommended and was tailored according to individual's tendency. Therefore, weight loss is an effective remedy for NAFLD in children. Thus, diet with exercise can be suggested as the first–line of safeguard for preventing the onset of hepatic steatosis and NASH.

(Wang *et al*., 2008) suggested that low calorie diet with exercise is effective rather than Vitamin E therapy alone. It is first approved approach in managing obesity and related its disorders i.e. NAFLD, metabolic syndrome.

(Pacifico *et al*., 2013) found in their study that lifestyle intervention plan improved the visceral fat (central adiposity), improved arterial functions related with NAFLD but LDL, HDL and cholesterol were insignificant within one year of follow up. (Reinehr *et al*., 2009) demonstrated that lifestyle modification improved liver enzymes and had weight loss with follow up of one year intervention. (Moschen *et al*., 2010) found that weight loss of 7% with Hypocaloric diet and exercise was an effective treatment in NASH but not effective for IR in NASH patients in 12 months period follow up. (George *et al*., 2009) found that mild and moderate lifestyle modification was effective for improving simple steatosis and its risk factors. (Verduci *et al*., 2015) demonstrated that lifestyle behavior modification was significantly efficacious for reducing TG and increase in HDL-c but not significantly effective for LDL-c, total cholesterol in one year follow up in obese children. (Kelishadi *et al*., 2014) showed that lifestyle intervention i.e. dietary habits and eating pattern changes improved HDL-c not cholesterol or LDL-c and had no significant changes in anthropometric measurements in obese children. (Vos *et al*., 2016) found that long term follow up of diet and exercise was fruitful for controlling central adiposity including blood sugar and not significant changes were seen in inflammatory biomarkers.

(Ranucci *et al*., 2017) demonstrated that multidisciplinary family based strategy was effective for improving children's health including change in their eating pattern and

increased physical exercise. (Reinehr *et al*., 2009 and Promrat *et al*., 2010) showed that lifestyle intervention was efficacious for weight loss and might improve the metabolic syndrome risk factors and another study also suggested in RCT that combined therapy of diet with exercise including behavior changes regarding eating habits in 48 weeks improved the severe form of NAFLD/NASH and also changed NAFLD grading scores. Decrease in weight promoted the reduction in the prevalence of "X" syndrome. (Jiao *et al*., 2018) conclude that dietary intervention with exercise reduced BMI, WC, blood pressure and other lipid profile i.e TG, cholesterol except blood glucose fasting in obese boys aged from 11-13 years. (Jeon *et al*., 2016) showed that combined exercise of walking and band exercise was effective approach for treating markers of metabolic syndrome in obese children. (Iniguez *et al*., 2014) suggested that nutrition education and behavior modification strategy was potent for motivating the mothers about healthy cooking practices to inculcate such a good dietary habits in obese children with NAFLD that prevents its risk factors and another study (Brownell *et al*., 1982) defined that school based nutrition counseling is effective for modifying the behavior related to dietary pattern in obese children. Recently, (Brandon *et al*., 2018) suggested that supplementation of Vit E is effective with dietary intervention for treating NAFLD and Adamo *et al*., 2013) showed that combine therapy of Vitamin E with lifestyle intervention was potent therapy for managing hepatic steatosis and cardio- metabolic dysfunctions in obese children.

2.7. Obesity hormones; Leptin and Ghrelin

Food intake and weight gain are mediated by Leptin and ghrelin hormones in humans. These hormones have major influence on energy balance. Leptin basically reduce food intake, prevent weight gain and maintain the energy levels for long time (Klok *et al*., 2006 and Schwartz *et al*., 1996). On the contrary, ghrelin is a hunger hormone works fastly also called orexigenic hormone (Levie *et al*., 1999). In obese person this hormone generally has been found lower than to normal healthy individuals. It is a peptide hormone and associated with insulin sensitivity and commonly seen its lower levels in NAFLD patients (Marchesini *et al*., 2003 and Estep *et al*., 2011). On the other hand, leptin is increased in obese person and lower in healthy person.

Leptin is an OB gene product and also called anorexigenic hormone: lack of appetite. Leptin hormone helps in reducing the appetite while ghrelin increases (Pellymounter *et al*., 1995). Abnormalities in these hormones leads to various obesity related degenerative diseases. Leptin influence on various internal systems such as metabolism, immunity, inflammation process, wound healing and formation of bones (Schwartz *et al*., 1996 and Montague *et al*., 1996). Adipose tissues release leptin hormone which binds on to the receptors of hypothalamus by crucifix blood brain barrier. Ghrelin hormone secreted by stomach and food intake mediates through hypothalamus. Abnormalities in these hormones possibly increase the obesity and its related disease. Leptin levels are commonly seen higher in females as compared to males. It increases within age and higher sugar and fatty foods intake increases the serum leptin levels and enhances energy stores in the body. Energy intake equals to energy expenditure. (Schwartz *et al*., 1996) showed that serum leptin was higher in patients who had higher BMI and excess body fat percentage. Leptin decreases by exercise, low fat with low carbohydrate diet. Another study resulted that overeating caused the excess of adipose tissues and increased the levels of leptin in blood stream. Most interestingly, leptin signals the brain barrier center to inhibit food intake and maintained the energy equilibrium. Studies in mice and humans showed that leptin involved in maintenance of energy intake and stimulate the basal metabolic rate. (Montague *et al*., 1997) showed proved the scientific fact that leptin regulates the food intake through sending signals to hypothalamus and avoid an excessive eating in humans. (Farooqi *et al*., 2001) concluded that serum leptin was increased in obese volunteer because they had leptin deficiency as compared to controls. Treatment with leptin could reduce appetite including exercise which prevents weight gain and improved endocrine functions and its disorders. Furthermore (Weigle *et al*., *2003)* showed that restriction in intake of dietary fat reduced the leptin levels and body and percentage in humans. Most recent studies indicated that leptin plays a role in meal initial and also controls the meal portion size. Fasting for 36 hours indicated that decrease the levels of leptin hormone (Pico *et al*., 2003). Ghrelin is also called orexigenic hormone which is affected by various factors i.e. age, gender, food intake, BMI and growth hormones. It has been seen that ghrelin decreased after postprandial and increased in preprandial (Kolaczynski *et al*., 1996). A recent study showed that leptin and ghrelin worked independently in maintaining the energy levels and leptin did not regulate the ghrelin levels. (Chan et al., 2004 and Tschop *et al*., 2000)

reported that ghrelin seemed to be involved in the regulation of food intake in mice. (Cummings *et al*., 2004) demonstrated that a preprandial level of ghrelin was correlated with BMI levels and hunger scores. This hormone also deploys pro-inflammatory and pro-fibrogenic in liver to toxic hepatic chemicals. Many studies found that leptin and ghrelin could be a potential therapeutic treatment for energy homeostasis and food intake regulation (Westerterp-Plantenga *et al*., 2001). Unfortunately, Leptin treatment showed less beneficial effects on leptin deficit obese people (Heymsfield *et al*., 1999). (Rosenbaum *et al*., 2005) showed that daily dose of leptin in addition to diet could prevent the weight gain and reduced food intake. Prospective beneficial effects of ghrelin administration are under discussion in obesity (Tschop *et al*., 2001). Portion of meal with composition of dishes and extra serving sizes influence leptin and ghrelin hormones levels. Obese people have leptin resistance and no control on their diet and no satiety value for food. High carbs with lower fat also increase the circulating levels of leptin despite high fat diet with low carbs meal lower the circulating levels of leptin within 24 hours (Havel *et al*., 1999). Some high protein diet and guar gum fiber are also famous for weight loss programme but it has not shown any effect on the circulating levels of leptin concentration (Heini *et al*., 1998). It has been seen that low fat diet is effective and suppress the ghrelin levels (Monteleone *et al*., 2003). A similar study has been resulted that low fat and high carbs diet reduced weight without exerts the orexigenic hormone levels (Kennedy *et al*., 1997). Another study showed that high carbohydrate diet was sufficient to reduce the ghrelin levels and gave satiety and reduced hunger pangs (Roberts *et al*., 1997 and Aprath-Husmann *et al*., 2001). It's a controversial that leptin and ghrelin are dependent to each other or independent hormones act differently related to meal size and composition (Attele *et al*., 2002). High protein diet gives satiety especially in morning promotes weight loss also but found some conflicts that it had no effect on ghrelin. High fiber diet promoted weight loss in various studies suggested especially soluble fiber that have water holding capacity i.e. guar gum, psyllian reduced the hunger hormone and maintained blood sugar levels (Anania *et al*., 2018). Weight loss through lifestyle intervention is first prime importance for everyone if it's not working for some person and less compliance in children so they can take leptin and ghrelin as drug therapy for controlling their satiety and hunger, which is sometime out of control.

Agouti related protein in hypothalamus which responds to feeding behavior and also called appetite stimulator. It has been suggested that AgRP decreases satiety and slow down the metabolism. It is basically suppressed by leptin and generated by ghrelin hormone. During fasting period AgRP levels are high. AgRP has role in obesity. Over secretion of this peptide in mice causes excessive hunger and obesity (de Piano *et al*., 2010). It is commonly found in obese male. Surprisingly, on acute stress moment it has been found to be regulated down.

Most importantly, obese patients are leptin deficient and excessive ghrelin secretion in them. Their body has no control on these hormones. They follow high calorie diet with mixture of high carbs and fat that imbalances various hormones related to obesity in the body. It increases the size of fat cells in adipose tissues. If it is not dealt with time then leads to obesity related various degenerative diseases. Healthy diet with combination of exercise regulated these hormones and body weight. Reduction in body weight and controlling the hormone imbalance is possibility of changes in gut microbiota composition. Gut health improves and maintain the gut integrity including reduction of intestinal porosity. Excessive acetate production is very harmful for body it happens generally when person is obese. Most of the ethanol break down in the liver by alcohol dehydrogenase (ADH) enzyme which changes in to aldehyde and convert it to carcinogen substances. HFCS is sweet toxin for human body and highly processed found in soft drinks and other junk food. Excessive fructose thought to be not metabolizing by liver perhaps it leads to liver inflammation and steatosis. Change in gut microbiota composition is a new effective defined treatment for managing gastrointestinal diseases. In fact, it depend the diet of person enriched with prebiotics and also probiotics.

CHAPTER-3

MATERIALS AND METHODS

The Present study was taken out in the community of Punjab, and carried out under the School of Agriculture, Lovely Professional University, Phagwara, Punjab.

3.1. Study Design: A Randomized controlled trial (RCT) was performed in an area of Punjab, India from January, 2017 to October, 2018.

3.2. Sampling:

Sample size formula for medical studies (Pouhoseingholl *et al*., 2013):

$$n = Z^2 \frac{P(1-P)}{d^2} \quad (1)$$

Equation (1) state that Z is the statistic corresponding to level of confidence, P is expected prevalence and d is precision (corresponding to effect size) & 95% confidence interval (CI).

3.3. Settings: 500 children were defined by BMI in schools and households of Jagraon and Daudhar and 340 were excluded because of not matching the inclusion criteria and also refused to give blood sample. All defined anthropometric, biochemical parameters and ultrasonography (USG) were performed in fasting at Babe Ke Medical Hospital Daudhar, Moga, Punjab, India. Finally, 160 obese children were identified for study in which 54 obese children without NAFLD were excluded by USG.106 obese children diagnosed with NAFLD out of 160 obese children in our study. The scheme of study (Consort flow chart) is presented in Fig-3.1.

3.4. Inclusion criteria: Obese children age ranging from 5-18 years with BMI of $\geq 95^{th}$ percentile for age and gender using WHO standard reference for standard deviation Z score and without alcohol intake were selected in present study.

3.5. Exclusion criteria: Secondary obesity, wilson disease, patient on medications, hepatitis B(HBV), hepatitis C(HCV),hepatitis A&E, autoimmune hepatitis(AIH).

3.6. Intervention duration: 4 months.

3.7. Ethics: Ethical committee of Post Graduate Institute of Medical Education and Research Chandigarh, India ethically approved the study on 27 October, 2016 (protocol no. 2016/2608) and registered in Clinical Trials Registry India (CTRI/2017/12/010997). Informed written consents were taken from the parents/children selected for the study before assessment and all principles in Declaration of Helsinki were followed for conducting all concerned clinical investigations during entire study. Confidentiality of the data and privacy of children was maintained as per ethical norms.

3.8. Diagnosis: Ultrasonography (USG) (convex transducer 2-5MHz probe) was performed by expert radiologist by using the following standard criteria: Mild steatosis (Grade-1), Moderate steatosis (Grade-2,) Severe steatosis (Grade-3). USG detected (n=106) obese children with NAFLD, they were randomly divided in to four groups to receive interventions: **1**. VSL#3 capsule (De Simone Formulation) with lifestyle intervention (Diet +physical activity) (n= 26), **2**. VSL#3 capsule only (n=27), **3**. Lifestyle Intervention only (n=26) and **4**. Placebo (corn flour) (n=27).

3.9. Nutritional Assessment:

a) Anthropometric measurements:

Digital weighing scale was used to the nearest 0.1kg with the capacity of 180. Standard non-flexible measuring tape was used for measuring height, mid arm circumference (MAC) and waist circumference (WC) to the nearest of 0.1cm. BMI (body mass index) was calculated by weight (kg)/height (m^2). Z-BMI (Z score) was calculated from WHO reference data (WHO reference; 5-19 years, 2007).

1SD-2SD = Overweight

2SD-3SD = Obese

>3 SD = Extreme Obese

TSF (triceps skinfold) thickness was measured to the nearest 0.2 mm by skinfold caliper.

b) Following biochemistry parameters were tested	**Reference range**
1. Serum alanine aminotranferase (ALT)	8-40 U/L
2. Serum aspartate aminotranferase (AST)	5-35 U/L
3. Gamma-glutamyl-transferase (GGT)	5-40 U/L
4. Uric acid	3-6.0 mg/dl
5. Blood glucose fasting	<110 mg/dl
6. Cholesterol	150-200 mg/dl
7. High-density lipoprotein (HDL)-cholesterol	30-65 mg/dl
8. Low-density lipoprotein (LDL)-cholesterol	<100 mg/dl
9. Triglycerides	<160 mg/dl
10. C-reactive protein	5-9 yrs : <2.8 mg/l >10 yrs : < 5 mg/l
11. Leptin	10-16 ng/ml
12. Ghrelin	3.7-120 ng/ml

c) Clinical investigation:

Tulip diagnostics (P) Ltd. kits were used for diagnostic procedure (LFT, Lipid, FBG, uric acid, CRP) using Tulip Biochemistry analyzer (Biochemical Systems International Srl made in Italy, model: 3000 evolution, Cod: RM4030, Serial no. 36636). 5 ml fresh fasting blood sample was drawn from each child by lab technician at Babe Ke Medical Hospital Daudhar, Moga, Punjab, India. CETEC Software was used for calculating the readings of each test. Obesity hormones: serum ghrelin was estimated by using ELISA (enzyme-linked immunosorbent essay) kit; Qayee bio for life sciences and serum leptin was also estimated by DRG Leptin. Tests were performed before and after intervention during study.

d) Dietary recall:

Three day dietary recall was taken to evaluate their dietary intake. Average three days intake of calorie, protein, fat, carbohydrate (CHO) and fiber was calculated before and after intervention by using manually and DietCal.exe software a tool which is used for dietary assessment and planning (version-1).Their baseline calorie and after calorie

was compared with revised RDA calorie (recommended dietary allowances for Indians) released in 2010 (RDA for Indians, 2010).Self-reported questionnaire was used to collect data on their lifestyle pattern: food habits and physical activity of participant's were examined which indicated the consumption of junk food especially soft drinks (coke, sprite mountain dew, fanta, pepsi), french fries or fried chips and others (pizza, burger, noodles) before intervention on daily basis. Physical exercise was examined among obese children before and after intervention.

Table 3.1. ICMR, Recommended dietary allowances kilocalorie per day for Indian children and adolescents released in 2010

Group	Category	Average Energy(kcal)/day
Children	7-9 yrs	1690
Boys and Girls	10-12 yrs	2100
Boys and Girls	13-15yrs	2540
Boys and Girls	16-17 yrs	2730

3.10. Lifestyle Intervention:

Hypocaloric diet was suggested consisting carbohydrate of 50-60%, fat 23-30%, protein 15-20% of total calories referring the protocol described by (Africa *et al*., 2016) Refined carbohydrate and excess saturated or trans-fatty acids were restricted including recommendation of ample amount of fresh fruits and vegetables. Lifestyle interventional group and VSL#3 plus lifestyle intervention group advised to follow diet plans and diet counseling session were also conducted for these groups. Diet was tailored to individual's economic status, preference and according to children age group as per guidelines of ICMR for Indians in 2010 with moderate programme of aerobic exercise (swimming, brisk walking, skipping rope, dance & playing tennis) and 30-45 minutes/day exercise was suggested. It was recommended initially for at least 3 days a week and gradually increased to 5 days a week.

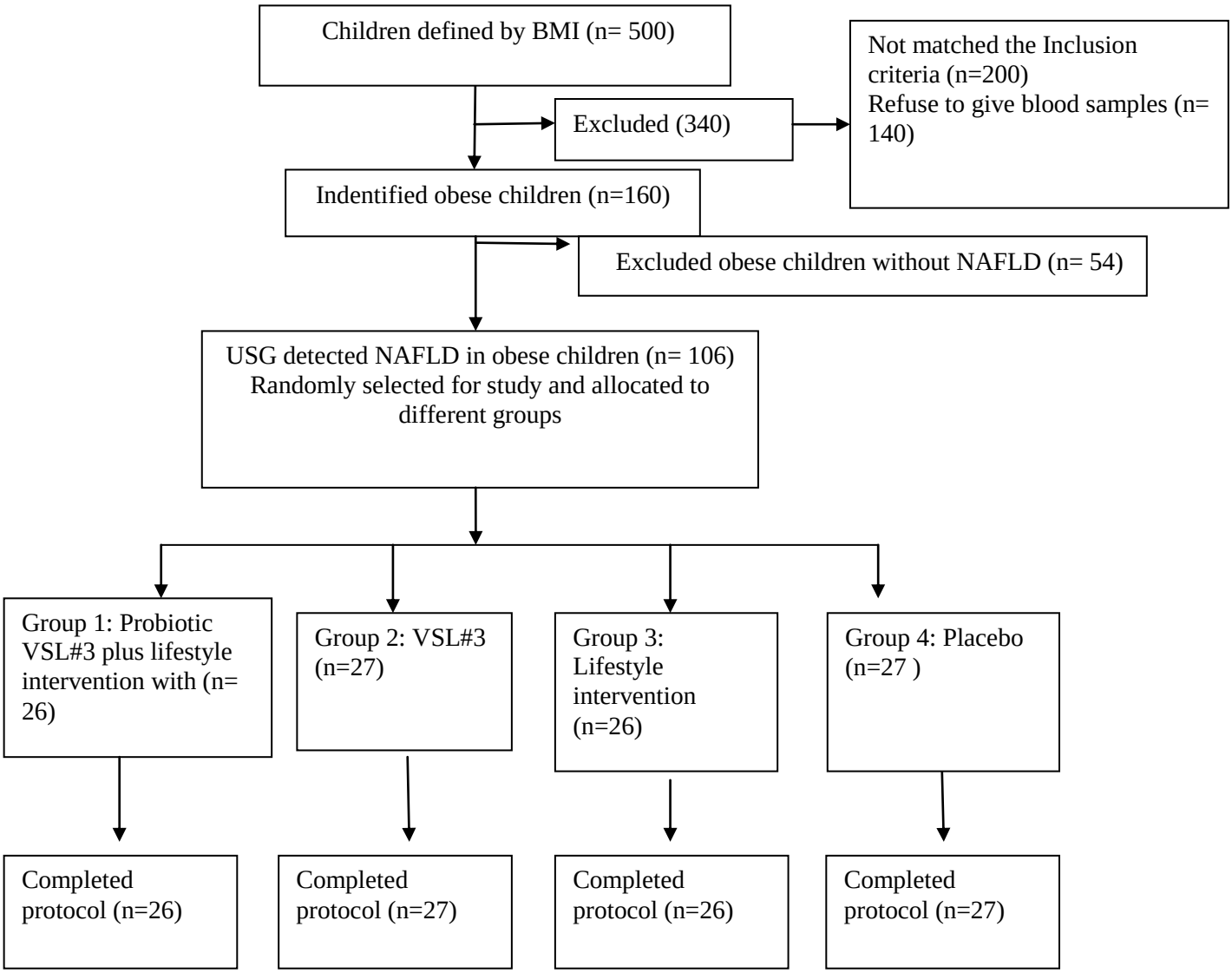
3.11. Probiotic VSL#3 (De Simone Formulation) intervention:

Probiotic VSL#3(De Simone Formulation) contains 112.5 billion colony forming units (CFU) of following 8 bacterial strains; live freeze-dried lactic acid bacteria and bifidobacteria. Dosage form contained lyophilized powder in individual capsules which is a mixture of 8 probiotic strains: *Streptococcus thermophilus DSM24731,*

Bifidobacterium breve DSM24732, Bifidobacterium longum DSM24736, Bifidobacterium infantis DSM24737, Lactobacillus acidophilus DSM24735, Lactobacillus plantarum DSM24730, Lactobacillus paracasei DSM24733, Lactobacillus delbrueckii ssp. bulgaricus DSM24734. It can be stored at room temperature for up to two weeks without adversely affecting their potential and stored at 2-8°C (refrigeration) and 2 years of shelf life from manufacturing date. Parents were instructed to keep capsules in refrigerator and avoid any other probiotic or antibiotic medications. Oral dosage of VSL#3 capsules prescribed by physician according as per requirement of child before meals or placebo capsules (corn flour) was identical of experimental capsules in shape, color and size. 40 mg of corn flour was used for each placebo. Children were randomized to receive treatment in each group. Duration of treatment was four months. No side effects or health hazards of VSL#3 in children during study were observed.

3.12. Special advice: All groups were instructed to avoid all junk food, curd and yogurts and other medications during study period for avoiding bias in the study. Home-based diet was advised to everyone. Specific intervention groups were advised to follow diet plans with exercise and take probiotic VSL#3 for 16 weeks only.

3.13. Statistical analysis: Data has been demonstrated in mean±SD and SPSS software version-21 used for statistical analysis. Kruskal-wallis test was applied for assessing the difference in anthropometric variables of different intervention group, similarly it is applied to check pre and post levels of obesity hormones in obese with NAFLD in different interventional groups. Pearson chi-square test used to find out relation between various variables: gender, liver grades, and frequency of consumption with type of junk food item and physical exercise among obese children with NAFLD amid various interventional groups in before and after treatment. Multinomial logistic regression analysis was used for variables associated with prediction of fatty liver among obese children. Wilcoxon signed rank test was also applied to analyze before and after treatment outcomes. Changes in various anthropometric and biochemical variables in probiotic and lifestyle similarly, in other groups probiotic and placebo were compared by using Mann-whitney U test. P value <0.05 was contemplated statistically significant and 95% confidence interval (CI) considered for statistical analysis.



CONSORT Flow Chart for Study Protocol

Fig 3.1 SCHEME OF STUDY

CHAPTER- 4

RESULTS & DISCUSSION

The current research was taken out in the community of Punjab, and associated with the School of Agriculture, Faculty of Technology & Sciences, Lovely Professional University, Phagwara, Punjab during 2015-2019. The results and discussion have been explored under following headings:

4.1. Demographic and anthropometric details of obese children in different interventional groups at baseline

4.2. Distribution of obese children according to different junk food items consumed over a week

4.3. Variables associated with prediction of fatty liver in obese children

4.4. Liver grades in percentage before and after intervention

4.5. Anthropometric parameters of obese children with non-alcoholic fatty liver disease

4.6. Biochemical parameters of obese children with non-alcoholic fatty liver disease

4.7. Pre and post levels of obesity hormones in obese children with non-alcoholic fatty liver disease

4.8. Nutrient intake of obese children per day with non-alcoholic fatty liver disease in different intervention groups

4.9. Comparison of RDA calorie Vs before calorie (kcal) intake in different interventional groups

4.10. Comparison of RDA calorie Vs after calorie (kcal) Intake in different interventional groups

4.11. Before intervention of physical exercise among obese children with non-alcoholic fatty liver disease

4.12. After intervention of physical exercise among obese children with non-alcoholic fatty liver disease at least 3 days a week

4.13. Comparison of changes in anthropometric and biochemical parameters for probiotic VSL#3 and lifestyle interventions

4.14. Comparison of changes in anthropometric and biochemical parameters for probiotic and placebo interventions

4.15. Comparison of changes in leptin and ghrelin parameters for probiotic and lifestyle interventions

4.16. Comparison of changes in leptin and ghrelin parameters for probiotic and placebo interventions

Table 4.1. Demographic and anthropometric details of obese children in different interventional groups at baseline

VARIABLES	VSL#3 plus lifestyle intervention n= 26	VSL#3 alone n= 27	lifestyle intervention n= 26	Placebo n= 27	p value*
Age(yrs)	12.06±1.76	11.7±2.21	11.4±1.05	11.0±1.20	0.181
Height (cm)	150±8.31	149±9.37	148±8.12	145±8.02	0.315
Weight (kg)	61.6±12.2	60.7±12.3	59.2±7.92	57.5±8.33	0.664
BMI (kg/m^2)	27.2±3.74	27.1±4.07	27±3.57	27±3.23	0.997
Female n (%)	10(38.5%)	11(40.7%)	11(42.3%)	11(40.7%)	0.994**
Male n (%)	16(61.5%)	16(59.3%)	15(57.7%)	16(59.3%)	
Liver grades in n (%)					
Mild	10(38.5%)	10(37%)	10(38.5%)	11(40.7%)	1.000**
Moderate	13(50%)	14(51.9%)	13(50%)	13(48.1%)	0.996*
Severe	3(11.5%)	3(11.1%)	3(11.5%)	3(11.1%)	

Where BMI= Body mass index,

Data represented in mean±SD & n (%)

*Kruskal-Wallis Test, p value <0.05

**Pearson Chi-Square P<0.05

In our study, Jagraon and Daudhar, area under Punjab were selected for survey on children. 500 children were defined by BMI in schools and households and 340 were excluded because of not matching the inclusion criteria and also refused to give blood sample. All defined anthropometric, biochemical parameters and USG were performed in fasting at Babe Ke Medical Hospital Daudhar, Moga, Punjab. Finally, 160 obese children were identified for study in which 54 obese children without NAFLD were excluded by USG.106 obese children diagnosed with NAFLD out of 160 obese children in our study. In current study, NAFLD prevalence rate was 66.2% in obese children as showed in (Fig 4.1).

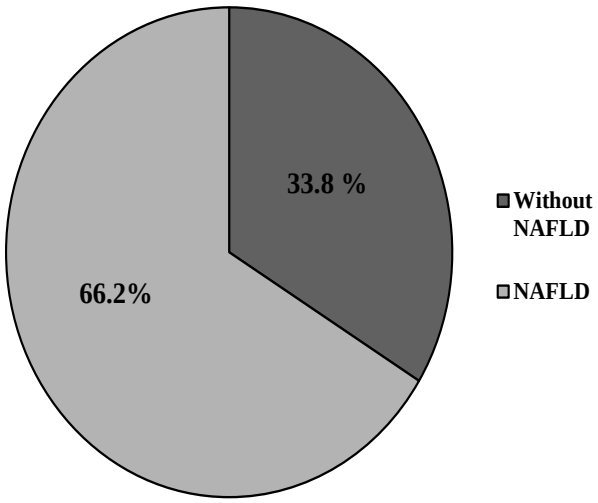


Fig 4.1 Percentage of obese children with NAFLD and without NAFLD at baseline. Values are in percentage (%).

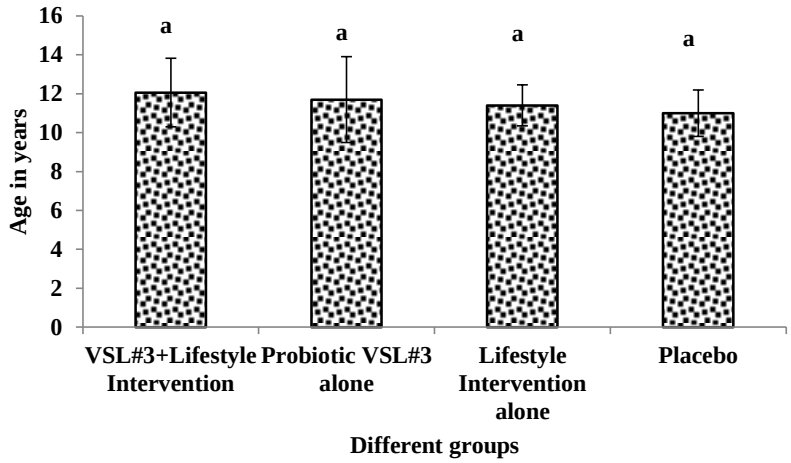


Fig 4.2 Distribution of age in different groups at baseline. Values are in mean±SD. Age was non significant in different groups at before treatment. Bars with same letter (a) defines non significant by Kruskal-wallis test, p value <0.05.

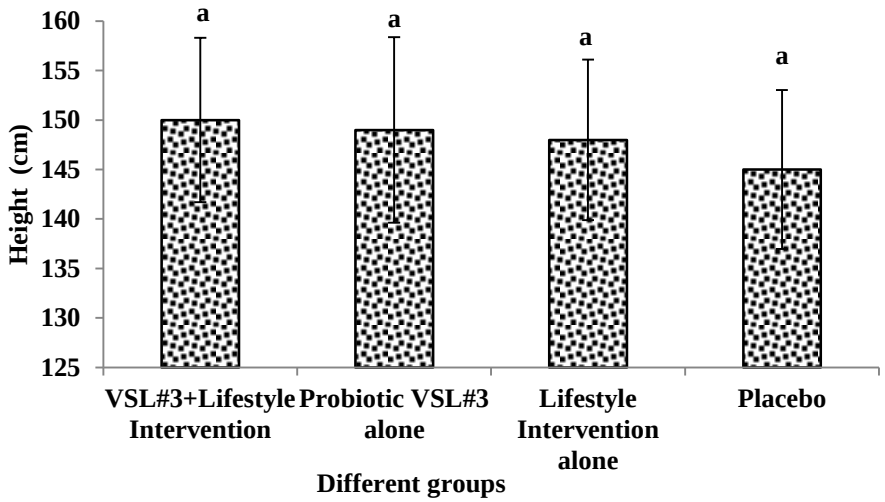


Fig 4.3 Distribution of height in different groups at baseline.Values are in mean±SD. Height was non significant in different groups at baseline. Bars with same letter (a) defines non significant by Kruskal-wallis test, p value <0.05.

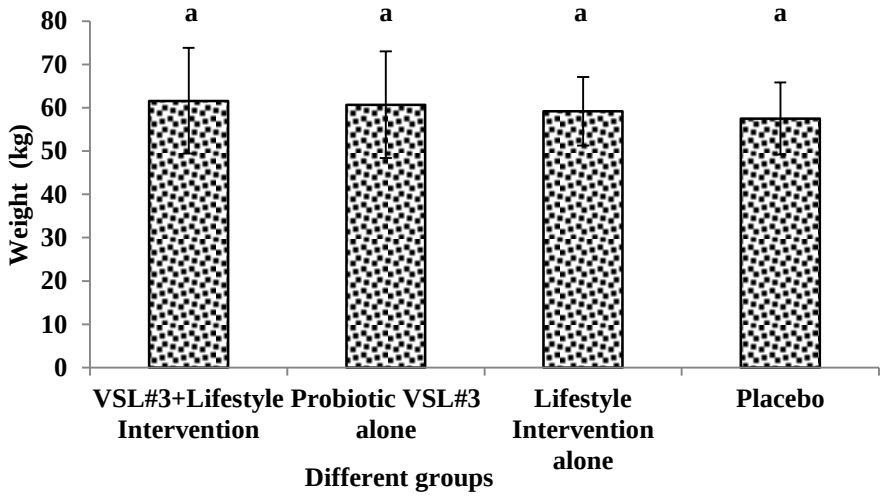


Fig 4.4 Distribution of weight in different groups at baseline Values are in mean±SD.Weight was non significant in different groups at before treatment. Bars with same letter (a) defines non significant by Kruskal-wallis test, p value <0.05.

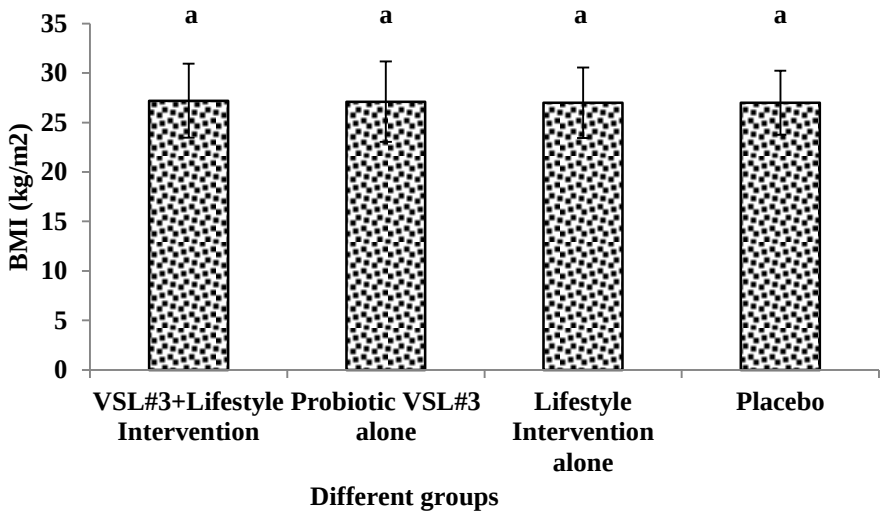


Fig 4.5 Distribution of BMI in different groups at baseline.Values are in mean±SD. BMI was non significant in different groups at before treatment. Bars with same letter (a) defines non significant by Kruskal-wallis test, p value <0.05.

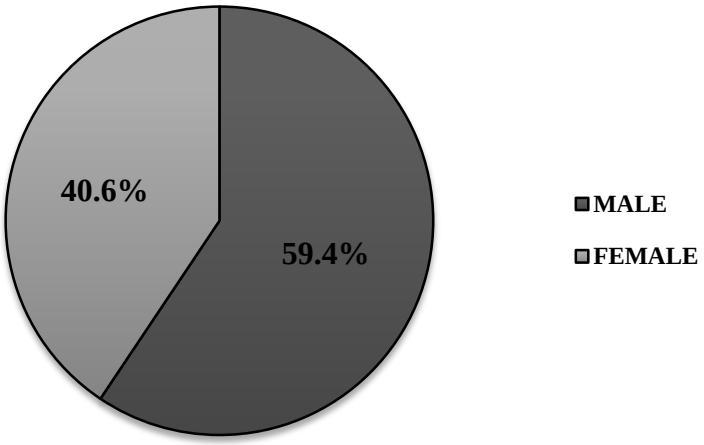


Fig 4.6 Overall male and female with NAFLD.
Values are in percentage (%).

NAFLD was present in 43(40.6%) females and 63(59.4%) in males respectively (Fig 4.6). After the screening of NAFLD in obese children they were enrolled in study and randomly allocated to different interventional four groups: **1.** VSL#3 plus lifestyle intervention, **2.**VSL#3, **3.** Lifestyle intervention and **4.** Placebo. In our study, minimum and maximum age range was 8-16 years. Average age in years was non significant at baseline in group 1. (12.06) group 2. (11.7), group 3. (11.4), group 4. (11.0) (p= 0.181) (Fig 4.2). Height, weight and BMI were non significant at baseline (Fig 4.3-4.5). In current study, overall at baseline obese children had 41(38.7%) mild, 53 (50%) moderate and 12(11.3%) severe form of NAFLD. NAFLD grades in numbers (n) among different interventional groups mentioned (Fig 4.7-4.11).

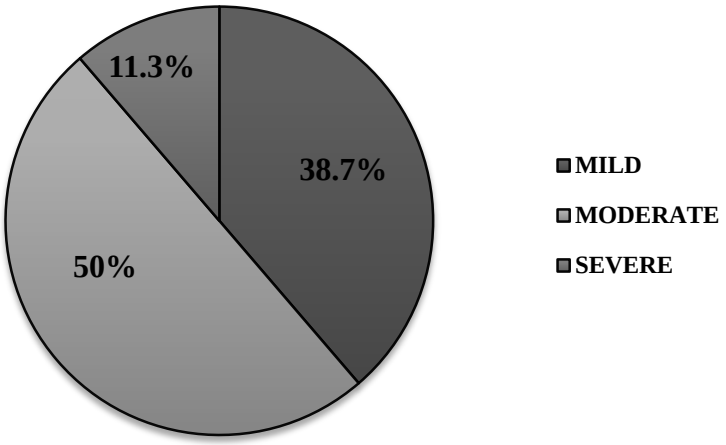


Fig 4.7 NAFLD grades among obese children.

Values are in percentage (%).

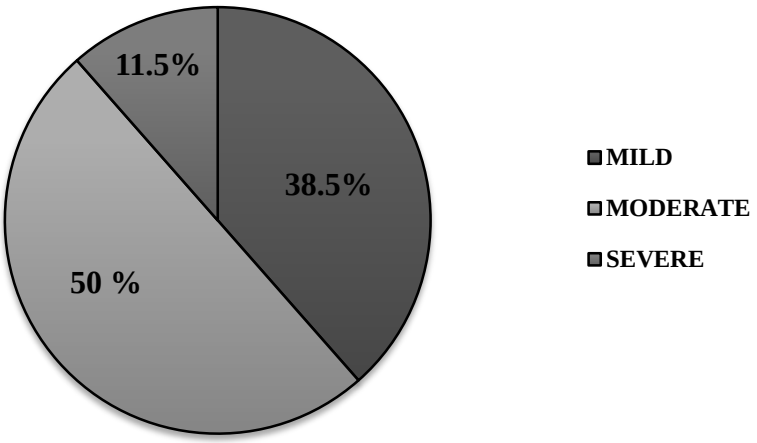


Fig 4.8 NAFLD grades in VSL#3+lifestyle intervention group at baseline.

Values are in percentage (%).

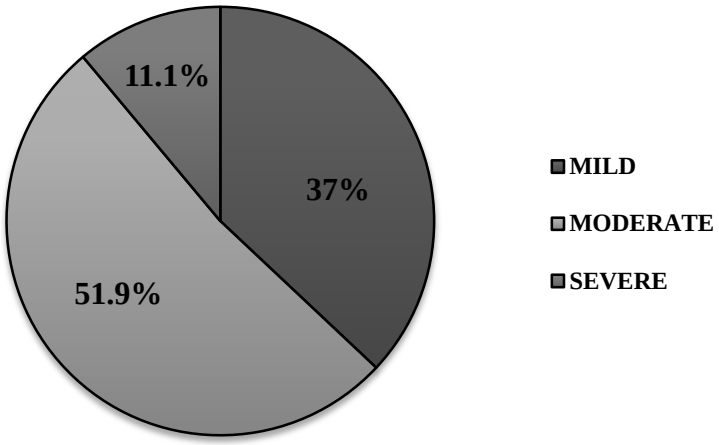


Fig 4.9 NAFLD grades in probiotic VSL#3 alone at baseline. Values are in percentage (%).

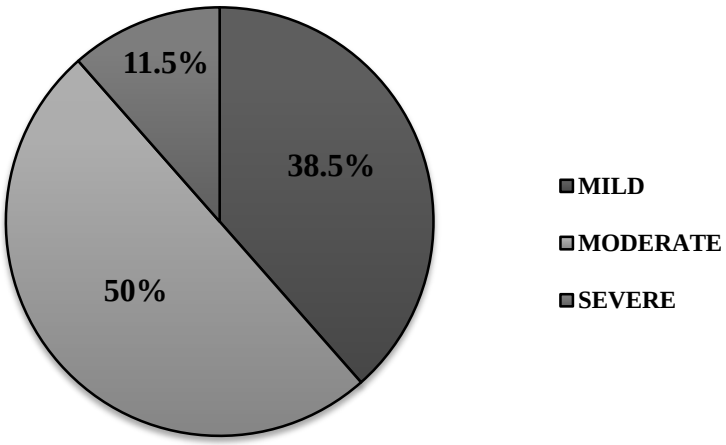


Fig 4.10 NAFLD grades in lifestyle intervention at baseline. Values are in percentage (%).

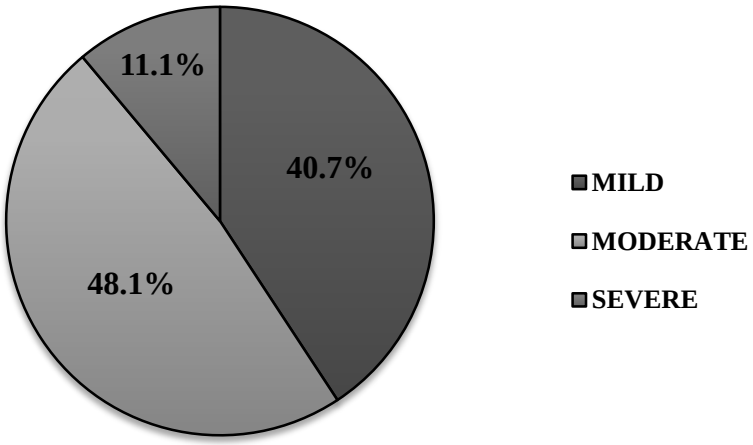


Fig 4.11 NAFLD grades in placebo group at baseline.
Values are in percentage (%).

(Freedman *et al*., 2007) concluded in his study that 69.9% overweight and obese children had fatty liver disease and its risk factors i.e. insulin resistance, dyslipidemia including high blood pressure. Another study defined 43 children with fatty liver by USG. NAFLD occurrence is 5-19% common among Asian obese children (Deeb *et al*., 2018). (Clemente *et al*., 2016) showed that overall occurrence of NAFLD has been reached about 39-70% amid obese children.

Table 4.2. Distribution of obese children according to different junk food items consumed over a week

Soft Drink Consumption			**French Fries or Fried Chips**		**Others(**Pizza, Burger, Noodles**)**	
Consumption	Without NAFLD (n=54)	NAFLD (n= 106)	Without NAFLD (n=54)	NAFLD (n=106)	Without NAFLD (n=54)	NAFLD (n=106)
Daily (%)	37	60.4	38.9	58.5	25.9	30.2
Twice (%)	24.1	16	24.1	17	42.6	15.1
Thrice (%)	38.9	23.6	37	24.5	31.5	54.7
p value	0.020*		0.063		<0.001**	

Pearson Chi-Square test p value<0.05, values are given in percentage (%)

p<0.05*

p<0.001**

Table 4.2. explains the obese children with NAFLD and without NAFLD who consumed junk food items daily, twice and thrice a week. Comparison of both the groups has discussed in this table. 60.4% children with NAFLD significantly consumed soft drinks (coke, sprite, mountain dew, fanta, pepsi) on daily basis as compared to without NAFLD group 37% respectively ($p<0.05$) (Fig 4.12). Consumption of fried chips or french fries was not statistically significant between both the groups (Fig 4.13). Others food items (pizza, burger and noodles) were significantly more consumed by NAFLD children as compared to without NAFLD ($p<0.001$) (Fig 4.13). According to findings obese children with NAFLD were significantly more inclined to take soft drinks with pizza, burger and noodles ($p<0.05$). This shows that faulty dietary eating habits and poor lifestyle leads to fatty liver disease and its risk factors. They consumed simple carbohydrates in the form of soft drinks and excess fat in the form of refined fried food.

Fructose is a simple sugar commonly found in honey and fruits but HFCS is sweetener generally made from corn starch that is broken down by some enzymes to make it in syrup form and highly processed by isomerism of glucose, lastly convert to glucose and fructose. It is available and cost effective and also approved by Food Drug Administration (FDA) for the purpose of use in soft drinks and other processed foods (FDA, 2017 and Coca Cola Mannual, 2016). HFCS-55 is commonly used in soft drinks and HFCS-65 is used in coca cola machines of freestyle (Coca Cola Mannual, 2016). HFCS-42 is commonly used in breakfast porridges (Coca Cola Mannual, 2016 and Lindsay, 2016). (Jensen *et al*., 2018) in his study suggested that HFCS is responsible for denovo-lipogenesis and blocking the β-fatty acid oxidation with decline in ATP and more uric acid levels because of increased intestinal porosity and endotoxemia. It is the best predictor of fatty liver disease. (Kim *et al*., 2017) suggested fast food diet might influence steatohepatitis and also metabolic syndrome in mice.

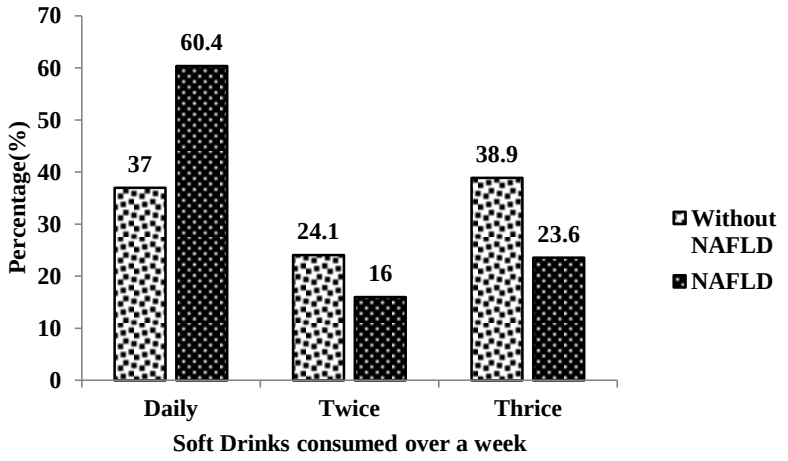


Fig 4.12 Distribution of children consumed soft drinks.
Values are given in percentage (%) for categorical data.

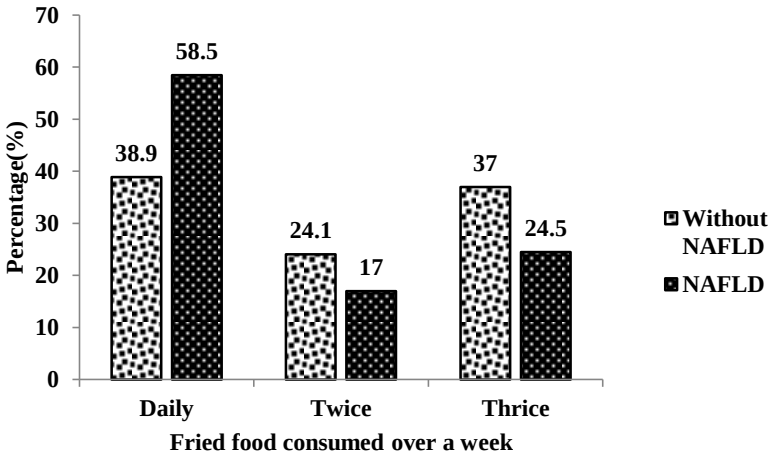


Fig 4.13 Distribution of children consumed french fries or chips. Values are in percentage (%) for categorical data.

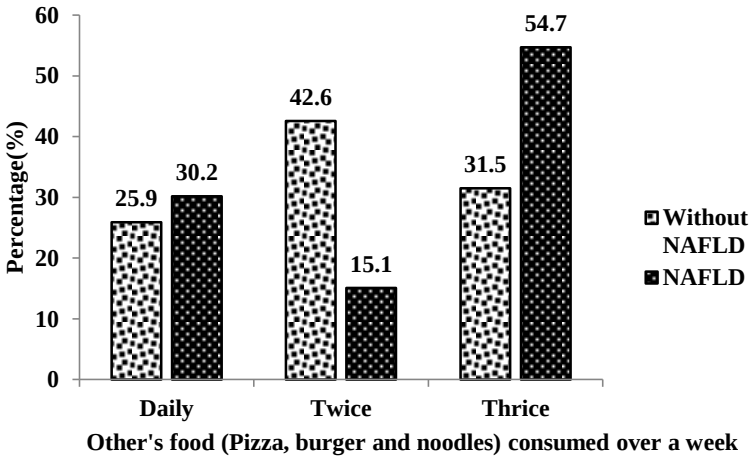


Fig 4.14 Distribution of children consumed others food. Values are given in percentage (%) for categorical data.

Table 4.3. Variables associated with prediction of fatty liver in obese children

Variables	OR*	95% CI	p value
Uric Acid (mg/dl)	2.98	1.05- 8.45	0.04
Triglyceride (mg/dl)	30.68	6.31- 149.1	< 0.001
ALT (U/L)	7.84	1.41- 43.4	0.018
BMI 2 SD	7.20	1.84- 28.1	0.005
3 SD	11.91	2.23- 63.4	0.004
Soft Drink (daily consumption)	4.11	1.40- 12.0	0.010

Where ALT= Alanine aminotransferase, BMI= Body mass index

Multinomial Logistic Regression Analysis. Abbreviations: OR*; odds Ratio, CI; Confidence Interval, p value <0.05 considered statistically significant.

Table 4.3. Predicts variables associated the fatty liver disease in obese children. In our current research, Triglyceride (OR= 30.68) is the crucial predictor of fatty liver among obese children another one best predictor was BMI- 2SD, 3 SD (OR=7.20, 11.91). These are more significant associated risk factors of NAFLD. Children who consumed daily soft drinks (1-2, standard glass-250-500ml) had higher chances of NAFLD (OR= 4.11). ALT and uric acid (OR=7.84 and 2.98) were also good predictor of fatty liver disease among obese children. (Silaghi *et al*., 2016) showed in his study that ALT, LDL-c, HOMA-IR were the independent associated markers of NAFLD through model of regression analysis. (Braticevici *et al*., 2011) suggested that CRP, age, BMI and GGT were the most potent factors to forecast biopsy proven NASH through multivariate analysis. In another study anthropometric indices i.e. WC, BMI, waist to hip ratio were cost effective, non invasive and easily accessible in Indian community studies and also predictors of NAFLD but BMI was the most effective tool to define fatty liver (Naide *et al*., 2018). Nowadays, obesity is more prevalent in young adolescents and their GGT with uric acid levels were found higher revealed by multivariate logistic regression analysis before USG investigation (Kim *et al*., 2018). In our study uric acid is the predictor of NAFLD. In some cross-sectional and prospective studies elaborated that higher serum uric acid level was related with increased level of ALT and liver damage. Fatty liver is one of the major risk factors of hyperuricemia but its mechanism is not clear yet and studies are ongoing (Zhang *et*

al., 2018). High intake of fructose sugar increases the level of uric acid after some minutes of ingestion because of ATP depletion. Some refined sugars sensitive adults or children may prone at risk of higher uric acid in blood.

Table 4.4. Liver grades in percentage before and after intervention

Fatty Liver Grades	**VSL#3 plus lifestyle intervention (n= 26)**		**Probiotic VSL#3 (n= 27)**		**Lifestyle intervention (n=26)**		**Placebo (n= 27)**	
	Before	After	Before	After	Before	After	Before	After
None (0) (%)	0.0	38.5	0.0	33.3	0.0	30.8	0.0	0.0
Grade (1) (%)	38.5	53.8	37	51.9	38.5	53.8	40.7	44.4
Grade (2) (%)	50.0	7.7	51.9	14.8	50.0	15.4	48.1	44.4
Grade (3) (%)	11.5	0.0	11.1	0.0	11.5	0.0	11.2	11.1
*****p value	p<0.001**		p<0.001**		p<0.001**		p= .317^{Ns}	

Where None= No fatty liver, Grade 1= Mild steatosis, Grade 2= Moderate steatosis, Grade 3= Severe steatosis. Values are given in percentage (%) for categorical data.

*p value calculated from Wilcoxon Signed Ranks Test

Pearson Chi-Square Test p value<0.05

p<0.001** (highly significant)

p>0.05^{Ns} (non significant)

In our current research, combined therapy of VSL#3 plus lifestyle intervention was novel treatment for managing the NAFLD in obese children. Primary outcome showed in table 4.4, in combined therapy after intervention 38.5% children had none fatty liver, 53.8% had mild and 7.7% had moderate form of fatty liver. Probiotic VSL#3 group only had 33.3% had none fatty liver, 51.9% had mild and 14.8% had moderate form of fatty liver after intervention. Lifestyle intervention only had 30.8% had none fatty liver, 53.8% had mild, 15.4 % had moderate form of fatty liver disease. Placebo group had 0.0% had none fatty liver disease, 44.4% had mild, 44.4% had moderate and 11.1% had severe form of NAFLD at the end of study. According to our findings liver grades reduced significantly (p<0.001) in three groups except placebo (p=.317) (Fig 4.15-4.18).

Overall at baseline obese children had 41(38.7%) mild, 53(50%) moderate and 12(11.3%) severe form of NAFLD and after intervention they had 27(25.5%) none fatty liver, 54(50.9%) had mild, 22(20.8%) had moderate and 3(2.8%) had severe form of fatty liver disease ($p<0.05$). In similar study (Alisi *et al*., 2014) showed that after 16 weeks of intervention of VSL#3 in Italian obese children had 21% none, 70% mild, 9% moderate and 0% severe form of fatty liver. Another study (Famouri *et al*., 2017) found that probiotic therapy was effective for improving fatty liver grades in Iran obese children, after intervention of 12 weeks, they had 53.1% none fatty liver, 25% had mild and 21.9% had moderate form of fatty liver disease .

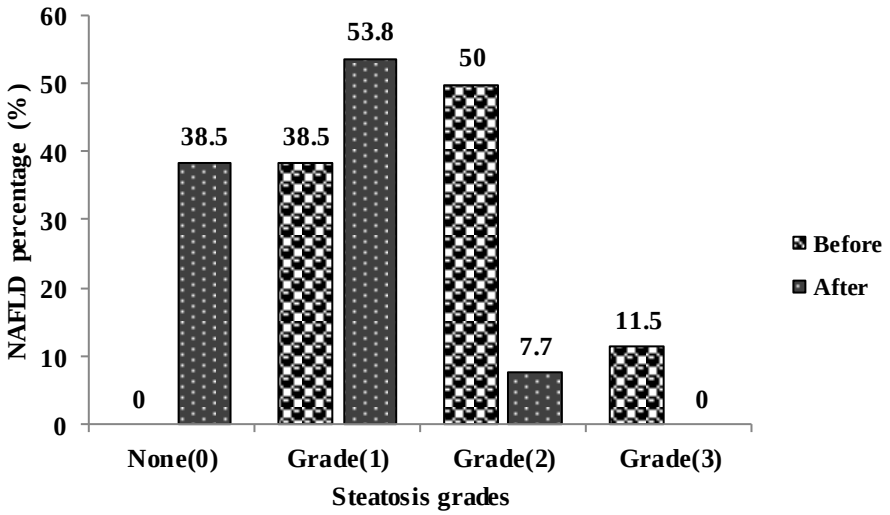


Fig 4.15 Fatty liver grades in VSL#3 + lifestyle intervention. Values are given in percentage (%)for categorical data. Where= NAFLD (non alcoholic fatty liver disease)

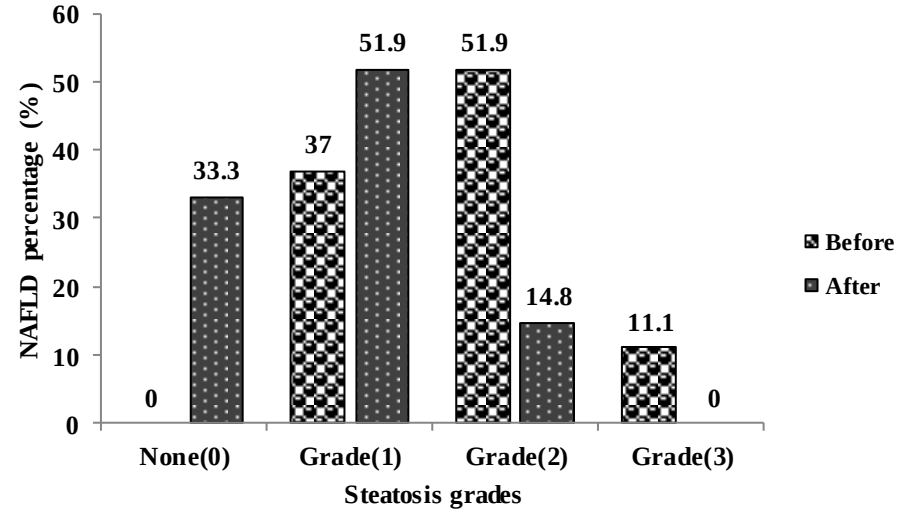


Fig 4.16 Fatty liver grades in probiotic VSL#3 alone. Values are given in percentage (%) for categorical data. Where= NAFLD (non alcoholic fatty liver disease)

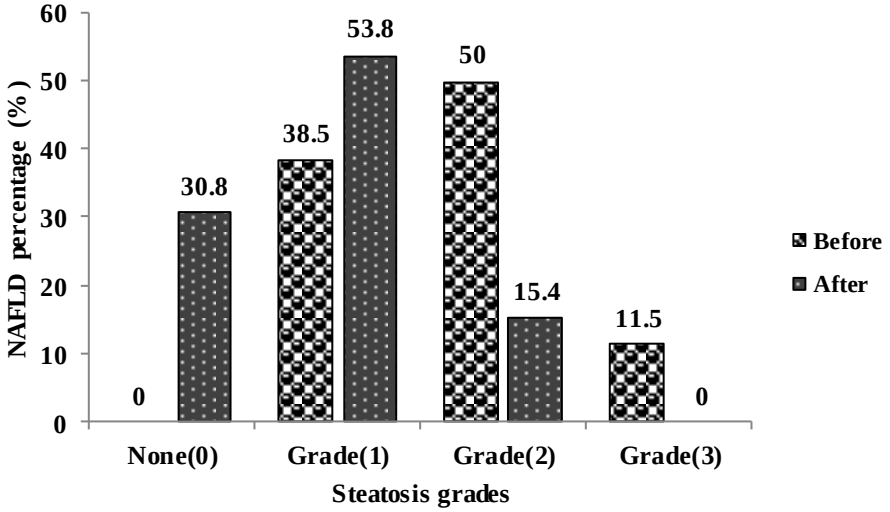


Fig 4.17 Fatty liver grades in lifestyle intervetion alone. Values are given in percentage (%) for categorical data. Where= NAFLD (non alcoholic fatty liver disease).

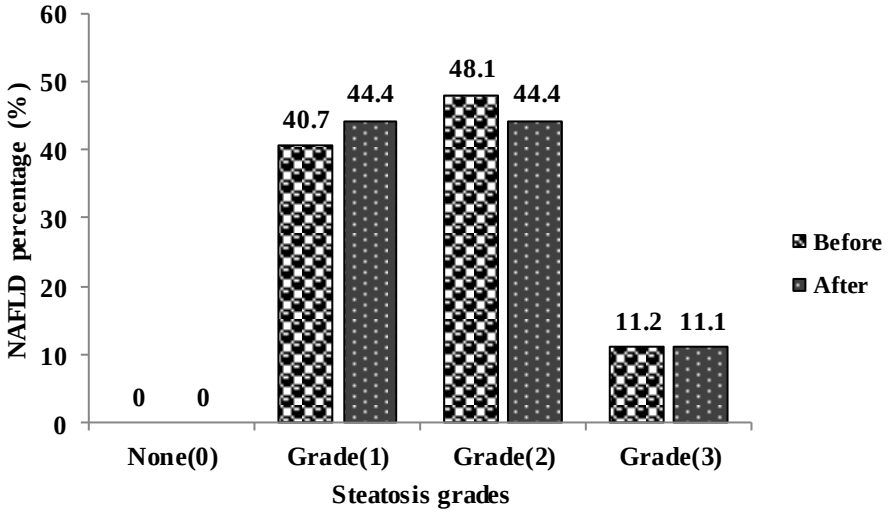


Fig 4.18 Fatty liver grades in placebo. Values are given in percentage (%) for categorical data. Where= NAFLD (non alcoholic fatty liver disease).

Table 4.5. Anthropometric parameters of obese children with non-alcoholic fatty liver disease

Variables	VSL#3+ Lifestyle (n=26)		Probiotic VSL#3 (n=27)		Lifestyle intervention (n= 26)		Placebo (n=27)	
	Before	After	Before	After	Before	After	Before	After
Weight(kg)	61.6±12.2	56.1±11.7**	60.7±12.3	58.2±12.1**	59.2±8.12	56.1±8.00**	57.5±8.33	$57.3±8.43^{NS}$
BMI(kg/m^2)	27.2±3.74	24.7±3.83**	27.1±4.07	26.0±4.06**	27±3.57	25.6±3.46**	27±3.23	$26.9±3.17^{NS}$
Zscore	2.61±0.30	2.15±0.34**	2.64±0.40	2.52±0.38**	2.63±0.39	2.49±0.39**	2.63±0.44	$2.63±0.43^{NS}$
MAC (cm)	33.7±2.39	32.0±2.52**	33.3±1.93	32.4±2.06**	33.8±3.23	32.8±3.47**	32.8±2.81	$32.8±2.81^{NS}$
TSF (mm)	20.7±1.97	18.7±2.04**	20.4±2.00	19.0±2.29**	20.7±2.51	19.2±2.75**	19.3±2.32	19.4±2.38*
WC (cm)	87±1.54	84.5±1.68**	86.4±1.55	85.3±1.46**	86.3±1.94	84.8±1.92**	85.3±2.23	85.6±2.28**

Where= BMI= Body mass index, MAC= Mid arm circumference, TSF= Triceps skinfold thickness, WC= Waist circumference

Wilcoxon Signed Ranks Test, Data are manifested in ean±SD

$p<0.001$**

$p<0.05$*

$p>0.05^{NS}$ (non significant**)**

Table 4.6. Biochemical parameters of obese children with non-alcoholic fatty liver disease

Variables	VSL#3+ Lifestyle (n=26)		Probiotic VSL#3 (n=27)		Lifestyle intervention (n= 26)		Placebo (n=27)	
	Before	After	Before	After	Before	After	Before	After
AST (U/L)	47.3±5.22	37.3±3.08**	46.5±5.97	40.2±4.68**	49.5±5.99	45.5±5.20**	46.4±6.45	47.1 ± 6.55^{NS}
ALT (U/L)	58.1±7.36	45.2±4.95**	50.9±8.36	44.4±9.22**	51.5±9.72	47.1±9.18**	47.6±10.4	47.5 ± 10.4^{NS}
GGT(U/L)	21.4±3.07	18.0±2.48**	21.7±3.28	18.6±3.44**	21±2.29	20.1±2.61**	20.8±2.22	20.8 ± 2.18^{NS}
LDL-c mg/dl	102±9.64	97.3±8.35**	100±8.09	96.9±7.95**	106±10.3	106 ± 10.1^{NS}	99.4±10.6	99.3 ± 10.4^{NS}

Where= AST= Alanine aminotranferase, ALT= Aspartate aminotransferase, GGT= Gamma glutamyl transferase, LDL-c= Low density lipoprotein cholesterol, HDL-c= High density lipoprotein cholesterol, Wilcoxon Signed Ranks Test, Data are manifested in mean±SD $p<0.001$**

$p<0.05$*

$p>0.05^{NS}$ (non significant)

Table 4.6. Biochemical parameters of obese children with non-alcoholic fatty liver disease

HDL-c mg/dl	38.9±3.90	41.9±3.49**	39.9±4.02	42.1±3.51**	40.2±5.02	40.3±4.92^{NS}	40.1±3.37	40.2±3.30^{NS}
Cholesterol mg/dl	172±8.45	164±7.06**	170±9.51	165±8.42**	169±8.14	169±7.58^{NS}	173±6.73	174±6.55^{NS}
TG mg/dl	147±7.15	137.2±5.38**	151±3.82	146±3.81**	150±6.53	147±7.33*	150±4.07	149±4.08^{NS}
CRP mg/l	2.46±0.40	1.33±0.41**	2.39±0.46	1.28±0.55**	2.36±0.30	2.11±0.13**	2.34±0.33	2.44±0.41*
FBG mg/dl	95.1±7.45	88.4±7.22**	98.4±5.73	94.7±5.66**	102±13.1	99.8±11.7*	99.14±5.46	98.10±4.83^{NS}
Uric acid mg/dl	5.51±0.85	3.50±0.48**	5.10±0.90	3.84±0.81**	5.08±0.78	4.15±0.45**	5.08±0.94	5.15±0.94^{NS}

Where= HDL-c= High density lipoprotein cholesterol, CHOLE= Cholesterol, TG= Triglyceride, CRP= C- reactive protein, FBG= Fasting blood glucose

Wilcoxon Signed Ranks Test, Data are manifested in mean±SD $p<0.001$**

$p<0.05$*

$p>0.05^{NS}$ (non significant)

In current study, VSL#3 plus lifestyle intervention has been proved effective therapy for managing NAFLD in obese children as compared to single therapy of Probiotic VSL#3 alone and lifestyle intervention alone. Probiotics targets the gut liver axis; change in gut microbiota composition reduced the harmful bacteria overgrowth (SIBO) such as enterobacteria and clostridia. VSL#3 is live lactic acid bacteria strains are effective for maintaining the proportion of gut microbes firmicutes and bacteriodetes. Probiotics therapy improved the SCFA hormonal axis through gut microbiota. Combined therapy group had much significant weight loss average (5.5 kg), BMI: 27.2 to 24.7 kg/m^2 and also had 38.5% none fatty liver disease at the end of study as shown in table 4.4, 4.5($p<0.001$). Another single therapy of probiotic VSL#3 had average (2.5kg) significant weight loss, BMI: 27.1 to 26.0 kg/m^2 and single therapy of lifestyle intervention had average (3.1 kg) significant weight loss, BMI 27 to 25.6kg/m^2($p<0.001$) (Fig 4.19-4.21). In similar study showed that probiotic VSL#3 was effective for decline in BMI 27.1 to 25 kg/m^2 and increased in satiety hormone (Alisi *et al*., 2014).

Z score, MAC, TSF and WC were significantly reduced in three groups ($p<0.001$) and weight, BMI, Z score and MAC were non significant in placebo group but TSF and WC were significantly increased ($p<0.05$) in placebo group at the end of study (Fig 4.22-4.24).Increase in TSF basically predicts energy stores and calorie reserves in human and WC predicts belly fat (visceral fat) is major risk factor of fatty liver. Various studies have shown that lifestyle intervention such as diet and exercise is effective for reduction in WC and defined therapy for initial weight loss without any medications but it's strenuous to achieve desired weight loss. (Pacifico *et al*., 2013) showed that WC was reduced from 87 to 84cm within 1 year of lifestyle modification in fatty liver children.

Table 4.6. explains about the biochemical parameters of children with NAFLD. All blood parameters; AST, ALT, GGT, LDL-c, cholesterol, TG, CRP, uric acid, FBG were significantly most reduced and increased HDL-c in VSL#3 plus lifestyle intervention group($p<0.001$) as compared to VSL#3 alone and lifestyle intervention group($p<0.05$) (Fig 4.25-4.31). In lifestyle intervention group HDL-c, LDL-c and cholesterol were non significant. Wong and his colleagues concluded there were non significant changes found in HDL-c, LDL-c and cholesterol as compared to placebo after 1 year of lifestyle intervention but changes seen in interahepatic triglycerides (Wong *et al*., 2013).In meta- analysis probiotic therapy is cornerstone management of

NAFLD with the reduction of AST, ALT, total cholesterol, ameliorate IR and tumor necrosis factor- alpha (Ma *et al*., 2013). Another meta-analysis of RCT resulted that probiotic therapy is effective for reducing lipid profile and FBG and it is a promising treatment option for managing NAFLD in future defined by meta-analysis of 12 RCT's (Ma *et al*., 2013; Anurag *et al*., 2017 and He *et al*., 2017). Another studies concluded that probiotic is efficacious for treating NAFLD with reduction of WC, AST, ALT, lipids in pediatrics (Famouri *et al*., 2017 and He *et al*., 2017) In our study probiotic therapy is potent for reduction in lipid profile because of modulation in inflammatory pathways and improvement in absorption of nutrients. One study observed in 48 patients in 10 weeks with consumption of 200g yoghurt enriched with potential bacteria *Lactobacillus acidophilus* L1 had hypocholesterolemic effect and showed significantly beneficial effects in lipid profile (Anderson and Gilliland, 1999). Moreover, another study showed significantly increased in HDL-c levels with long time consumption of 300g yoghurt enriched with *B. Longum and Lactobacillus acidophilus* strains (Kiessling *et al*., 2002) and similar study found *L. plantarum* reduced fibrinogen and pro-atherogenic biomarkers (Rajkumar *et al*., 2014). In current research we have used probiotic with mixture of 8 bacterial strains and each capsule contains 112.5 billion bacteria potent for reduction of inflammation and also effective for change in gut microbiota framework. (Rajkumar *et al*., 2014) observed VSL#3 was effective for reducing HSCRP, cholesterol, and increased HDL-c in overweight adults.They also opined that BSH (bile salt hydrolase) gene might responsible for reduction of cholesterol but its concerned action mechanism is unknown. They also found that their patient's fecal concentration of total anaerobes, aerobes, *Bifidobacteria, Lactobacillus* and *Streptococcus* in VSL#3 group increased and omega-3 did not effect on gut microbiota. These results are clearly defined that probiotics treatment is a novel approach to maintain the gut constitution. In our study VSL#3 probiotic contain multibacterial live strains which help in surviving the gastric juices, bile and pancreatic secretions and colonize the GI tract and also increased beneficial bacteria that prevent growth of harmful gram negative bacteria. In existing research probiotic improved CRP and blood glucose levels that might be possible due to improved absorption and enhanced barrier function (Fig 4.32, 4.33) as another studies showed similar effects of probiotics *Bifidobacteria lactis* Bb12 and *Lactobacillus acidophilus* (Ejtahed *et al*., 2012). Another study in mice model used VSL#3 demonstrated reduction in blood glucose and pro-inflammatory cytokines that

cause insulin sensitivity (Nestel *et al*., 1984). In liver TNF-α, IL-6 are major regulators of CRP indicated in adipose tissue (Rohde *et al*., 1999). In one Study used *Lactobacillus rhamnosus* GG in high fed fructose mice with NAFLD enervated inflammatory markers and declined in fatty infiltration including ALT levels (Ritze *et al*., 2014).In current research uric acid levels of obese children significantly reduced after intervention with probiotic therapy and lifestyle intervention but most declined in combined therapy of probiotic plus lifestyle. Uric acid is product of purine metabolism; in NAFLD and insulin resistance generally it is disturbed because of fructose enriched drinks and poor lifestyle. Recent study suggested probiotics *Lactobacillus* and *Bifidobacteria* managed the purine nucleotides through the degradation of their intermediate guanosine and inosine that leads to remission of uric acid in individuals (Prasad *et al*., 2017) and lifestyle changes with hypocaloric diet (low fat dairy, avoid red meat, including fruits and veggies enriched with fiber and less purine content food) and moderate exercise was also fruitful for controlling excess purine content endogenously. Another study defined probiotics contain uricolytic bacteria that helped in reducing higher uric acid in blood of animals with higher consequences of renal disease (Garcia-Arroyo *et al*., 2018).

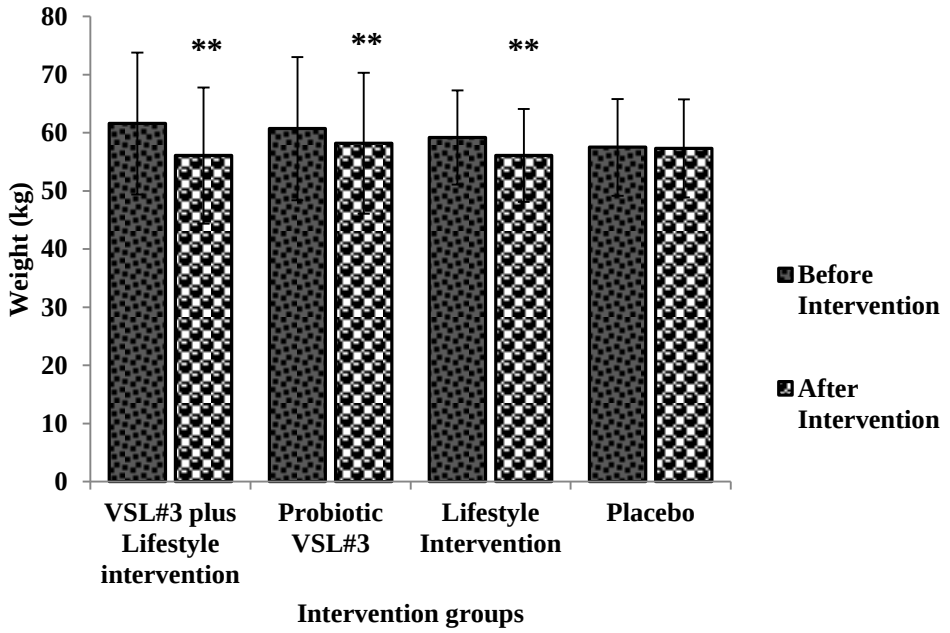


Fig 4.19 Distribution of weight in different intervention groups.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in weight in three groups (p<0.001) except placebo after treatment**.

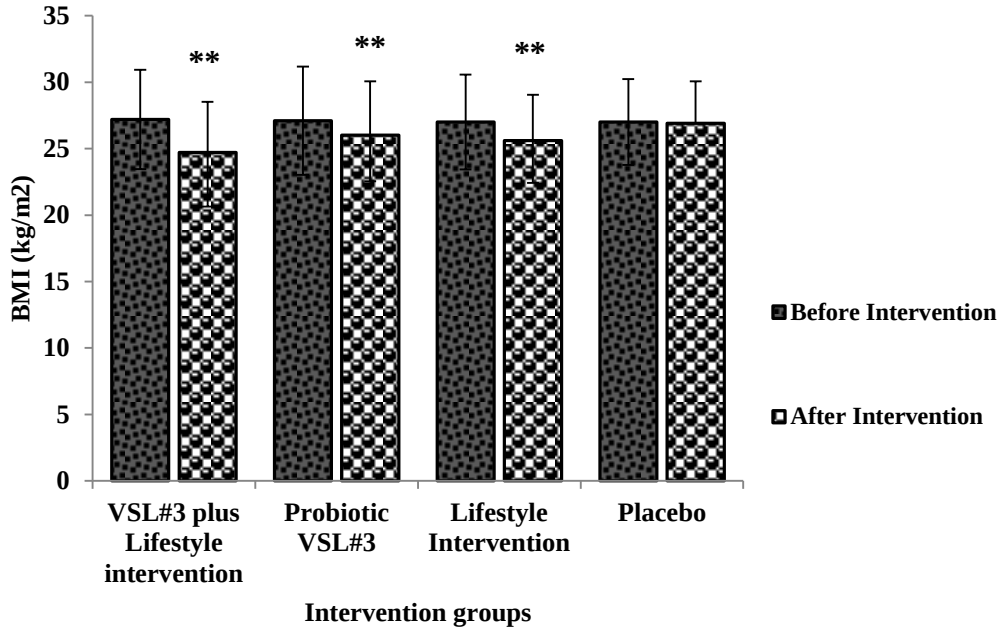


Fig 4.20 Distribution of BMI in different intervention group. Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in BMI in three groups (p<0.001) except placebo.**

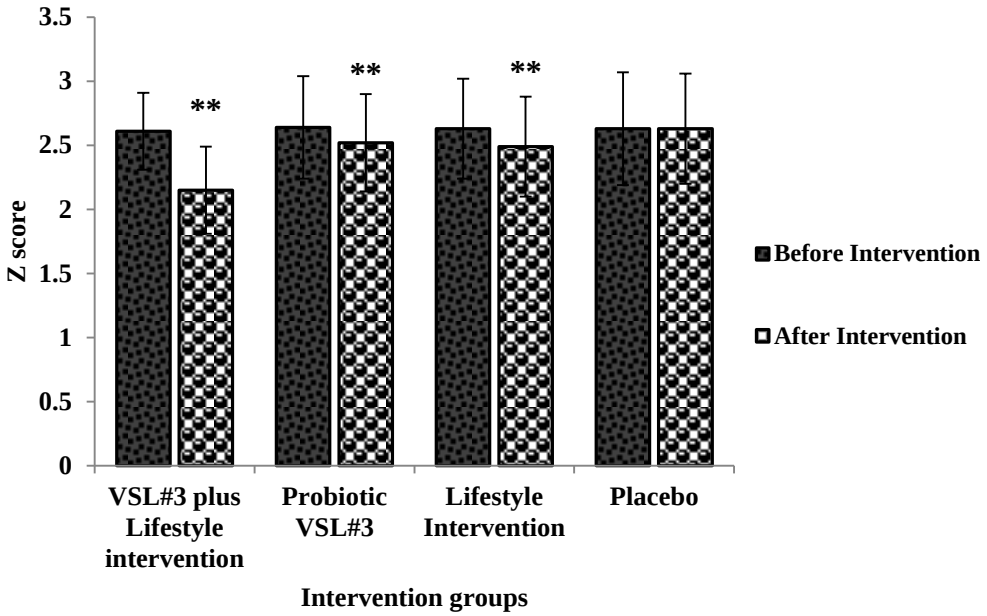


Fig 4.21 Distribution of Z score in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in Z score in three groups (p<0.001) except placebo.**

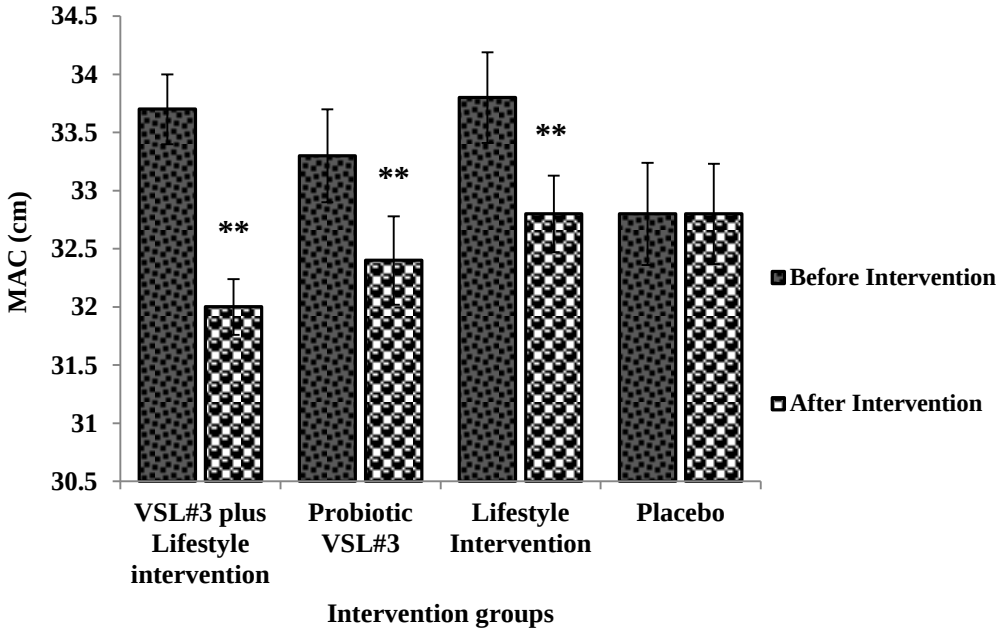


Fig 4.22 Distribution of MAC in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in MAC in three groups (p<0.001) except placebo.**

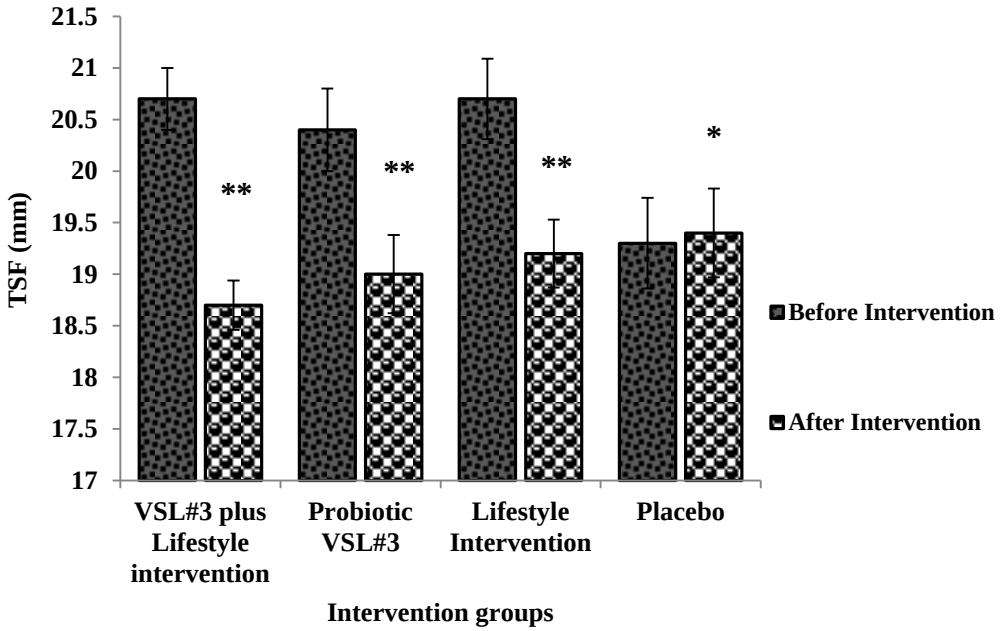


Fig 4.23 Distribution of TSF in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. TSF significantly decreased in three groups (p<0.001) and significantly (p<0.05*) increased in placebo.**

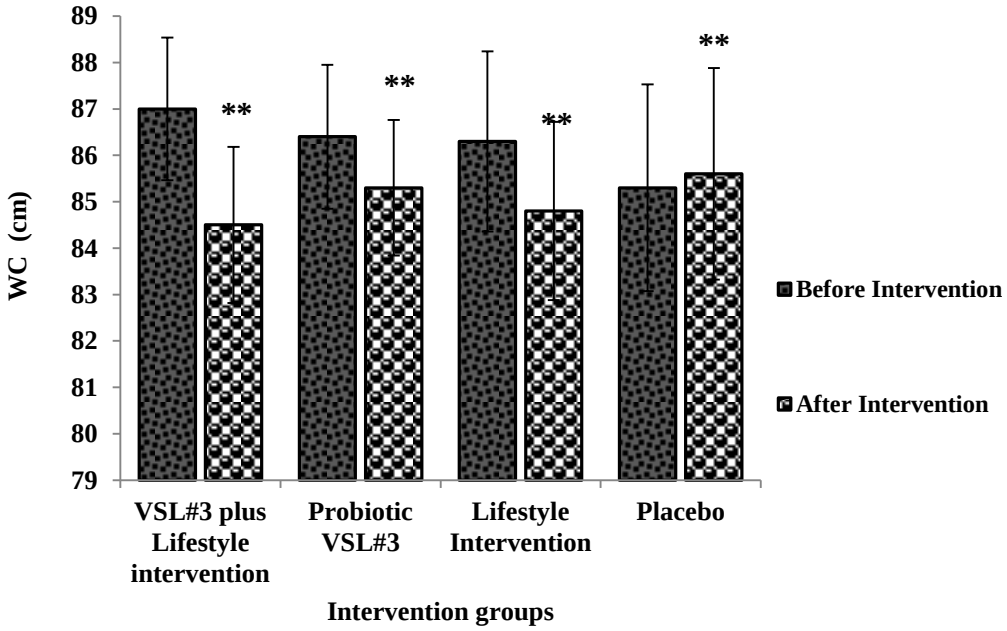


Fig 4.24 Distribution of WC in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. WC significantly decreased in three groups (p<0.001) and significantly (p<0.001**) increased in placebo after the treatment.**

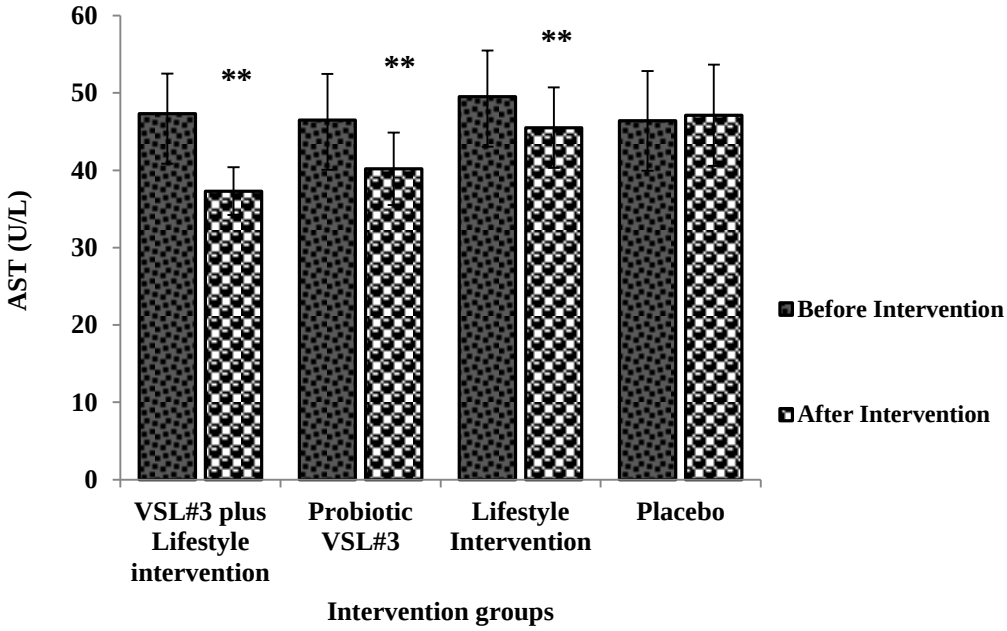


Fig 4.25 Distribution of AST in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in AST in three groups (p<0.001) except placebo after the treatment.**

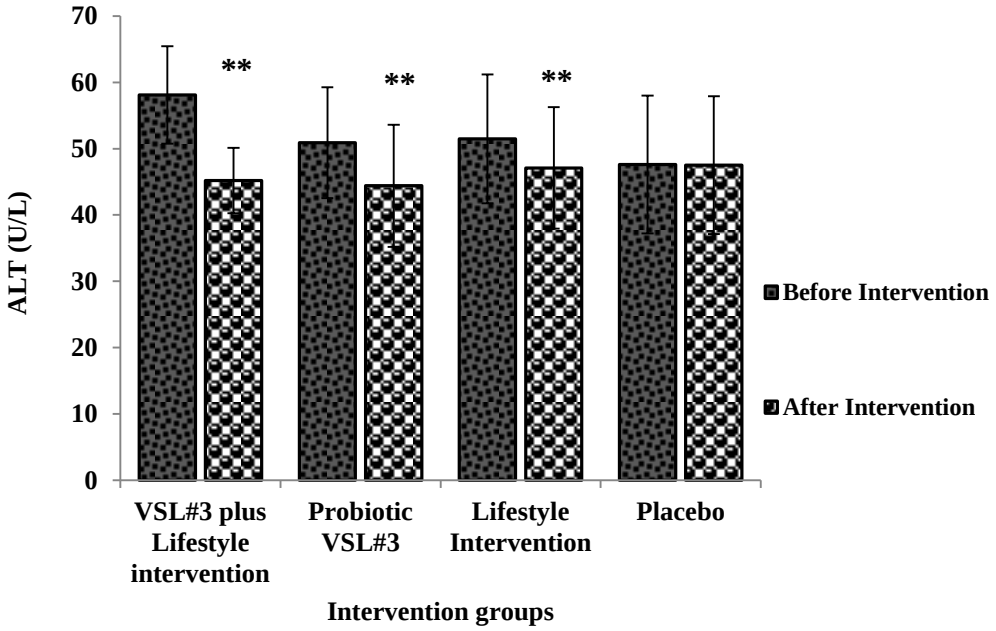


Fig 4.26 Distribution of ALT in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in ALT in three groups (p<0.001) except placebo after the treatment.**

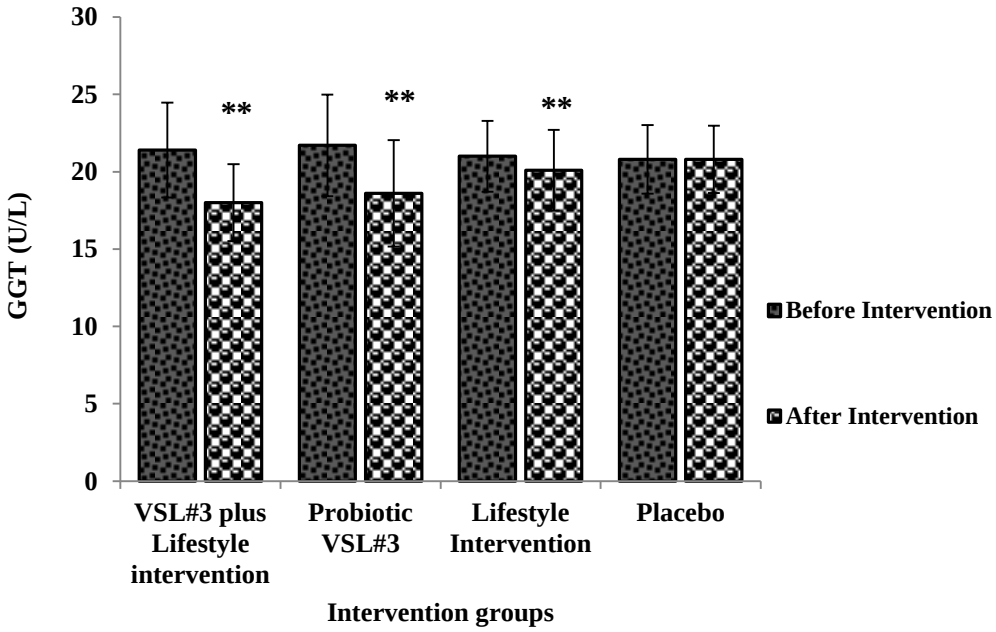


Fig 4.27 Distribution of GGT in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in GGT in three groups (p<0.001) except placebo after the treatment.**

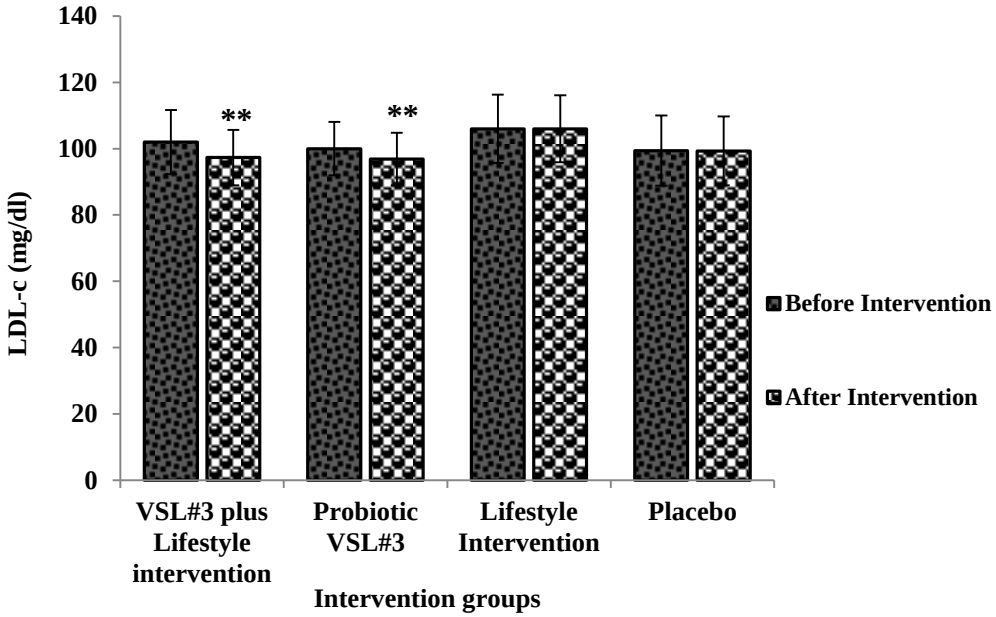


Fig 4.28 Distribution of LDL-c in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in LDL-c in two groups (p<0.001) except lifestyle and placebo groups after the treatment.**

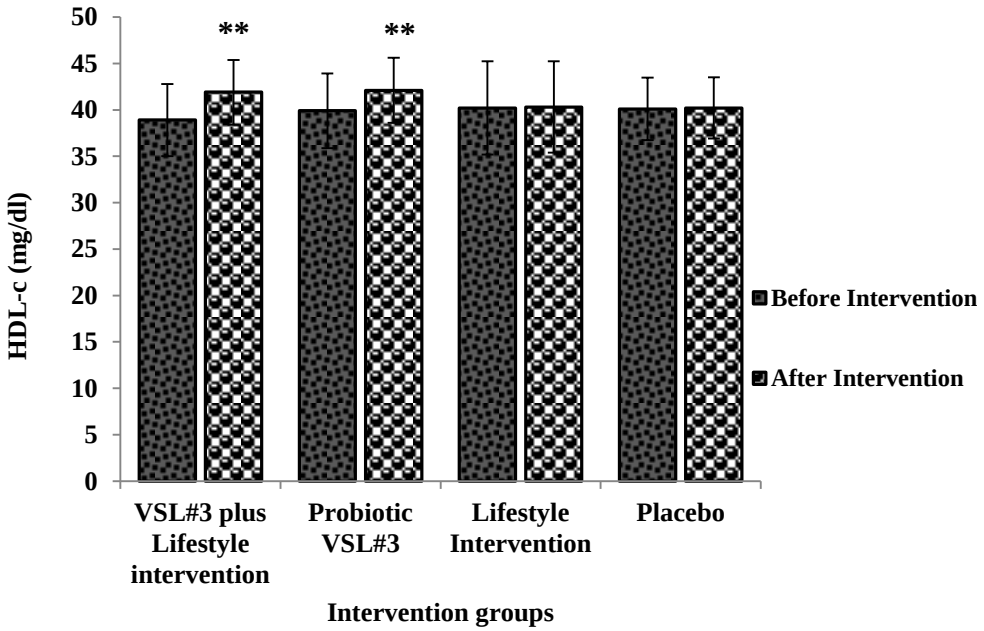


Fig 4.29 Distribution of HDL-c in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in HDL-c in two groups (p<0.001) except lifestyle and placebo groups after the treatment.**

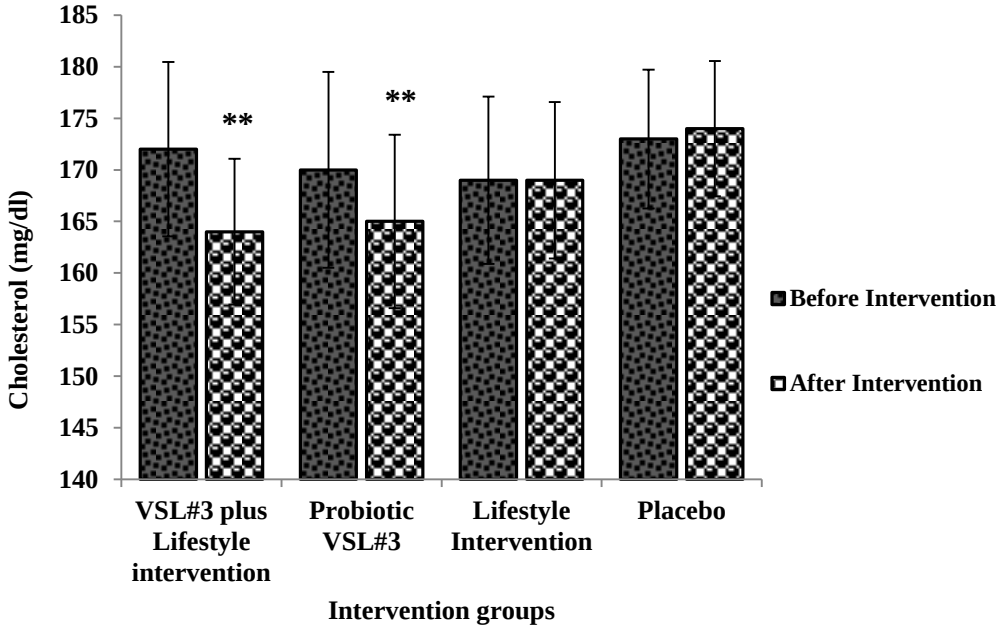


Fig 4.30 Distribution of cholesterol in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in cholesterol in two groups (p<0.001) except lifestyle and placebo groups after the treatment.**

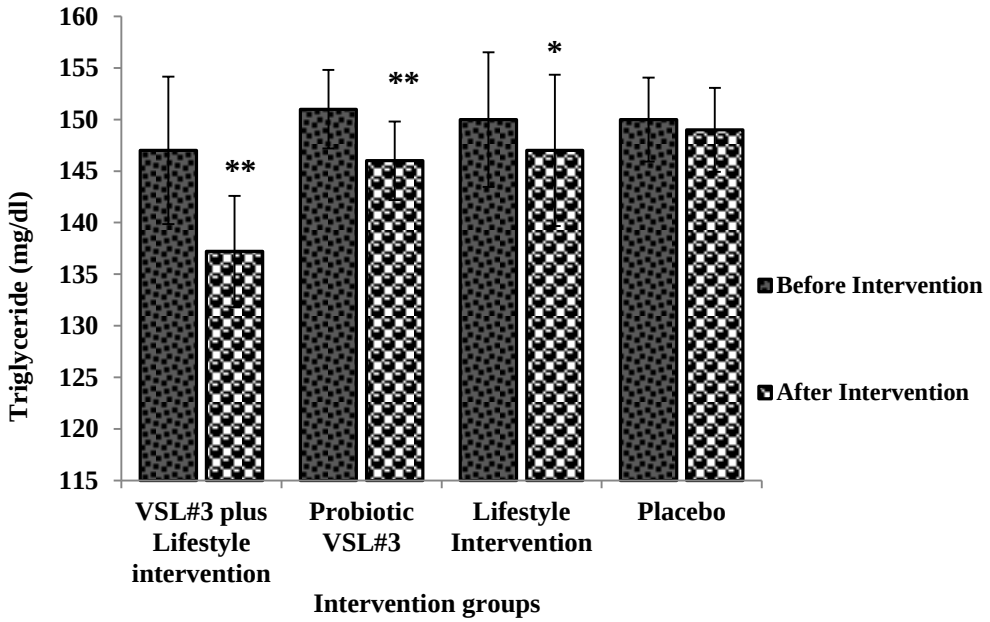


Fig 4.31 Distribution of triglyceride in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Significant difference found in triglyceride in two groups (p<0.001) and in third group (p<0.05*) except placebo after the treatment.**

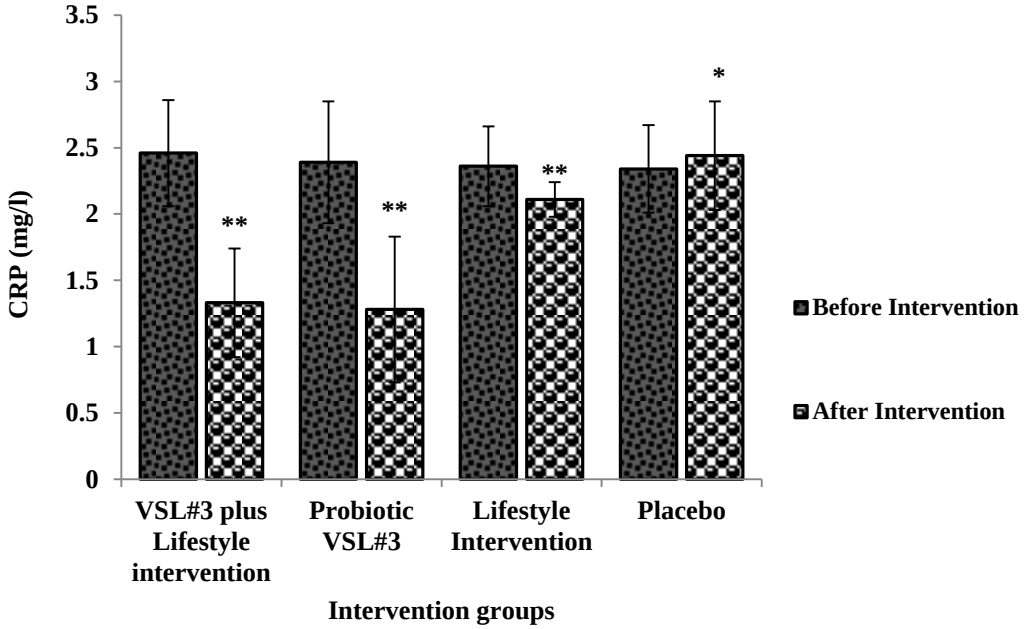


Fig 4.32 Distribution of CRP in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. CRP significantly decreased in three groups (p<0.001) and significantly increased in placebo (p<0.05*) after the treatment.**

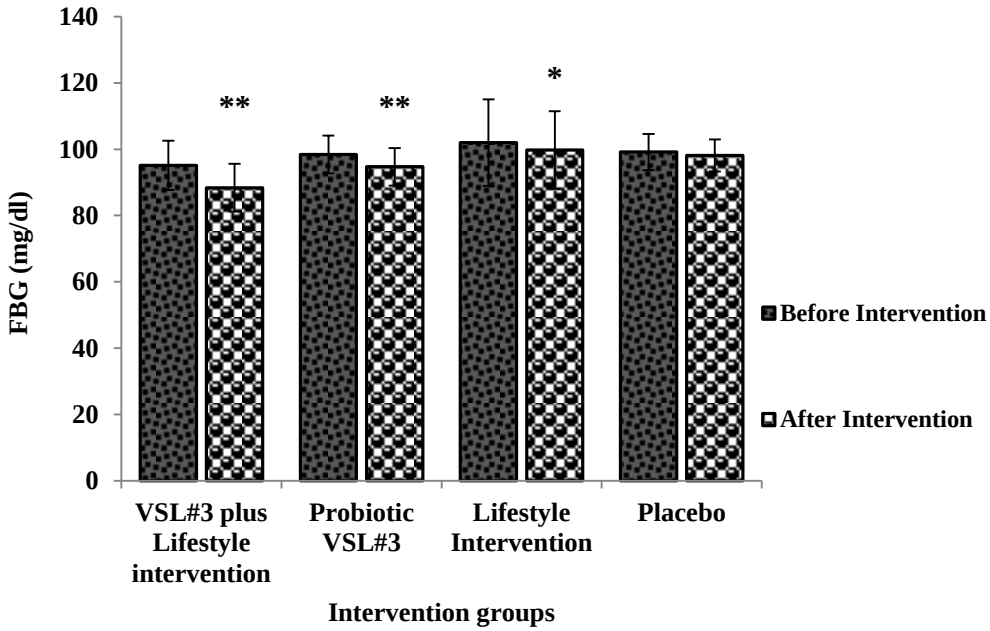


Fig 4.33 Distribution of FBG in different intervention group.Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. FBG significantly decreased in two groups (p<0.001) and in third group (p<0.05*) except placebo after the treatment.**

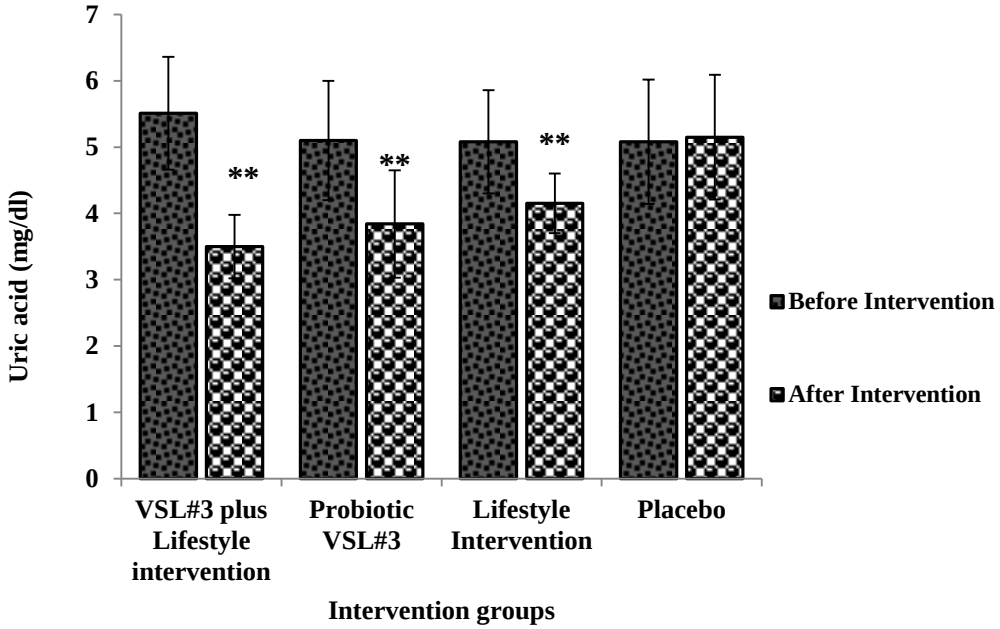


Fig 4.34 Distribution of uric acid in different intervention group. Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Uric acid significantly decreased in three groups (p<0.001) except placebo after the treatment.**

Table 4.7. Pre and post levels of obesity hormones in obese children with Non- alcoholic fatty liver disease

Interventional Groups	Pre Ghrelin	Post Ghrelin	Pre Leptin	Post Leptin	Changes in Ghrelin	Changes in Leptin
VSL#3+Lifestyle Intervention	25.1±3.12	31.8±3.05	23.0±5.24	18.6±4.96	6.71±1.81	-4.40±0.66
Probiotic VSL#3	25.1±2.80	29.2±3.51	22.5±3.01	19.7±2.96	4.11±3.10	-2.75±0.46
Lifestyle Intervention	25.5±2.87	30.7±3.27	23.8±3.68	21.0±3.54	5.20±1.44	-2.85±1.33
Placebo	25.5±3.07	24.3±3.16	23.5±3.83	24.7±3.89	-1.22±2.08	1.24±1.75
P value	0.824	<0.001**	0.490	<0.001**	<0.001**	<0.001**

Kruskal- Wallis Test p<0.05, Data are manifested in mean±SD, p<0.001** (higly significant)

Pre and post levels of ghrelin and leptin hormones are shown in table 4.7. In obese children serum leptin was increased and ghrelin decreased. Leptin has anorexigenic effect and ghrelin has orexigenic effect, hormones maintain the energy intake, generally liver stellate cells expressed and nourished the leptin, it also triggers the pro-inflammatory and other cytokines. Some studies suggested leptin has crucial role in NAFLD disease severity and major predictor of fibrosis and also increased in insulin sensitivity (Potter *et al*., 1998; Ikejima *et al*., 2001; Aleffi *et al*., 2005 and Garcia-Mayor *et al*., 1997). On the other hand, low level of ghrelin is associated with insulin resistance in NAFLD patients with obesity and higher BMI (Marchesini *et al*., 2003). In our study leptin levels were increased than normal range in different interventional groups at baseline. At the end of trial leptin levels were significantly decreased and ghrelin levels were increased in probiotic group alone and lifestyle alone but most significant changes were seen in combined therapy of probiotic plus lifestyle intervention ($p<0.05$). Before intervention levels of leptin and ghrelin were non significant between groups and after intervention levels of leptin and ghrelin significantly changed between groups ($p<0.001$) (Fig 4.35-4.38).Recent study in Iran revealed combined therapy of probiotic with prebiotic along with lifestyle intervention had better impact on leptin levels and also on blood glucose parameters among adults (Behrouz *et al*., 2017). In placebo group ghrelin was decreased and leptin was increased at the end of treatment.

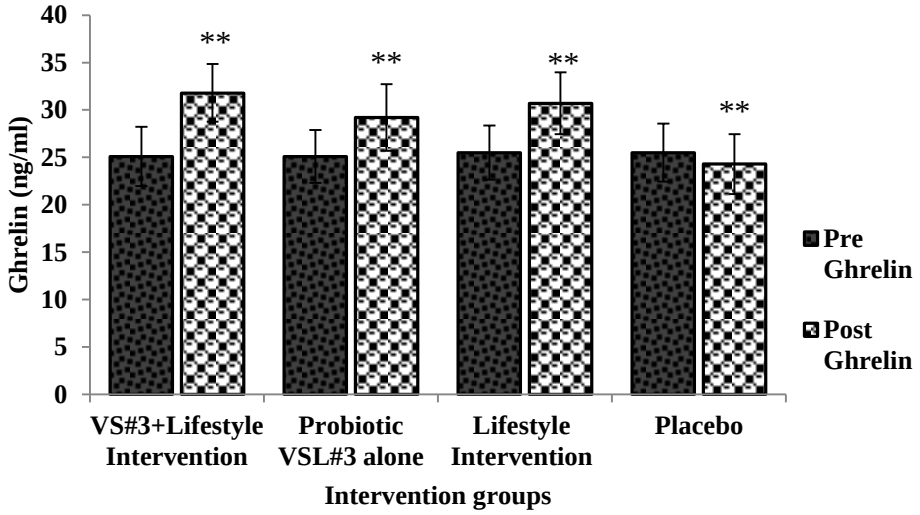


Fig 4.35 Distribution of ghrelin in different intervention group.

Values are in mean±SD, p<0.05 by Kruskal wallis test, ghrelin was increased in three groups and decreased in placebo (p<0.001) after intervention.**

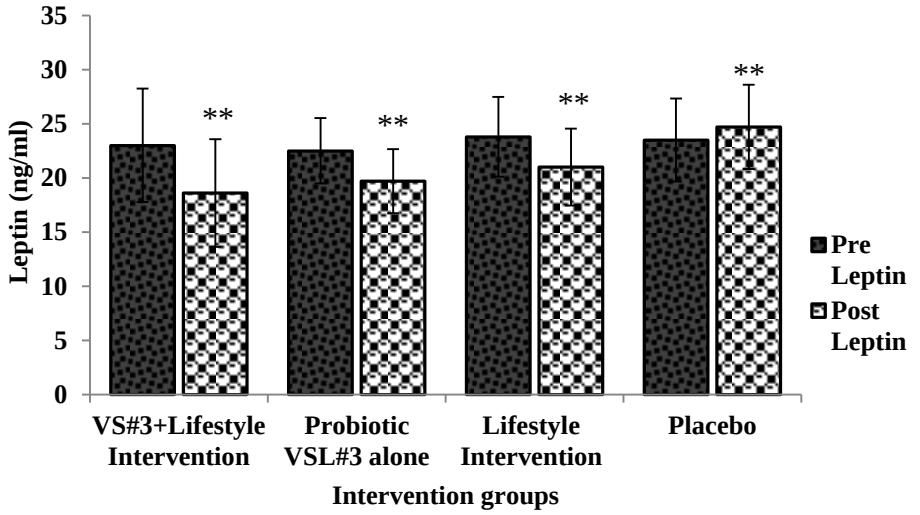


Fig 4.36 Distribution of leptin in different intervention group.

Values are in mean±SD, p<0.05 by Kruskal wallis test, leptin was decreased in three groups and increased in placebo (p<0.001) after intervention.**

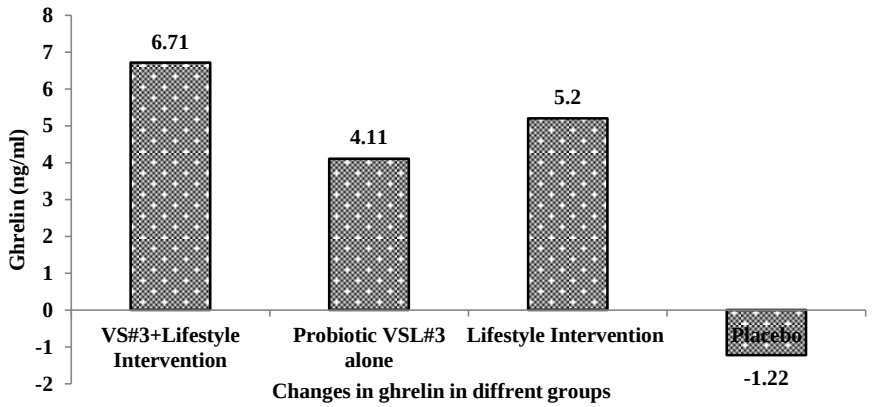


Fig 4.37 Distribution of changes in ghrelin in different intervention group.

Values are in mean±SD, p<0.05 by Kruskal wallis test, Significantly difference (p<0.001) found in different groups after intervention. After treatment ghrelin was increased in three groups and decreased in placebo group.

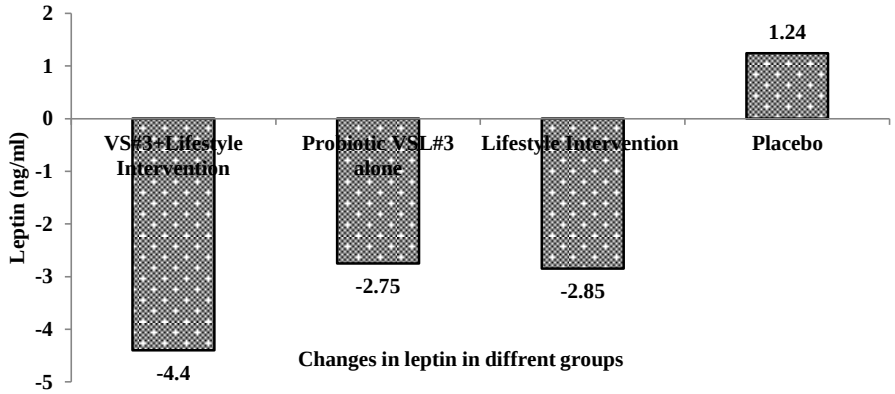


Fig 4.38 Distribution of changes in leptin in different intervention group.

Values are in mean±SD, p<0.05 by Kruskal wallis test, significantly difference (p<0.001) found in different groups after intervention. After treatment leptin was decreased in three groups and increased in placebo group.

Table 4.8. Nutrient intake of per day of obese children with non-alcoholic fatty liver disease in different intervention groups

	VSL#3 +Lifestyle Intervention		Probiotic VSL#3		Lifestyle Intervention		Placebo	
Nutrients	**Before**	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
Energy (kcal)	2496±317	2045±269**	2475±370	2269±346**	2435±357	2134±357**	2420±366	2400±367**
Protein(g)	62.4±7.55	56.2±7.42**	61.8±9.27	59.8±9.66**	62.5±5.35	58.6±9.83*	60.5±11.7	60.7±11.1*
Fat(g)	91.8±9.63	70.9±9.35**	90±13.0	82.3±13.2**	88.9±3.93	71.1±11.9**	89.1±5.62	87.8±5.82*
CHO(g)	369±40.9	295±38.9**	354±52.3	324±52.4**	370±29	314±52.7**	383±69.2	389±71.6*
Fiber(g)	13.42±1.81	20.0±2.11**	12.44±1.28	15.77±2.11**	13.23±1.79	18.53±2.08**	12.05±1.13	12.40±1.36ns

Where= CHO= Carbohydrate, (g) = gram, (kcal) = Kilocalorie

Data are manifested in mean±SD, Wilcoxon Signed Rank Test, $p<0.05$

$p<0.001$**

$p<0.05$*

ns (non significant)

In our study, actual per day intake of obese children were more in different intervention groups at baseline and after the trial with the help of diet plan and counseling session their calorie intake was reduced. Probiotic plus lifestyle group most decreased their calorie intake as compared to single therapy of VSL#3 probiotic and lifestyle. Mean calorie difference between before and after intervention in probiotic plus lifestyle group; 451kcal, in VSL#3 alone; 206kcal, in lifestyle intervention; 301kcal and placebo; 20kcal respectively($p<0.001$). Fat and CHO intake were also significantly reduced in probiotic plus lifestyle had 20.9g and 74g, in VSL#3 alone had 7.7g and 30g and lifestyle group had 17.8g and 56g respectively($p<0.001$). In Placebo group fat was reduced 1.3g but CHO was increased 6g respectively ($p<0.05$) (Fig 4.39-4.42).

In VSL#3 group reduced their food intake because of improved metabolic efficiency of nutrients, ameliorate gut microbiota functions and much better impact on leptin and ghrelin hormones. Interestingly, Probiotic therapy also prevented the food cravings of children in our study. In one study postulated that higher GLP-1 levels are connected to higher energy expenditure in resting state with increased fat oxidation rates in humans (Pannaciulli *et al*., 2006). It is well recognized that VSL#3 consistent with production of short chain fatty acid specially butyrate, increased GLP-1 hormone from L cells of intestine and effective for weight reduction and also protects from deteriorate effect of obesity and diabetes by high fat diet. The proposed mechanism underlying that in mice model VSL#3 improved diabetes, obesity and declined the leptin levels conciliated through hypothalamus. It basically suppressed food intake that regulated the genes that is POMC, (promelanocortin), AgRP and neuropeptide (NpY) in hypothalamus (Yadav *et al*., 2013). Alteration in gut flora is a new treatment of Obesity and NAFLD. A protein fasting induced adipocycte factor (fiaf) produced by liver and have antagonist role to lipoprotein lipase enzyme which ultimately stores energy in form of fat (triglycerides). Genes influence hunger (AgRP and NpY) were reduced and while another satiety influence gene POMC most effectively improved and provided satiety value. Therefore, this mechanistic role of probiotic VSL#3 on preventing obesity, diabetes and gut satiety hormones provides strong efficacious results in different mice models (Yadav *et al*., 2013).

In Lifestyle intervention group and probiotic plus lifestyle group significantly more increased their fiber intake after intervention ($p<0.001$) that is beneficial for gut health and less increase in probiotic group alone ($p<0.001$). In placebo group it was non significant (Fig 4.43) Fibers are non digestible carbohydrate that benefits the health of the host, improves digestion and produce bulky stools. Prebiotics; specific fibers are basically food for probiotics and increase the friendly bacteria in gut but its dosage should be defined under supervision of registered dietician (Behrouz *et al*., 2017 and Parnell *et al*., 2012).

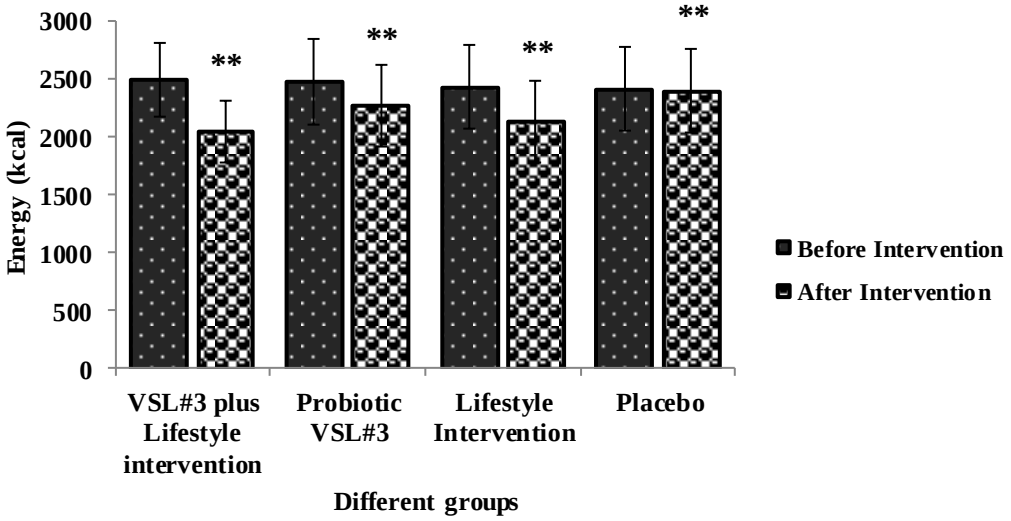


Fig 4.39 Distribution of calorie intake in different interventional group.

Values are in mean±SD, $p<0.05$ by Wilcoxon signed rank test. Significant difference found in calorie intake in all different groups ($p<0.001$) after treatment.**

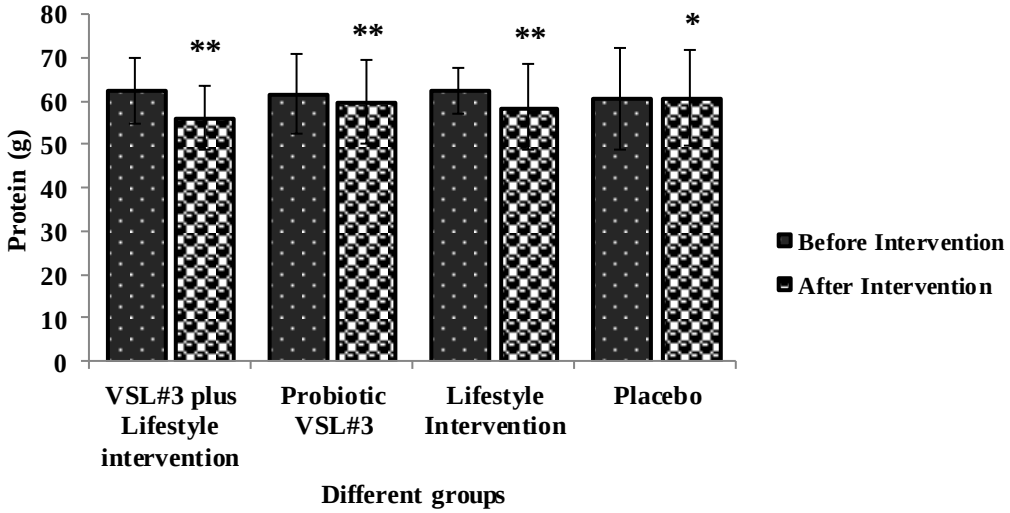


Fig 4.40 Distribution of protein intake in different interventional group.
Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Protein intake was significantly decreased in three groups (p<0.001) and increased significantly in placebo (p<0.05*) after treatment.**

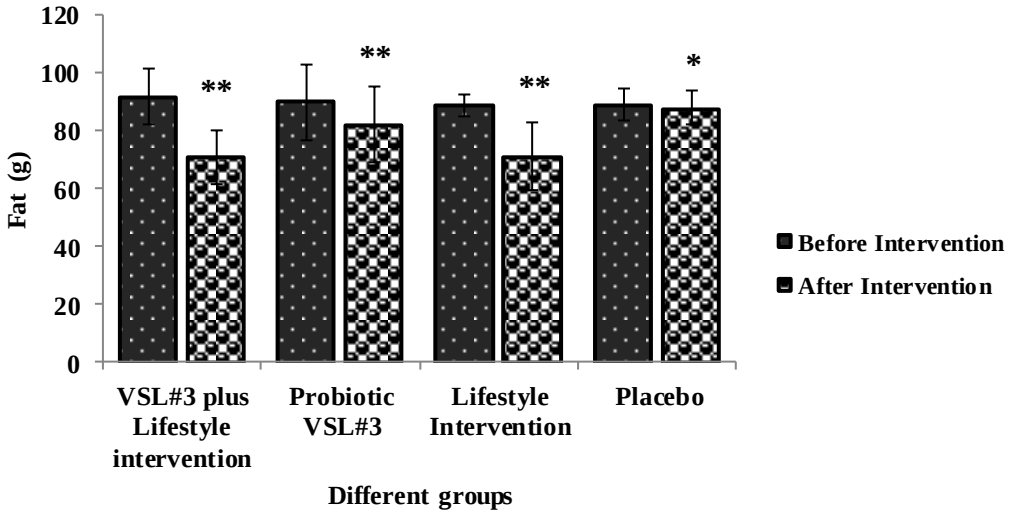


Fig 4.41 Distribution of fat intake in different interventional group
Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Fat intake was significantly decreased in three groups (p<0.001) and also significantly decreased in placebo (p<0.05*) after treatment.**

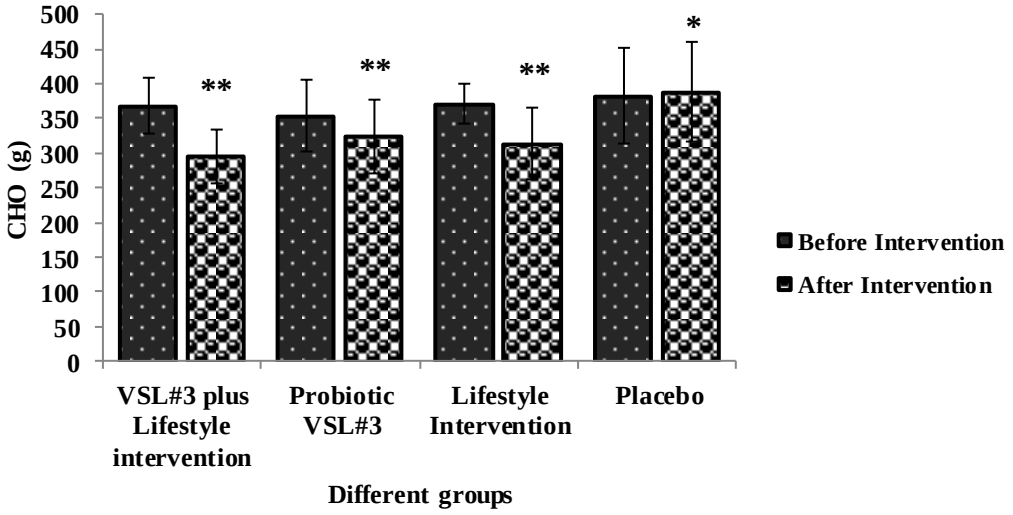


Fig 4.42 Distribution of CHO intake in different interventional group.

Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. CHO intake was significantly decreased in three groups (p<0.001) and significantly increased in placebo (p<0.05*) after treatment.**

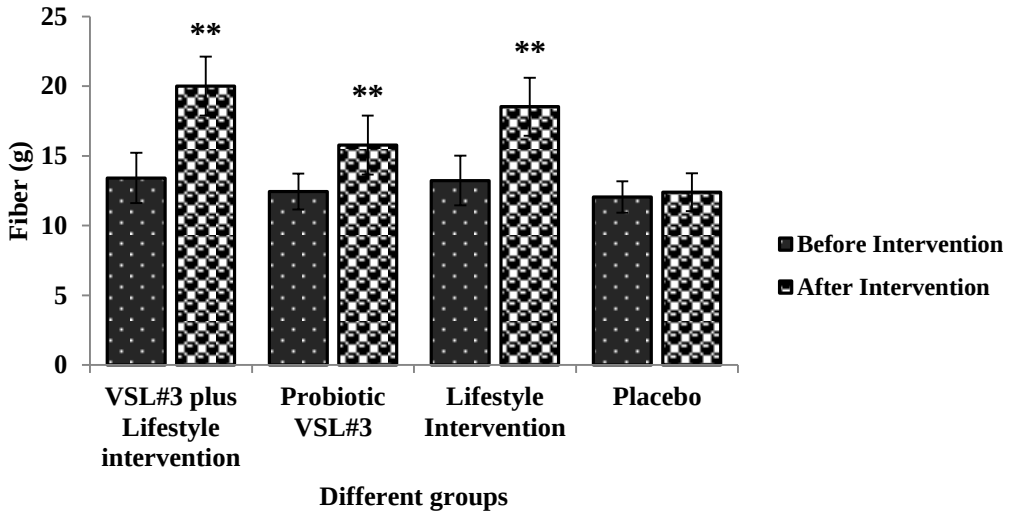


Fig 4.43 Distribution of fiber intake in different interventional group

Values are in mean±SD, p<0.05 by Wilcoxon signed rank test. Fiber intake was significantly increased in three groups (p<0.001) and fiber intake in placebo was non significant after treatment.**

Table 4.9. Comparison of RDA calorie Vs before calorie intake in different interventional groups

Groups	RDA[#] (Kcal/day) 2010	Mean calorie intake before (Kcal/day)	Mean Difference
VSL#3 + Lifestyle intervention (n= 26)	2100	2496	396**
VSL#3Alone (n=27)	2100	2475	375**
Lifestyle Intervention (n=26)	2100	2435	335**
Placebo (n=27)	2100	2420	320**

#RDA (recommended dietary allowances) according to average age in different groups

One Sample T-test, $p<0.05$, $p<0.001$** (highly significant)

Table 4.10. Comparison of RDA calorie Vs after calorie (kcal) intake in different interventional groups

Groups	ICMR RDA[#] 2010 (Kcal/day)	Mean calorie intake after intervention (Kcal/day)	Mean Difference	P value
VSL#3 + Lifestyle intervention (n= 26)	2100	2045	55	.312[ns]
VSL#3Alone (n=27)	2100	2269	169	.018*
Lifestyle Intervention (n=26)	2100	2134	34	.630[ns]
Placebo (n=27)	2100	2400	300	<0.001**

#RDA (recommended dietary allowances) according to average age in different groups

One Sample T-test, $p<0.05$*, $p<0.001$** (highly significant), ns= non significant

In current study, baseline calorie was compared with RDA calorie released in 2010. Calorie intake was significantly more than their RDA average calorie value in all different intervention groups at baseline ($p<0.001$). After intervention calorie intake of children were close to RDA calorie in lifestyle intervention and probiotic plus lifestyle intervention group respectively. Calorie comparison of probiotic group alone and placebo group was significant with RDA calorie at the end of intervention ($p<0.05$).

Table 4.11. Before intervention of physical exercise among obese children with non-alcoholic fatty liver disease

Groups	0 (None Exercise)*	1(15-30 Minutes Exercise)
VSL#3 + Lifestyle intervention (n= 26)	23 (88.5%)	3(11.5%)
VSL# 3Alone (n=27)	23(85.2%)	4(14.8%)
Lifestyle Intervention (n=26)	21(80.8%)	5(19.2%)
Placebo (n=27)	24(88.9%)	3(11.1%)
Total (n= 106)	91(85.8%)	15(14.2%)
Pearson Chi-Square <0.05	**p value = 0.882**	

*None Exercise: (watching TV, video games and surfing on internet) (no fixed hours)

Table 4.12. After intervention of physical exercise among obese children with non-alcoholic fatty liver disease at least 3 days a week

Groups	(0) None Exercise	(1)15-30 Minutes Exercise	(3)Exercise >30 Minutes (up to 1 hour)
VSL#3 + Lifestyle intervention (n=26)	0(0.0%)	8(30.8%)	18(69.2%)
VSL#3 Alone (n=27)	21(77.8%)	6(22.2%)	0(0.0%)
Lifestyle Intervention (n=26)	0(0.0%)	10(38.5%)	16(61.5%)
Placebo (n=27)	27(100%)	0(0.0%)	0(0.0%)
Total (n= 106)	48(45.3%)	24(22.6%)	34(32.1%)
Pearson Chi-Square <0.05	**p value = <0.001**		

Exercise= aerobics (Skipping rope, brisk walking, tennis, dance & swimming)

In our research, at baseline overall 85.8% NAFLD children were physically inactive and 14.2% were actively indulge in sports, aerobics and running. Physically inactive children were more inclined to play video games, watching television and surfing on internet. After diet counseling with diet plans their activity level was increased and Overall 22.6% children did 15-30 min/day exercise and 32.1% children did >30min up to 1 hour/day at least 3 days a week ($p<0.001$) as explained in table 4.11,4.12.

Diet plus physical activity is very essential for desired weight loss. In general, it has been seen among overweight/obese children their energy intake is more as compared to their energy expenses. In today's era, they burn less calorie may be reason behind that of study stress, indulge in indoor activities (more sitting for several hours) as compared to outdoor activities (Ahmad *et al*., 2010 and Robinson *et al*., 2017). Now children consume calorie dense food as compared to nutrient dense food and they lacks in vital nutrient requirements and suffer from various gastrointestinal diseases. This is wide possible because of market exposure of processed food and carbonated beverages that are skyrocketed among every children. Unhealthy foods are cheapest and healthy foods are costly but health suffers later after consumption of these cheapest junk food but with advise of registered dietician everybody can change their food choices and according to their status. Lifestyle intervention (diet restriction plus 1hour moderate activity/day) reduced average 7.1 kg weight in 10-weeks (Wang *et al*., 2008). In our study lifestyle intervention group had 3.1 kg weight loss in 16 weeks and improved liver function and probiotic plus lifestyle group reduced average weight loss of 5.5kg. Weight management programme with diet and exercise is challenging for weight loss leads to improvements in hepatic, metabolic functions and also increase in BMR (basal metabolic rate) but under supervision of dieticians (Ho *et al*., 2012).

Table 4.13. Comparison of changes in anthropometric and biochemical parameters for probiotic VSL#3 and lifestyle intervention

Variables	ProbioticVSL#3	Lifestyle	P value
	n = 27	n = 26	
Weight (kg)	18.98	35.33	**
BMI (kg/m^2)	18.98	35.33	**
MAC (cm)	25.39	28.67	0.435^{ns}
TSF (mm)	26.00	28.04	0.629^{ns}
WC (cm)	22.87	31.29	0.045^{*}
AST (U/L)	28.31	25.63	0.524^{ns}
ALT (U/L)	31.94	21.87	0.017^{*}
GGT (U/L)	38.15	15.42	**
LDL-c (mg/dl)	40.00	13.50	**
HDL-c (mg/dl)	19.28	35.02	**
CHOLE (mg/dl)	38.22	15.35	**
TG (mg/dl)	32.81	20.96	**
CRP (mg/l)	36.22	17.42	**
Blood sugar (mg/dl)	34.11	19.62	**
Uric acid (mg/dl)	32.15	21.65	**

Where= BMI= Body mass index, MAC= Mid arm circumference, TSF= Triceps skinfold thickness, AST= Alanine aminotranferase, ALT= Aspartate aminotransferase, GGT= Gamma glutamyl transferase, LDL-c= Low density lipoprotein cholesterol, HDL-c= High density lipoprotein cholesterol, CHOLE= Cholesterol, TG= Triglyceride, CRP= C- reactive protein.

Values are in Mean Ranks. P values refer to comparisons between the Probiotic and Lifestyle Group

Mann-Whitney U test $p<0.05$

$p< 0.05^{*}$, $p<0.001^{**}$

ns= non significant

Table 4.14. Comparison of changes in anthropometric and biochemical parameters for probiotic and placebo interventions

Variables	Probiotic VSL#3	Placebo	Significance
	n = 27	n = 27	
Weight (kg)	41.00	14.00	**
BMI (kg/m^2)	41.00	14.00	**
MAC (cm)	40.63	14.37	**
TSF (mm)	40.98	14.02	**
WC (cm)	39.00	16.00	**
AST (U/L)	39.31	15.69	**
ALT (U/L)	40.59	14.41	**
GGT (U/L)	39.85	15.15	**
LDL-c (mg/dl)	40.74	14.26	**
HDL-c (mg/dl)	19.04	35.96	**
CHOLE (mg/dl)	38.96	16.04	**
TG (mg/dl)	41.00	14.00	**
CRP (mg/l)	39.69	15.31	**
Blood sugar (mg/dl)	37.56	17.44	**
Uric acid (mg/dl)	40.76	14.24	**

Where= BMI= Body mass index, MAC= Mid arm circumference, TSF= Triceps skinfold thickness, AST= Alanine aminotranferase, ALT= Aspartate aminotransferase, GGT= Gamma glutamyl transferase, LDL-c= Low density lipoprotein cholesterol, HDL-c= High density lipoprotein cholesterol, CHOLE= Cholesterol, TG= Triglyceride, CRP= C- reactive protein.

Values are in Mean Ranks. P values refer to comparisons between the Probiotic and Placebo Group Mann-Whitney U test, $p<0.05$, $p<0.001$**

Table 4.13 is showing the comparison of changes in anthropometric and biochemical parameters for probiotic VSL#3 and lifestyle interventions. Most significant changes in WT, BMI and WC were seen in lifestyle intervention group as compared to probiotic group alone ($p<0.05$) and most significant changes were seen in ALT, GGT, LDL, HDL, cholesterol, triglyceride, CRP, blood sugar and uric acid in probiotic group as compared to lifestyle intervention ($p<0.05$) because of enhance in gut barrier function, increased in friendly bacteria including increase in butyrate production that promotes secretion of GLP-1 hormone for giving satiety value. MAC, TSF and AST were non significant between both groups. Basically, lifestyle intervention is method of choice to adopt in daily routine to prevent obesity and its complications but in advanced disease condition probiotic, prebiotic or any antioxidant therapy is to be added with lifestyle treatment for a particular period.

These results are showing that probiotic therapy and lifestyle intervention work independently for management of NAFLD in obese children. If both treatments could be combined, its effects will be more promising for the treatment of NAFLD and also for advanced stage of NAFLD in children as we have already seen in our 1st group; probiotic plus lifestyle intervention.

Table 4.14 explains comparison of changes in anthropometric and biochemical parameters for probiotic and placebo interventions. Most of the significant changes have seen more in all variables given in table 4.14 in probiotic group alone as compared to placebo group ($p<0.001$). These results are showing probiotic VSL#3 is more potent and effective than placebo as our 2nd objective concluded by this analysis.

Table 4.15. Comparison of changes in leptin and ghrelin parameters for probiotic and lifestyle interventions

Variables	Probiotic	Lifestyle	p value	Significance
	n = 27	n = 26		
Leptin (ng/ml)	28.63	25.31	.426	ns
Ghrelin (ng/ml)	28.04	25.92	.618	ns

Values are Mean Ranks. P values refer to comparisons between the Probiotic and Lifestyle Group Mann-Whitney U Test $p<0.05$, ns= non significant

In our study, changes in leptin and ghrelin between both the groups were non significantly because of both interventions have notable impact on obesity hormones. Change in metabolic functions led to decrease in weight and increase in satiety value. Earlier study reported that *L. plantarum* declined the leptin levels in animal research (An *et al*., 2011). Other research group demonstrated *Bifidobacterium and B. longum* decreased circulatory level of leptin in morbid obese mouse (Takemura *et al*., 2010). Leptin deficiency was improved in probiotic group and in lifestyle intervention, weight loss achieved through low calorie diet followed with exercise, their leptin level decreased and ghrelin was increased concomitantly at the end of study.

Table 4.16. Comparison of changes in leptin and ghrelin parameters for probiotic and placebo interventions

Variables	Probiotic	Placebo	Significance
	n = 27	n = 27	
Leptin (ng/ml)	40.41	14.59	**
Ghrelin (ng/ml)	16.91	38.09	**

Values are Mean Ranks. P values refer to comparisons between the Probiotic and Placebo Group Mann-Whitney U Test $p<0.05$, $p<0.001$** (highly significant)

Significant higher changes were seen in leptin, ghrelin hormones in probiotic VSL#3 group ($p<0.001$) as compared to placebo, basically leptin was increased (1.24) and ghrelin was decreased (-1.22) in placebo group as shown in table 4.7.

CHAPTER-5

CONCLUSION, FUTUTRE SCOPE & RECOMMENDATION

In today's era NAFLD has emerged among obese children which are one of the upcoming causes of chronic liver disease in developing countries where faulty dietary habits and poor lifestyle is more prevalent. In Punjab, obesity including central adiposity is widespread in children because of adoption of junk food items that leads to pathogenesis of NAFLD and its risk factors.

In current research an attempt has been made to evaluate the potential effects of Probiotic VSL#3 and Lifestyle intervention in obese children with NAFLD in community of Punjab. Nutritional assessment was first step to identify nutritional status of children. Obese children were defined by BMI considering WHO standard reference, diagnosed fatty liver grades by USG and their dietary intake which was calculated before intervention. After all relevant screening, obese children were enrolled for treatment and divided in to four groups. The results of study are as below:

- Our study demonstrated NAFLD prevalence rate 66.2% in obese children age ranged 8-16 years that was more comparable to some studies. Dietary habits of children were found poor. The consumption of fructose rich soft drinks with fried food items were more pervasive among obese children with NAFLD. Most of them were taking calorie from fat and carbohydrate which leads to pathogenesis of NAFLD. In our research most associated variable with prediction of NAFLD were triglyceride (OR= 30.68) and Z-BMI 2and 3SD (OR= 7.84 and 11.91) among obese children ($p<0.05$).

- Combined therapy of VSL#3 plus lifestyle intervention was found most effective than single therapy of probiotic VSL#3 alone and lifestyle intervention. Combined therapy had 9%, single therapy of probiotic had 4.2%, lifestyle intervention had 5.3% and placebo group had 0% weight loss after intervention. 5-10% weight loss is crucial for decline in fatty liver grades as suggested by researchers. We also observed the liver grades that improved after treatment significantly in three groups ($p<0.001$) as compared to placebo. Anthropometric measurements were also reduced after intervention in three groups significantly ($p<0.001$) as compared to placebo. Biochemical parameters such as ALT, AST, GGT, LDL, cholesterol, triglyceride, CRP, FBG and uric acid were significantly declined with subsequent increase HDL as

a result of combined therapy of VSL#3 plus lifestyle and probiotic VSL#3 alone ($p<0.001$). In lifestyle intervention all biochemical variables were reduced ($p<0.05$) except LDL, cholesterol and HDL which were non significant. Change in gut microbiota framework is new evidence from various meta-analyses of RCT's and emerged in management of NAFLD because gut micro flora is involved in gut permeability, enhance gut barrier function, prevent bacterial overgrowth in intestine (SIBO), prevention of inflammation, and maintain immune system that indulges in metabolism of bile acid, choline and also production of ethanol. Probiotics therapy declined the calorie availability from nondigestible carbohydrates. Probiotics basically targets the gut liver axis.

- Lifestyle intervention is cornerstone modality for the prevention of NAFLD and choice method for treatment. Lifestyle intervention could be defined as under supervision of Registered Dietician before NAFLD occurrence. Behavior modification for weight loss programme in children needs tact and time. Children in lifestyle intervention (decreased 301 kcal) and VSL#3 plus lifestyle group (decreased 451 kcal) followed diet plan and exercise at least three days a week as much as possible and reduced their calorie intake including decreased fat and carbohydrate intake as compared to previous intakes of these and their fiber intake was also increased as compared to their baseline.

- Normal home-based diet was advised to children in VSL#3 alone group but their food intake decreased (200 kcal after 16 weeks) after supplementation of probiotic which improved their cravings of junk food item and better their gut microbiota composition and also maintained their gut hormones. Proposed mechanism of VSL#3 targets SCFA hormonal axis, promotes butyrate production and release of GLP-1 from intestinal L cells for increasing satiety of obese person and prevent weight gain.VSL#3 contain eight potential strains of friendly bacteria and balanced the gut bacterial proportion and also decrease intestinal permeability. Not any side effects of probiotic were seen in our study.

- Normal home-based diet in placebo group also advised but not significant reduction was seen in parameters at the end of study because there was not any effect of placebo capsule.

- VSL#3 and Lifestyle intervention both were found effective for managing the NAFLD but these interventions work independently for changing in most variables. Sometimes less compliance in children to follow diet plans and programme of exercise was observed because of study pressure which induces more sittings and less time for physical activity for children moreover is strenuous to achieve desired weight loss. In this condition multi-target therapy (probiotic plus lifestyle intervention) can be advised by pediatrician and dietician, so that obesity and NAFLD risk factors could be controlled as early as possible.

- In our study serum leptin and ghrelin hormone were evaluated that regulates energy homeostasis in body especially leptin maintains energy intake and expenditure having anorexic effect. After intervention serum leptin levels were decreased in three groups and ghrelin levels were increased in three groups as compared to placebo. Because of weight loss at the end, their metabolic efficiency is improved with increased BMR, their leptin deficiency recovered with decrease in BMI and TSF. According to our knowledge in India, there is no any pertinent study that has done for seeing the effect of probiotic VSL#3 on leptin and ghrelin hormones in obese children with NAFLD.

- Limitations of the study: Presently we did not perform the following test NEFA(non-esterified fatty acid), OGTT(oral glucose tolerance test) and HOMA(homeostasis model assessment) which can indicate the substantial mechanistic link between obesity and insulin resistance and metabolic dyslipidemia and hence, predicting the risk of NAFLD. The diagnosis of NAFLD was based upon USG; we could not take written consent for liver biopsy.

- Future scope of the study: Probiotic VSL#3 is significantly effective for managing the NAFLD in obese children and we have not seen any adverse effects of probiotic in the study. Lifestyle intervention is first line safeguard treatment for managing NAFLD. Sometimes less compliance in children for behavior modification to adopt good eating habits and it may take time in modification. In this condition multi-target therapy of VSL#3 plus lifestyle intervention is novel treatment approach for managing NAFLD in obese children. In advanced stage of NAFLD/NASH combined therapy can be used for treatment. Probiotic therapies are defined for change in gut microbiota composition and also enhance the intestinal mucosal barrier function that improves metabolic efficiency of nutrients. VSL#3 and lifestyle intervention have

beneficial impact and also positive effects on leptin and ghrelin in obese children with NAFLD and these are new investigations in our study.

- **Future recommendations is to evaluate the effect of probioticVSL#3 (De Simone Formulation) potent with all 8 bacterial strains should be used on all hormones responsible for obesity and inflammatory markers in obese children with advanced stage of biopsy proven NAFD/NASH.**

CHAPTER-6

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Chapter-7
APPENDIX- 1
PROFORMA/QUESTIONNAIRE

Study Title: **The Effect of Probiotic VSL#3 and Lifestyle Intervention in Obese Children with Non-Alcoholic Fatty Liver Disease (NAFLD)**

Groups coded: VSL#3 plus lifestyle (1) VSL#3 alone (2) Lifestyle alone (3) Placebo (4)

1. GENERAL INFORMATION

Sr No. DOA: Father Name Mother Name:

Complete Diagnosis: (a) **About the subject**

Name: Age/Sex:

School:

Class: Residential Area:

2. ANTHROPOMETRIC MEASUREMENTS: Before After

Weight (kg): Height (cm):

Body Mass Index (BMI):

weight (Kgms)/ Height (m^2) =

SD Z-score=

Waist circumference (cm)

MAC (cm):

TSF thickness (mm):

3. BLOOD PARAMETERS:

Parameters
ALT U/L
AST U/L
Uric acid mg/dl
GGT U/L
Glucose mg/dl fasting
Cholesterol mg/dl
HDL cholesterol mg/dl
LDL cholesterol mg/dl
Triglycerides mg/dl
C-reactive protein mg/l
Leptin ng/ml
Ghrelin ng/ml

4. ULTRASONOGRAPHY DIAGNOSE STEATOSIS

Liver Ultrasound Grades:	Before Intervention (0 month)	After Intervention(4months)
(0) Normal absent Steatosis		
(1) Grade 1		
(2) Grade 2		
(3) Grade 3		

5. DIET HISTORY:

3-day dietary recall history before and after intervention:

DAY 1		DAY 2		DAY 3
Cereal				
Milk products				
Meat				
Fruits				
Vegetables				
Fats				
Sugar				

Consumption of food items

leads to NAFLD **Daily** **Twice** **Thrice**

1.Soft Drinks (coke, sprite, pepsi, fanta and mountain-dew),

2. Fried Chips or French Fries

3. Others(pizza, burger, noodles)

Before intervention & After intervention Nutrients Calculated:
Energy (kcal), protein, fat, carbohydrate and fiber

Dietary Guidelines for NAFLD patients:

Hypo-calorie modified diet according to age group consisting Protein:
15-20%, Fat: 23-30%, carbohydrate: 50-60%

6. PHYSICAL ACTIVITY:

No exercise: (watching TV, video games and surfing on internet) (no fixed hours)

Physical exercise: aerobics (Skipping rope, brisk walking, playing tennis, dance & swimming) (minimum 3 days a week) or (maximum 5 days a week) Recommended Exercise: 30-45 min/day

Exercise min/ day	**Before Intervention (0 month)**	**After Intervention (4 months)**
(0) No exercise		
(1) 15-30 min		
(2) >30 min(up to 1hour)		

IEC, PGIMER,Chd; Assent Form

PROTOCOL NO: 2016/2608

SPONSOR: Sant Kapoor Singh Ji, Babe Ke Medical Hospital Moga, Punjab

PRINCIPAL INVESTIGATOR: POOJA GOYAL

Title: **"The Effect of Probiotic VSL#3 and Lifestyle Intervention in Obese Children with Non-Alcoholic Fatty Liver Disease (NAFLD)"**

We are doing a research study. I am Pooja Goyal (Registered Dietician) doing doctoral degree from Lovely Professional University Phagwara, Punjab.
Prof. BR Thapa, Head Gastroenterology PGIMER, Chd (Pediatrician) and Dr. Anmol Bhatia, Dept. of Gastroenterology PGIMER, Chd (Radiologist) will examine you.
We are doing this study to find out fatty liver in obese children and will prevent it by using probiotic VSL#3 Vs Placebo for the management of fatty liver.
We are asking you to take part in this study because you are obese children (age group 5-18 years) but we will only take you if you allow us. If you do not want to do so your treatment will continue as usual. If you decide to take part now but wish to discontinue later, you can tell us and we will take you out of the study.
Once you agree to take part, ultrasound will be done for diagnosis of fatty liver. You will have to give your blood samples and anthropometric measurements and dietary intake. Low calorie diet and physical activity will be prescribed to you for treatment. It will be better for your health.
No risks & discomfort to you. If you feel you can terminate from the study.
It is possible that the study will help you feel better. It can also occur that you do not get any benefit but the information we get from you may help other children in future.
We have asked your parents or guardian their permission and it is all right with them.
Do not hesitate to ask questions. You can also ask us about anything later on if there are no questions right now.

Assent form

Parent signature:

	Child's signature
I have been explained about the study and I agree to take part in it.	

Child’s Name: Date:

Certificate by the Investigator (his/her representative obtaining assent):

	Tick one	Signature of the Investigator / representative
The child can read the assent form and was able to understand it		
The child was not capable of reading the assent form, but I verbally explained the information.		

Name of Investigator / representative: Date:

APPENDIX- 2

Diet Chart for 7-9 yrs Obese Child with Non Alcoholic Fatty Liver Disease

RDA Energy = 1690 Kcal

Modified Energy = 1490 Kcal

Early morning:	lukewarm water 4-5 almonds/ 1-2 walnuts
Breakfast:	broken wheat/oats/kelloggs/muselli low fat milk dalia- 1 bowl/ 2-besan/ragi puda + 1 Fruit or 2-missi stuffed chapati without frying + 1tsp butter homemade+egg white-1/paneer- 20g
Mid morning:	Fruit-1 or Sprouts- 1 katori
Lunch:	1 Plate green salad 2 missi chapatti + 1 katori leafy veg+ 1 katori beans (rajma/ black chana/white chana/soyabean/peas)
Evening snack:	1cup (150ml) cow's milk roasted chana/ soyabean/makhana/chidwa- 1 handful or brown bread sandwich-1/wheat veg pasta/vegetable upma/wheat veg vermicelli- 1 katori/ marie gold biscuit-2
Dinner:	Fruit chat- 1 small bowl 1 chapati+ 1 katori veg+ 1 katori dal soup/ chicken soup 3-4 tsp/day (olive, sunflower, soyabean, groundnut, mustard oil, peanut butter)
Exercise	30-45min/day at least 5 days a week aerobics (Skipping rope, brisk walking, playing tennis, dance & swimming)

Diet Chart for 10-12 yrs Obese Child with Non Alcoholic Fatty Liver Disease

RDA Energy = 2100 Kcal

Modified Energy= 1900Kcal

Early morning:	lukewarm water 4-5 almonds/ 1-2 walnuts
Breakfast:	broken wheat /oats/kelloggs/muselli low fat milk dalia- 1 bowl/ 2-brown bread slices + 1 cup milk or 2- missi stuffed chapati without frying + 1tsp butter homemade +egg white-1-2/ paneer- 25g
Mid morning:	Fruit-1 or Sprouts- 1 katori coconut water/lemon water/fruit juice homemade- 1 glass
Lunch:	1 Plate green salad + rice /2 missi chapatti+ 1 katori leafy veg+ 1 katori beans (rajma/black chana/white chana/soyabean/peas)
Evening snack:	1cup (150ml) cow's milk roasted chana, soyabean, makhana, chidwa- 1 handful or brown bread sandwich-1/wheat veg pasta/vegetable upma/wheat veg vermicelli- 1 katori/marie biscuit-2
Dinner:	Fruit chat- 1 small bowl 1 chapati+ 1 katori veg+ 1 katori dal soup/ chicken
Oil :	3-4 tsp/day (olive, sunflower,soyabean, groundnut, mustard, peanut butter)
Exercise	30-45min/day at least 5 days a week aerobics (Skipping rope, brisk walking, playing tennis, dance & swimming)

Diet Chart for 13-15 yrs Obese Child with Non Alcoholic Fatty Liver Disease

RDA Energy= 2540 Kcal

Modified Energy = 2340Kcal

Early morning:	lukewarm water 4-5 almonds/ 1-2 walnuts
Breakfast:	broken wheat/oats/kelloggs/muselli low fat milk dalia- 1 bowl/ 4-brown bread slices + 1 cup milk or 3missi stuffedchapati without frying + 1tsp butter homemade +egg white-1-3/ paneer- 25g
Mid morning:	Fruit-1 or Sprouts- 1 katori coconut water/lemon water/fruit juice homemade- 1 glass
Lunch:	1 Plate green salad Rice- 1 plate medium/ 2 missi chapatti + 1 katori leafy veg+1 katori beans (rajma/black chana/white chana/soyabean/peas)
Evening snack:	1cup (150ml) cow's milk roasted chana/soyabean/makhana/chidwa- 1 katori or brown bread sandwich-1 wheat veg pasta/ vegetable upma/wheat veg vermicelli- 1 quarter plate
Dinner:	Fruit chat- 1 small bowl 2 chapati+ 1 katori veg+ 1 katori dal soup/ chicken soup
Oil : -	4 tsp/day (olive, sunflower, soyabean, groundnut, mustard, peanut butter)
Exercise	30-45min/day at least 5 days a week aerobics (Skipping rope, brisk walking, playing tennis, dance & swimming)

Diet Chart for 16 yrs Obese Child with Non Alcoholic Fatty Liver Disease

RDA Energy= 2730 Kcal

Modified Energy = 2530Kcal

Early morning:	lukewarm water 4-5 almonds/ 1-2 walnuts
Breakfast:	brokenwheat/ oats/kelloggs/muselli low fat milk dalia- 1 bowl/ 4-Brown bread slices + 1 cup milk or 3 missi stuffed chapati without frying + 1tsp butter homemade +egg white-2-3/ paneer- 25g
Mid morning:	Fruit-1 or Sprouts- 1 katori coconut water/lemon water/fruit juice homemade- 1 glass
Lunch:	1 Plate green salad Rice- 1 plate/ 3 missi chapatti + 1 katori leafy veg+ 1 katori beans (rajma/black chana/white chana/soyabean/peas)
Evening snack:	1cup(150ml) cow's milk roasted chana/ soyabean,/makhana/chidwa- 1 katori or brown bread sandwich-1/wheat veg pasta/ vegetable upma/wheat veg vermicelli- 1 quarter plate
Dinner:	Fruit chat- 1 small bowl 3 chapati+ 1 katori veg+ 1 katori dal soup/ chicken soup
Oil : -	4-5 tsp/day (olive, sunflower, soyabean, groundnut, peanut butter)
Exercise	30-45min/day at least 5 days a week aerobics (Skipping rope, brisk walking, playing tennis, dance & swimming)

Foods beneficial for liver & healthy tips

Grapefruit, beets, carrot, apples, orange, broccoli, lemom/lime, argula, walnuts, cabbage, cauliflower, avocado, spinach, garlic, turmeric, curry leaves, kali mirch cleans the liver. Add also these veggies in the diet: mushroom, ghia, tori, pumpkin, onion, tomato, kheera, lettuce, capsicum, brinjal, bhindi, karela (3-5 servings/day).

- use homemade green leafy, coconut, peanut chutney
- Drinks: homemade juice, lemon water, coconut water, jal jeera & homemade shakes.
- Take fruits instead of fruit juices (2 servings/day).
- Use gur, shakkar if required instead of white sugar.
- use whole grain atta: bajra, ragi, jowar, soyabean , chana, besan.
- Add also pulses in the diet at least one time in a day.
- Daily glass of water: 8-10/day.
- Follow your meal plan & stick to it for 4 months.

1. **Fortified Atta:**

Wheat	60g
Besan	40g

Mixed it well and use without sieved

Foods to be restricted:

- Cold drinks: coke, pepsi, maaza, spirit, mountain dew, diet cola etc. sweetened juices, instant energy drinks market made.
- avoid trans fat like pizza, burger, noodles, momos, instant foods, fried foods.
- Colored sweets, preservatives, baking powder, baking soda, ajinomoto, maida, candy, chocolates, ice creams, white rice, pastry, cake, muffins, white bread, naan.
- Restrict more starchy foods like aloo, zimikand, kacahlu, arbi.
- Don’t use more table salt and sugar and avoid ketchups, creamy spreads, jellies.

APPENDIX-3

LIST OF PUBLICATIONS

1. Nutritional Assessment in Obese Children with and without Non-Alcoholic Fatty Liver Disease (NAFLD) in an urban area of punjab, India (Accepted) Indian Journal of Public Health Research and Development (Vol. 9, No. 12, Dec 2018). DOI Number: 10.5958/0976-5506.2018.01833.8.
2. Goyal P, Thapa BR, Sharma NR, Bhatia A. The Effect of Probiotic and Lifestyle Modification in Obese Pediatrics with Non-Alcoholic Fatty Liver Disease. Indian J Comm Health. 2019; 31, 1: 50-56.

APPENDIX-4

LOVELY PROFESSIONAL UNIVERSITY
Transforming Education Transforming India

School of Research Degree Programmes

LPU/RDP/EC/160922/31

Dated: 22nd Sep 2016

Pooja Goyal

3509 Kapoor Street

Jagraon, Ludhiana

Punjab

Subject: Letter of Candidacy for Ph.D.

We are very pleased to inform you that the Department Doctoral Board has approved your candidacy for the Ph.D degree on 4th March 2016 by accepting your thesis research proposal titled: "THE EFFECT OF PROBIOTIC VSL#3 AND LIFESTYLE INTERVENTION IN OBESE CHILDREN WITH NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)" supervised by Professor, Dr. Neeta Raj Sharma, Lovely Professional University, Phagwara.

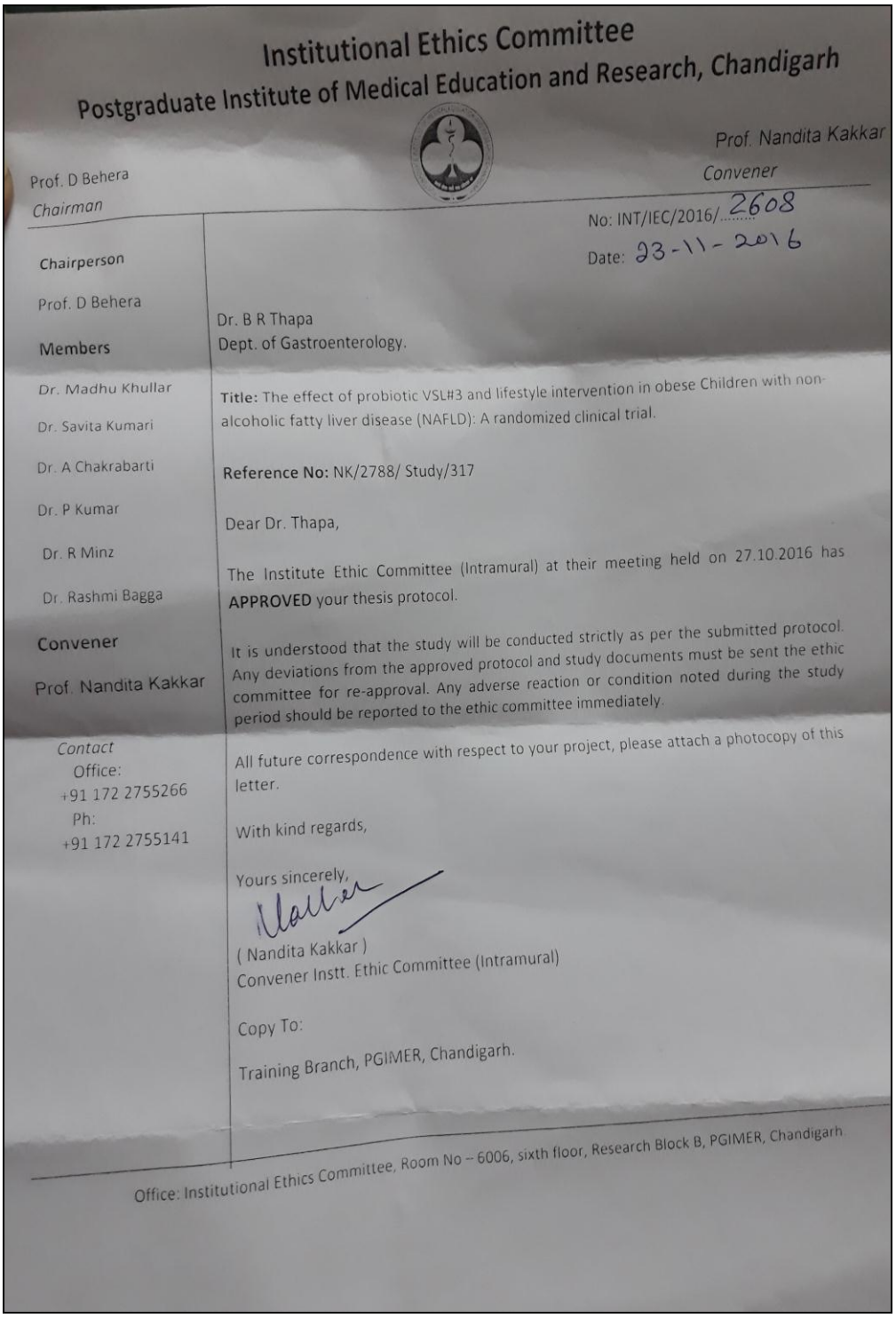
As a Ph.D. candidate you are required to abide by the conditions, rules and regulations laid down for Ph.D. degree students of the University, and amendments, if any, made from time to time.

We wish you the very best in completing your thesis research requirements in the near future. Please do not hesitate to contact us in case you have questions about the rules and regulations of the University.

Signature of HOS (Research Degree Programmes)

Jalandhar-Delhi G.T.Road, Phagwara, Punjab (India) - 144411
Ph : +91-1824-444594 Fax : +91-1824-240830 E-mail : drp@lpu.co.in website : www.lpu.in

APPENDIX-5



Institutional Ethics Committee
Postgraduate Institute of Medical Education and Research, Chandigarh

Prof. D Behera
Chairman

Prof. Nandita Kakkar
Convener

No: INT/IEC/2016/2608
Date: 23-11-2016

Chairperson
Prof. D Behera

Members
Dr. Madhu Khullar
Dr. Savita Kumari
Dr. A Chakrabarti
Dr. P Kumar
Dr. R Minz
Dr. Rashmi Bagga

Convener
Prof. Nandita Kakkar

Contact
Office:
+91 172 2755266
Ph:
+91 172 2755141

Dr. B R Thapa
Dept. of Gastroenterology.

Title: The effect of probiotic VSL#3 and lifestyle intervention in obese Children with non-alcoholic fatty liver disease (NAFLD): A randomized clinical trial.

Reference No: NK/2788/ Study/317

Dear Dr. Thapa,

The Institute Ethic Committee (Intramural) at their meeting held on 27.10.2016 has **APPROVED** your thesis protocol.

It is understood that the study will be conducted strictly as per the submitted protocol. Any deviations from the approved protocol and study documents must be sent the ethic committee for re-approval. Any adverse reaction or condition noted during the study period should be reported to the ethic committee immediately.

All future correspondence with respect to your project, please attach a photocopy of this letter.

With kind regards,

Yours sincerely,

(Nandita Kakkar)
Convener Instt. Ethic Committee (Intramural)

Copy To:

Training Branch, PGIMER, Chandigarh.

Office: Institutional Ethics Committee, Room No – 6006, sixth floor, Research Block B, PGIMER, Chandigarh

APPENDIX-6

KEY TO MASTER SHEET

Sr no.	Serial number
WT	Weight
HT	Height
BMI	Body mass index
Z	Z score
MAC	Mid arm circumference
TSF	Tricep skinfold thickness
AST	Alanine aminotransferase
ALT	Aspartate aminotransferse
GGT	Gamma glutamyl transferase
LDL	Low density lipoprotein
HDL	High density lipoprotein
CHOL	Cholesterol
TG	Triglyceride
CRP	C-reactive protein
BS	Blood sugar
UR	Uric acid
KCAL	Kilocalorie
PROT	Protein
FAT	Fat
CHO	Carbohydrate
FB	Fiber
BEX	Before excercise
AEX	After excercise
BLG	Before liver grade
ALG	After liver grade
SFD	Soft drinks
FD	Fried food
OT	Others

EX	Exercise
0	None
1	15-30 min
2	>30 min up to 1 hour
LG	Liver grade
0	None
1	Mild
2	Moderate
3	Severe
L	Leptin
PL	Post leptin
G	Ghrelin
PG	Post ghrelin
SFD, FD & OT	
1	Daily
2	Twice a week
3	Thrice a week

NAFLD (n=106) and without NAFLD(n=54)

L	PL(1)	L	PL(2)	L	PL(3)	L	PL(4)	G	PG(1)	G	PG(2)	G	PG(3)	G	PG(4)
19	15	21	17.9	19.9	16.9	19	20	25	34	26	34	22	25	24	18
17	13	22	19	19.9	16.9	19	21	26	33	26	31.7	26	30	22	26
18	14	22	19.7	22	19	18	19	22	31	21	29.1	22	26	21	22
19	15	22	20	25	22	19	19	28	37	25	32	28	33	28	27
20	16	20	18	26	23	23	21	29	35	26	33	29	33.1	29	29
21	17	21	18.9	22	19	26	27	30	37	29	34	30	33.8	30	31
16	12	22	19.9	22	19	21	22	34	37	23	21	34	39	34	32
17	13	22	18.9	23	20	24	26	22	29.8	35	33	22	27.6	22	21
18	14	24	20.9	18	15	18	22	25	30.5	25	25.1	25	30	25	24
20	16	25	21.9	31	23	19	25	23	29.5	23	26	23	27	23	22
25	21	25	21.5	25	22.5	25	22	24	30.6	24	28	24	31.7	24	23
26	22	21	18	26	23.5	26	29	25	30.4	25	30	25	31.7	22	24
27	23	16	13	27	24.5	27	29	26	35	26	25	26	31.4	26	25
25	21	17	14.8	25	22.5	25	29	28	33.7	25	28	28	34.7	28	27
21	17.5	21	18.5	21	18.5	22	23	21	25.1	23	29	22	27	21	21
23	18	23	20.3	23	20.5	21	22	22	28.1	26	31.5	26	32	25	25
33	28	24	21.7	24	19	25	27	21	29	21	27.3	24	30	23	23
25	20	25	22.2	25	22.5	29	30	22	29.8	22	27.7	24	27	24	23
37	31	23	20.7	23	20.5	30	31	23	30	23	26.8	27	33.7	27	26
33	29	30	27	18.9	17	21	21	24	30	24	29.8	24	30.8	28	23
21	16	18	15	35	33.1	32	32	25	29	25	31.9	25	33.8	27	23
19.9	14.9	19.9	16.7	19.9	19.9	19.9	21	26	30.3	26	25	26	31.1	26	24
24	19	24	20.8	24	22.1	24	25	27	31.9	27	33.4	27	32	27	25
25	20	25	21.7	25	22	25	25	22	31.8	22	25	22	26	23	20
25	19	22.6	19.3	22.6	19	22.6	23	28	35	28	33.9	28	33.5	28	26
25	21	26	23	27	25.1	28	29	26	35	26	31.9	26	29.3	26	24
		27	24.7			26	28			26	26			28	24

Sr no.	SEX	AGE	WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	CRP	BS	UR	KCAL	PROT	FAT	CHO	FB
1	f	16	90	165	33.06	3.2	35	19	88	44	70	24	100	38	172	160	3	112	7	2700	67.5	99	385	13
2	m	12	59	149	26.58	2.3	30	23	87	44	53	18	99	38	166	150	3	106	6.7	2270	56.75	83.2	323	12
3	m	10	58	155	24.14	2.2	33	24	86	43	70	19	99	40	164	148	2	92	5.5	2450	61.25	89.83	349	14
4	m	12	55	145	26.16	2.7	33	21	86	45	54	26	99	38	168	140	1.5	94	5.9	2270	56.75	83.23	324	15
5	m	14	79	163	29.73	2.4	34	19	87	44	53	22	99	35	167	140	2.5	99	6.7	2590	64.75	95	369	11
6	m	8	56	144	27.01	2.7	33	21	84	45	54	27	99	43	166	135	2.5	98	3.7	1950	48.75	71.5	278	13
7	m	12	65	140	33.16	3	34	19	88	49	58	28	95	39	167	138	3	99	6.6	2450	61.25	89.83	349	12
8	m	15	69	150	30.67	3	35	22	87	56	60	28	89	35	181	151	3.2	95	4.5	3010	75.25	110.4	429	12
9	m	14	49	145	23.31	2.5	34.5	19.5	84	38	55	19	98	35	180	154	2.7	86	4.2	3002	75	110.4	429	13
10	m	12	48	140	24.49	2.5	34	19	85	45	55	20	99	37	179	153	2.8	97	5.3	2450	61.2	89.82	349	14
11	m	16	90	163	33.87	2.3	36	21	89	48	60	21	90	35	168	151	2.4	100	5	3280	82	120.3	467	15
12	m	12	55	155	22.89	2.5	35	20	88	54	62	23	90	41	150	150	2.4	100	5	2450	61.5	89.83	349	12
13	m	13	50	135	27.43	2.4	38	21	88	55	60	20	98	38	185	145	2.5	100	6	3000	67.5	88	400	14
14	m	11	41	133	23.18	2.3	30	20	87	50	59	19	111	32	184	145	2.2	89	5	2438	67	87	400	14
15	m	12	55	145	26.16	2.4	32	20.5	89	40	51	22	114	38	164	152	2.1	98	4.5	2260	69	88	366	13
16	m	11	50	142	24.80	2.8	33	19	86	49	58	23	112	48	181	155	2.3	97	4.3	2258	55	89	390	15
17	f	12	63	150	28.00	2.4	35	20	89	48	59	23	91	42	184	145	2	100	5	2257	59	88	357	17
18	f	12	65	145	30.92	3.4	34	19	84	55	70	21	89	38	184	148	3	78	5	2258	55	87	370	19
19	f	11	59	155	24.56	2.6	34	21	88	56	55	22	109	34	172	152	2.3	78	6	2262	55	89	350	13
20	f	11	66	155	27.47	2.4	31	23	89	44	55	21	118	40	176	152	2	89	5.5	2230	56	95	330	13
21	f	11	65	157	26.37	2.5	29.9	19.9	88	46	56	18	113	40	177	152	2.4	89	6	2290	59	90	320	13
22	f	11	65	155	27.06	2.8	31	18	88	42	55	19	111	45	170	130	2.5	95	6	2435	55	87	355	12
23	f	12	60	154	25.30	2.5	33	20	86	40	40	18	120	48	167	140	3	99	6	3009	60	95	400	14
24	m	11	55	155	22.89	2.8	38	23	88	48	76	18	94	40	171	144	2.4	90	6	2445	69	95	420	14
25	f	11	55	156	22.60	2.5	32	20	87	54	51	19	111	38	167	139	2.2	96	6	2438	65	89	380	11
26	f	11.8	81	150	36.00	3	39	27	86	50	62	20	114	38	164	155	2.3	97	6	2444	61	88	360	11

WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	CRP	BS	UR	KCAL	PROT	FAT	CHO	FB	BEX	AEX	BLG	ALG
83.1	165	30.52	2.5	32.5	15	84	34.1	50	22	95	41	164	150	1.9	106	3.8	2250	61.8	78	325.12	23	0	2	3	2
54	149	24.32	2.5	27.5	19.1	83	36	45	15	96	41	158	139	1.9	98	3.1	1820	50.1	63.09	262.99	21	0	2	1	0
50	155	20.81	2.2	30.8	21.8	83.8	37	55.1	16	97	40	161	139	0.9	86	4.1	2000	55	69.33	289	17	1	2	2	1
52	145	24.73	1.8	31.2	19.2	84.2	34	50	24	94	37	164	131	0.4	87	3.3	1980	54.4	68.64	286.11	14	0	2	2	1
73.2	163	27.55	2.4	31.6	16.6	82	37	40	20	95	44	165	132	1.4	92.3	3.4	2140	58.9	74.18	309.23	20	0	2	1	0
50	144	24.11	2	32.1	20.1	83.1	34	44	22	96	41	164	130	1.4	91.3	3.5	1500	41.3	52	216.75	20	1	2	2	1
64	140	32.65	2.5	33	18	84.5	37.2	44	20	93	36	165	132	1.9	92.3	3.1	2200	60.5	76.26	317.9	20	0	2	1	0
61	150	27.11	2	33	19	84.1	35.1	44	20	86	38	166	133	2.1	88.3	4	1770	48.6	61.36	255.76	20	0	2	2	1
43	145	20.45	2.1	32.6	17.6	82.1	34	41	16	94	41	168	140	1.6	79.3	3.5	1950	53.6	67.6	281.77	19	1	1	1	0
44	140	22.45	1.9	32.1	17.1	83.1	38	40	17	96	40	169	142	1.7	90	3.9	1900	52.3	65.86	274.55	19	0	1	1	0
81.5	163	30.67	2.6	34.5	19.5	87.5	42	42	16	85	44	161	142	1.3	93.3	3.5	2090	57.4	72.45	302	22	0	2	2	1
50	155	20.81	2	33.4	17.3	86.4	43	41	17	86	41	145	137	1.3	92	3.7	2300	63.3	79.73	332.35	19	0	2	1	0
43	135	23.59	2	37.5	20.5	86.1	43	42.1	19	95	42	179	137	1.4	92	3.5	2600	71.5	90.13	375.7	15	0	2	2	1
39	133	22.05	1.6	28.4	18.4	85.4	42	42	18	105	40	174	138	1.1	82.3	5.2	2050	56.3	71.06	296.2	20	0	2	2	1
51	145	24.26	2	31	19.5	88	40	44	20	106	42	160	141	1	91.3	3.1	1870	51.4	64.82	270.2	22	0	2	1	0
46	142	22.81	2.2	30.8	16.8	83.8	43	43	19	107	50	170	140	1.2	90.3	3.1	2190	60.2	75.92	316.4	18	0	2	2	1
56	150	24.89	2.2	34	19	86.5	39	49.1	20	87	44	171	130	1	94	3.7	1550	42.6	53.73	223.9	22	0	1	2	1
58.1	145	27.63	2	32	17	82	39	55	19	86	40	173	142	1.9	73	4	1999	54.9	69.29	288.8	21	0	2	1	0
50	155	20.81	2.1	32.6	19.6	86.6	37	47	18	104	36	165	137	1	72	3	2610	71.7	90.48	377.14	22	0	1	2	1
60	155	24.97	2.2	29.3	21.3	86.5	36	45	16	110	43	166	142	0.9	82.3	3.2	1870	51.4	64.82	270.21	21	0	1	2	1
62	157	25.15	2	28.5	18.5	85.2	35	45	15	106	43	169	141	1	82.3	3.5	2190	60.2	75.92	316.45	21	0	2	1	0
60	155	24.97	1.9	29.5	16.5	85.1	37	44	14	115	47	160	125	1	88.3	3.4	2000	55	69.33	289	22	0	2	2	1
55.2	154	23.28	2.4	32	19	83.2	35	35	15	110	50	160	135	1.9	94	3.2	2500	68.9	86.66	361.25	21	0	2	2	1
47	155	19.56	2.1	36.1	18.2	84.2	35.2	55	17	90	42	159	141	1.3	83.3	3	1900	52.3	65.8	274.55	20	0	1	1	0
49.9	156	20.50	2.2	29.8	17.8	83.1	35.1	45	18	99	43	157	133	1.3	88	3.1	2000	55	69.5	289	20	0	1	3	2
76.3	150	33.91	2.5	37.3	25.3	85	34	50	17	97	44	152	139	1	90.3	3.1	1950	53.6	67.6	281.77	21	0	1	3	1

Sr no.	SEX	AGE	WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	CRP	BS	UR	KCAL	PROT	FAT	CHO	FB
1	m	16	85	165	31.22	3.2	35	19	86	48	57	24	100	38	172	145	3	112	7	2700	67.5	99	384.75	13
2	m	10	55	149	24.77	2.3	30	23	88	44	53	18	99	38	166	150	3	91	6.7	2450	61.2	89.8	349.1	12
3	m	10	48	142	23.8	2.2	33	24	86	43	52	19	99	40	164	150	2	92	5.5	2269	56.75	83.2	323.4	14
4	m	9	45	138	23.63	2.7	33	21	86	45	54	26	99	40	168	150	1.5	94	5.9	2590	64.75	95	369.075	15
5	m	14	71	163	26.72	2.4	34	19	84	44	53	22	99	35	167	150	2.5	99	6.7	1950	48.75	71.2	277	11
6	m	8	38	133	21.48	2.5	33	21	84	45	54	27	99	43	167	148	2.5	98	3.7	2268	56.74	83.3	323.4	13
7	m	12	59	141	29.68	2.4	34	19	88	49	58	28	95	39	167	149	3	99	6.6	2450	61.25	89.8	349.125	12
8	m	15	69	152	29.86	2.4	35	22	87	56	45	28	89	35	181	151	3.2	95	4.5	2272	56.7	83.2	322.275	12
9	m	10	48	140	24.49	2.4	34.5	19.5	84	38	47	19	98	35	180	154	2.7	86	4.2	2270	56.8	83.2	323.5	13
10	m	10	48	141	24.14	2.3	34	19	85	45	42	20	99	37	179	153	2.8	97	5.3	3280	82	120	467.4	14
11	m	16	90	163	33.87	2.3	36	21	87	48	41	21	90	35	168	151	2.4	100	3.8	2268	56.73	83.3	324.175	15
12	m	11	55	155	22.89	2.4	35	20	88	54	62	23	90	41	150	150	2.4	98	5	2450	61.25	89.9	349.125	12
13	m	12	67	144	32.31	3.5	35	20	88	52	41	24	92	40	164	153	2	109	4.2	2450	61.24	89.8	349.123	12
14	m	15	70	158	28.04	2.5	34.5	19.5	86	56	42	23	99	43	179	152	2	97	5	3009	75.25	110	428.95	11
15	m	8	47	130	27.81	2.9	35	20	87	57	43	23	89	44	179	152	3	100	3.9	1949	48.74	70	277.75	11
16	m	9	47	145	22.35	2.1	34	20	84	37	45.9	27	95	46	161	149	2	96	4.7	1951	48.75	72	277.85	11
17	f	10	79	144	38.1	3.4	34.5	24	87	46	62	22	95	47	160	149	3	99	4.8	1952	48.76	73	278	11
18	f	11	65	140	33.16	3.7	34.5	25	86	45	61	21	99	47	171	165	2	96	5	2450	61.25	89.8	349.125	11
19	f	12	63	143	30.81	2.8	32	17	85	44	65.2	18	100	48	169	151	2	97	5	2267	56.72	83.2	323.45	12
20	f	11	63	155	26.22	2.9	35	20	84	45	41	17	122	40	179	149	2	98	4.9	2450	61.26	89.7	349.15	13
21	f	12	55	149	24.77	2.5	31	18	87	34	42	19	114	40	181	154	2	100	5.6	3010	75.24	111	428.91	12
22	f	12.9	57	155	23.73	2.6	30	18	88	44	45	18	109	43	145	155	2	94	5.4	3008	75.23	110	428.9	12
23	f	13	66	158	26.44	2.7	30	22	89	57	68	21	99	36	173.5	158	2	95	4.6	3000	75.2	111	429	11
24	f	12	59	153	25.2	2.4	29.9	22	88	45	52	22	112	40	175	155	2.5	111	4.7	2450	61.25	89	349.1	12
25	f	12.4	66	154	27.83	2.7	30	22	87	48	60	21	110	35	182.5	148	2.7	100	4.5	2451	61.22	89.8	358	13
26	f	12.5	58	159	22.94	2.3	33	18	88	48	45	19	105	36	173.5	154	2	99	4.6	2200	55	80.7	342	15
27	f	12.5	66	155	27.47	2.8	35	20	88	39	45	18	109	38	182.5	154	2.4	106	6	3011	75.26	91	428.25	13

WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	CRP	BS	UR	KCAL	PROT	FAT	CHO	FB	BEX	AEX	BLG	ALG
82	165	30.12	3.1	34.9	18	85	45	55	22	98	41	166	140	2	109	5.5	2502	65.62	90.27	356.25	20	0	0	2	1
52.7	149	23.74	2.17	28	22	86	40	49	14	95	42	160	145	2	88	5.4	2250	59	81.25	320.6	15	0	0	1	0
45.9	142	22.76	2.3	32.6	23.2	85.2	40	44	17	94	41	160	145	1.88	89	4	2070	54.3	74.75	294.95	15	0	0	1	0
42.6	138	22.37	2.57	32.1	19.2	83.9	40	46	23	98	42	162	146	0.99	91	4.8	2390	62.7	86.3	340.575	14	0	0	2	1
69	163	25.97	2.5	32	16.8	83	39	44	18	97	42	163	144	0.66	96	5.2	1760	46.2	63.55	250.8	14	1	1	2	1
35.6	133	20.13	2.37	32.6	20.6	83	40	42	24	97.5	44	164	143	0.55	95	3	2080	54.6	75	296.4	18	0	0	1	0
57.6	141	28.97	2.27	33.3	17.4	84.5	38	54	23	91	41	166	144	1.76	96	5.3	2260	59.35	81	322.05	19	1	1	1	0
66.6	152	28.83	2.27	34.2	21.7	84	44.5	40	25	85.6	36	175	146	0.78	92	3.3	2082	54.6	75.1	296.2	20	1	1	1	0
45.9	140	23.42	2.27	33.7	16.5	85	30	41	15	96	37	176	149	0.79	83	3.8	2079	54.3	74.9	296.4	18	0	0	1	0
45	141	22.63	2.17	33.6	16.9	86	40	34	17	95	37	170	148	1.5	94	3.8	3000	81.11	111.58	440.325	15	1	0	1	1
86	163	32.37	3	35.5	18	86	42	34	20	88	42	160	146	0.77	97	3.6	2078	54.6	75.1	296.4	18	0	0	3	2
52.2	155	21.73	2.27	34.4	18.4	87	51	57	20	89	43	145	144	0.87	95	3.5	2300	59.25	81.62	322.05	14	0	0	2	1
64.4	144	31.06	3	34.2	19.4	86	36	37	21	87.6	43	163	148	1.6	106	2.7	2302	59.35	81.59	322.05	15	0	0	1	0
67.1	158	26.88	2.1	33.9	18	85	44	36	22	95	44	170	147	1.6	94	3.5	2700	74	101.8	401.85	15	0	0	1	0
44.4	130	26.27	2.77	34.8	19	86	36	40	19	84	47	173	146	1	97	2.4	1750	46.2	63.5	250.1	16	0	0	2	1
44.5	145	21.17	2.3	33	19.1	83.3	35	43.9	23	91.6	46	158	145	2	93	3.2	1758	46.3	63.6	250.8	14	0	0	2	1
76.7	144	36.99	3.27	32.5	22.5	85	48	52	17	92.3	47	155	144	2	96	3.2	1761	46.2	63.5	250	14	0	0	2	2
62.8	140	32.04	3.57	32.5	24	84	44	60	19	95	46	166	160	0.88	91	3.5	2260	59.2	81.61	322.05	20	0	0	2	1
60	143	29.34	2.67	31.6	16.6	84.5	42	59.2	12	97	47	165	145	1.7	92	3.5	2083	54.6	75	296.4	17	0	1	2	1
60.1	155	25.02	2.77	34.1	18	83	38	32	16	117	49	175	145	0.99	93	3.2	2260	59.5	81.61	322.05	14	0	1	1	0
52.5	149	23.65	2.37	30.4	16.5	86.8	31	36	17	108	43	176	149	1.6	95	4.1	2805	73.6	101.29	399	15	0	1	2	1
54.3	155	22.60	2.47	29.5	16	86.9	41	38	15	105.6	44	143	149	1.9	89	3.9	2700	72.9	103	399.7	14	0	0	2	1
63.1	158	25.28	2.57	29.1	21.1	88	35	63	17	95	38	168	154	0.55	90	4.5	2805	74.4	100	399	14	0	0	2	1
56.6	153	24.14	2.27	29	21	86	41	45	19	110.5	36	169	150	0.67	106	3.1	2247	58.5	81.06	319	15	0	0	2	1
63.7	154	26.86	2.57	29	20.7	86	45	52	20	107.8	41	167	144	0.57	95	4.3	2245	59	81	319.2	14	0	0	3	2
56.1	159	22.19	2.17	32.1	16.3	88	41	33	14	102.4	39	162	149	1.9	94	3.1	1995	52.3	73	284.2	15	0	0	3	2
64.01	155	26.64	2.1	34	18.2	87	39.1	34	15	105.5	41	176	148	1.2	101	4.5	2750	73.6	101	399.25	14	0	0	2	1

Srno.	SEX	AGE	WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	BS	CRP	UR	KCAL	PROT	FAT	CHO	FB
1	m	9	50	135	27.43	2	38	21	88	55	64	20	98	38	185	150	91.82	2.5	6	1940	67	88	400	14
2	f	9	41	133	23.18	2.3	30	20	87	54	63	19	120	32	184	156	84.7	2.5	5	1942	67	87	400	14
3	m	12	55	145	26.16	2.4	32	20.5	89	39	45	22	114	38	164	152	84.7	2.1	4.5	2261	69	88	366	13
4	m	11	50	138	26.25	2.8	33	19	86	49	34	23	112	48	182	155	85	2	4.3	2259	55	89	390	15
5	m	12	60	149	27.03	2.4	35	20	89	48	46	23	91	42	184	146	86	2.2	5	2260	59	88	357	17
6	m	12	60	132	34.44	2.8	34	19	84	47	47	21	89	38	184	148	84.7	2.3	5	2258	55	87	370	19
7	m	11	59	145	28.06	2.6	34	21	88	55	44	22	109	34	172	152	102.5	2.3	6	2259	55	89	350	13
8	m	11	55	141	27.66	2.4	31	23	89	44	43	21	118	40	176	150	106	3.2	5.5	2260	56	95	330	13
9	m	11	55	142	27.28	2.5	29.9	19.9	88	46	42	18	113	42	177	152	113.1	2.1	6	2262	59	90	320	13
10	m	11	63	155	26.22	2.6	31	18	85	42	41	19	111	48	170	150	109.6	2.4	6	2440	55	87	355	12
11	m	10.11	56	151	24.56	2.5	33	20	85	56	33	18	120	48	167	154	95.38	2.4	6	2460	60	95	400	14
12	m	10.5	53	145	25.21	2.8	38	23	85	48	40	18	94	40	171	152	109.6	2.4	6	2420	69	95	420	14
13	m	10.6	58	142	28.76	2.9	32	21	85	52	52	17	120	44	167	158	112	2.5	4.8	1940	61	85	300	13
14	m	11	55	146	25.80	2	32	20	87	57	51	19	120	38	167	158	111	2.2	6	2450	65	89	380	11
15	m	11.8	81	146	38.00	3	39	27	85	51	62	20	114	38	163	152	120.3	2.1	6	2430	61	88	360	11
16	f	12	61	153	26.06	3	39	26	85	51	62	21	112	42	167	152	120.3	2.3	4.9	3000	63	89	370	13
17	f	11	69	148	31.50	2.6	39	21	88	54	64	22	108	46	159	158	113.18	2	5	1940	60	80	380	12
18	m	12	59	155	24.56	2.5	34	19	87	35	46	23	112	42	162	153	91.82	3	4.6	2580	65	85	350	13
19	f	12.5	62	157	25.15	3.8	30	19	84	41	52	23	99	42	172	146	109.6	2.3	3.7	3000	74	95	400	11
20	f	12	55	149	24.77	3	31	22	89	56	51	18	121	36	165	154	109.62	2.4	4.9	2440	65	95	340	11
21	f	13.5	52	162	19.81	2.6	37	22	86	54	65	25	95	32	162	125	84.7	2.4	5.9	3000	72	95	410	12
22	f	12.5	64	156	26.30	3	33	18	85	46	57	24	95	36	169	150	84.7	2.2	3.8	2998	67	89	360	13
23	f	12	59	155	24.56	2.8	38	26	84	45	56	23	100	44	163	148	106	3	3.9	2263	60	88	380	14
24	f	12.4	71	154	29.94	3	35	19	88	56	60	24	99	44	161	145	120.3	2.4	4.6	2999	60	85	400	14
25	f	12.5	72	156	29.59	2.25	35	19	88	56	60	24	99	44.6	162	145	119	2.4	4.8	3000	65	85	390	13
26	f	11	65	159	25.71	2	28	17	82	52	61	21	98	30	161	144	99	2	4	2250	61	86	360	12

WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	BS	CRP	UR	KCAL	PROT	FAT	CHO	FB	BEX	AEX	BLG	ALG
46.9	135	25.73	1.87	37	19	86.1	49	63	18.1	98.1	38	183	147	85	2	4.1	1641	45.1	54.7	242.04	23	0	1	1	0
38	133	21.48	2.17	29	18	85.2	50	51	18	120	32.2	183.5	155	83	2	4	1642	45.15	54.73	242.19	21	0	1	2	0
52.5	145	24.97	2.27	31.8	20.3	86.7	35	43	21.9	114	38	160	153	86	2.1	4.4	1961	53.92	65.36	289.24	17	1	1	1	0
47	138	24.68	2.67	32.2	17.2	85	46	33	22.6	112	48	182	154	85	2	3.9	1950	53.62	65	287.62	14	1	1	1	0
57.2	149	25.76	2.27	33	18	88	45	40	22	91	42	182	144	85	2	4	1960	53.9	65.33	289.1	17	0	1	2	1
57	132	32.71	2.67	33.7	18.7	83	44	39	20.9	89	38.9	181	141	84	2.3	4.9	1965	54.03	65.5	289.83	20	1	1	1	1
55	145	26.16	2.47	33.3	19.4	86.9	43	43	20.5	108.99	34	171.5	159	102.5	2	4.5	1950	53.62	65	287.65	19	1	2	2	1
51.3	141	25.80	2.27	30.7	22.7	87.4	52	33	19.7	118	40.1	171	156	103	2.5	4.2	1960	53.9	65.33	289.1	20	1	2	1	1
51.5	142	25.54	2.37	26.9	17.9	86.1	44	41	16.1	113	41.1	174	152	110	2.1	4.1	1961	53.92	65.36	289.24	19	0	2	1	1
59.9	155	24.93	2.47	28	15	83.6	39	39	17.9	111	48	170	155	98	2.3	4.9	2124	58.41	70.8	313.29	19	0	2	1	1
53.1	151	23.29	2.37	31	17	83.5	50	30	16.6	119.9	48	164	155	95.3	2.1	4.6	2140	58.85	71.33	315.65	18	0	2	2	1
50.4	145	23.97	2.67	37.4	21.4	83.7	44	39	17	94	40.1	173	150	102	2.2	5	2150	59.12	71.66	317.12	19	0	2	2	1
55.1	142	27.33	2.77	31.4	20.4	82.6	48	51	15.9	120	44	166.4	155	112	2	3.7	1641	45.12	54.7	242.04	15	0	2	1	0
51.8	146	24.30	1.87	31.7	18.5	84.9	53	43	17	119.9	38	165	142	110.5	2.2	4	2141	58.87	71.36	315.7	19	0	2	2	0
77	146	36.12	2.87	38.8	25	82.7	45	53	19	114	38	163.4	141	118	2.1	5	2140	58.85	71.33	315.65	19	0	2	1	0
57.8	153	24.69	2.87	38.1	25.1	83.1	46	52	20.2	112	42.3	166	141	119	2.3	4.1	2702	74.3	90.06	398.54	18	0	2	1	0
66.2	148	30.22	2.47	38	19.5	86.1	48	62	21.1	107.9	46.1	160	147	112	2	4.1	1641	45.12	54.7	242.04	20	0	2	2	1
56.9	155	23.68	2.37	32	17	85.3	32	43	22.3	111.8	43	162.4	144	91.8	2.1	3.9	2270	62.42	75.66	334.82	21	0	2	2	1
58.7	157	23.81	3	29.6	18.6	82.5	38	48	22.8	99	42	169	142	108	2.1	3.5	2700	74.25	90	398.25	20	0	2	2	1
52.6	149	23.69	2.87	30	20	86.7	51	46	16.9	121	37	166	144	106	2	3.8	2140	58.85	71.33	315.65	20	0	2	2	1
49.4	162	18.82	2.8	36.4	20.5	84.5	45	62	23.3	95	32	162.4	125	88	2.3	4.2	2700	74.25	90	398.25	19	0	1	2	1
59.5	156	24.45	2.87	32	16	84.5	44	52	23.8	95	36.1	170	150	87	2.2	3.6	2700	74.25	90	398.25	19	0	1	2	1
55.7	155	23.18	2.67	37.1	25.1	83.2	51.3	53	22.5	100	43	165	146	99	2	3.4	1960	53.9	65.33	289.1	18	0	1	2	2
68.5	154	28.88	2.9	34.1	18.1	86.5	51.2	57.7	23.9	99.1	43	162	144	110	2	4.5	2699	74.25	90	398.25	17	0	1	3	2
69.3	156	28.48	2	34	18	87.4	47	55	23.1	99	44.5	163	144	117	2	3.9	2701	74.25	90	398.25	14	0	2	3	2
61.1	159	24.17	2	27.1	15.3	80.2	43	54	20.8	98	30	162	140	98	2	3.8	1948	53.625	65	287.62	17	0	2	3	2

Srno.	SEX	AGE	WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	CRP	KCAL	PROT	FAT	CHO	FB	BS	UR
1	m	9	48	132	27.55	3.1	29	16	83	44	50	20	95	34	162	142	2.5	1930	45	83	290	12	110	6
2	m	9.5	40	133	22.61	2.1	32	17	88	44	40	19	90	40	171	152	2.5	1940	50	84	299	11	91	6.7
3	m	9.5	50	137	26.64	3	31	20	82	48	45	22	80	40	173	148	2.1	1920	72.3	94	390	12	93	3.9
4	m	10.5	48	138	25.20	2.5	39	24	85	57	43	23	99	38	175	152	1.5	2250	52	89.8	355	11	93	5.9
5	m	10	57	140	29.08	2.5	33	18	83	43	33	23	89	42	177	146	2.2	1931	45	86	320	11	100	6.7
6	m	9.8	60	141	30.18	3	28	17	89	41	35	21	90	40	173	150	2.3	2430	54	79	400	11	98	3.7
7	m	10	62	145	29.49	3.2	33	18	82	44	36	22	89	36	180	144	2.3	2432	53	80	380	12	105	6.6
8	m	10	55	141	27.66	3	32	17	86	45	37	21	98	36	182	150	3.2	2249	55	85	440	12	95	4.5
9	m	10	51	140	26.02	2.8	33	18	84	41	49	18	90	46	173	148	2.1	2250	55	85	400	12	105	4.2
10	m	10	58	140	29.59	3	33	18	86	47	50	19	112	42	173	152	2.4	2570	64	90	450	12	96	7
11	m	10.11	48	142	23.80	2.1	33	18	86	46	55	18	90	42	166	146	2.4	2990	75	97	480	11	105	3.8
12	m	10.5	53	143	25.92	3	33	18	82	54	70	18	91	40	162	150	2.4	3000	76	98	478	12	99	5
13	m	10.6	58	140	29.59	3	37	22	85	38	68	17	98	44	165	154	2.5	2989	58	95	476	12	99	4.2
14	m	11	58	146	27.21	2.5	34	19	86	54	53	19	112	44	175	150	2.2	2250	67	86	350	12	97	5
15	f	11.8	81	147	37.48	3.5	35	20	85	45	53	20	90	46	178	147	2.1	2990	76	95	480	12	98	3.9
16	m	12	62	153	26.49	2.5	33	18	86	45	50	21	113	40	173	154	2.3	2430	78	99	478	12	96	4.7
17	f	11	69	148	31.50	3	35	22	88	57	46	22	116	40	183.5	154	2	2570	60	86	380	13	105	4.8
18	f	11.5	59	150	26.22	2.5	33	18	84	37	37	23	90	42	180.5	148	3	2430	70	89	370	12	96	5
19	m	12.5	62	157	25.15	2.2	38	23	83	58	38	23	97	44	161	148	2.3	1925	40	85	275	12	97	5
20	f	12	54	151	23.68	2	36	23	84	51	39	18	109	40	168.5	155	2	1935	43	84	270	12	98	4.9
21	f	13.5	55	161	21.22	2	34	19	84	34	61	25	93	40	175.5	149	2.4	2430	62	89	370	13	93	5
22	f	12.5	63	155	26.22	2	30	22	89	57	62	24	117	36	173.5	158	2.2	2425	63	95	360	12	94	5.8
23	f	12	59	154	24.88	2.4	29.9	22	88	45	52	23	112	44	175	155	3	2250	61	89	320	13	95	4.6
24	f	12.4	68.2	154	28.76	2.7	29	22	87	48	60	24	110	40	182.5	148	2.4	2430	62	94	350	13	110	4.7
25	f	12.5	59	158	23.63	2	30	18	88	48	39	22	111	36	173.5	154	2.4	2990	78	95	450	14.6	100	5
26	f	12.5	68	156	27.94	2.4	35	20	88	39	35	19	109	38	182.5	154	2.1	2890	75	91	460	15	99	4.6
27	f	11	49	139	25.36	3.1	29	16	83	44	50	20	95	34	162	142	2.5	2539	45	83	290	9	110	6

WT	HT	BMI	Z	MAC	TSF	WC	AST	ALT	GGT	LDL	HDL	CHOL	TG	CRP	Bs	UR	KCAL	PROT	FAT	CHO	FB	BEX	AEX	BLG	ALG
47	132	26.97	3.1	28.9	15.9	83.5	44.1	50.1	20.1	94.7	34.1	161	142.3	2.6	105	6.1	1911	46.5	81	279	10	0	0	1	1
39.9	133	22.56	2.1	31.8	16.8	88	44.2	39.9	19.2	90.4	39.6	170	152.4	2.4	90	6.3	1912	51.5	82	284	12	0	0	1	1
49.2	137	26.21	3	30.89	19.8	82.25	48	44.9	20.2	80.1	40.1	172.7	148.1	2.3	92	4	1912	73.8	92	397	12	0	0	1	1
47	138	24.68	2.5	38.8	23.8	85.5	57.9	44	22	99.09	38.09	174	152.09	1.6	92.9	5.99	2231.1	53.5	87.8	362	13	0	0	1	1
56.6	140	28.88	2.5	32.9	17.9	83.3	43.1	33	22	89.01	42.01	176	146.01	2.3	102	6.71	1910.1	46.5	84	327	11	0	0	2	1
59.9	141	30.13	2.99	27.9	16.9	89.1	40.9	35.3	20	90.1	40.1	175	150.1	2.1	98	3.8	2411.2	55.5	77	407	10	0	0	1	1
61	145	29.01	3.2	32.7	18	82.1	44.2	36.1	22	88.9	35.9	171	143.9	2.2	106	6.5	2412.1	54.5	78	387	11.6	0	0	1	1
54.9	141	27.61	3	31.8	16.8	86.43	43	36.8	22	100.1	38.1	181.7	152.1	3.3	94.4	6.6	2230	56.5	89	447	12	0	0	2	1
50.9	140	25.97	2.8	32.9	17.7	84.4	42	48.7	18	90.2	46.2	171	148.2	2.1	101	4.4	2229	56.5	83	407	13	0	0	1	1
57.7	140	29.44	3	32.9	18.4	86.1	46.9	50.1	19.2	111.4	42	173	151.4	2.9	95	7	2550	50	88	457	12	0	0	1	1
47.7	142	23.66	2.1	33.2	18.2	86.4	45.9	49.9	18.2	90.1	42.1	166.1	146.1	2.9	104	3.9	2971	76.5	95	487	13	0	0	1	1
52.8	143	25.82	3	33.3	18.1	82.2	53.7	71	17.3	90.8	40	166	149.8	2.3	98	5	2970.2	77.5	96	485	11	0	0	2	2
57.3	140	29.23	3	37.2	22	85.3	38.9	68.5	19	97.6	43.9	174.8	153.6	2.8	97	4.1	2971.1	59.5	93	483	11	0	0	2	1
57.7	146	27.07	2.5	34.1	19	86.2	53	53.1	20.1	111.7	44	174.5	149.7	2.7	96	5	2230.2	68.5	84	357	12	0	0	1	2
80.7	147	37.35	3.5	35	20.2	85.2	45	53.1	21	89.99	45.99	179	146.99	2.3	96	3.89	2971.4	77.5	98	487	12	0	0	3	3
61.7	153	26.36	2.5	33.1	18.2	86	46	50.2	22.3	112.6	40	185	153.6	2.9	97	4.7	2409	70	97	485	11	0	0	2	2
68.9	148	31.46	3	35	22.4	89	57.1	46.4	23	114	40	183	152	2	103	4.8	2550	61.5	84	387	12	0	0	2	2
58.8	150	26.13	2.5	33.1	18.3	84.1	57.3	37.1	23.1	89.9	41.9	181	147.9	3	97	4.9	2408	71.5	90	377	12	0	0	2	2
62.2	157	25.23	2.2	38	23.4	83.2	58.5	38.2	23.1	97.3	43.9	162	148.3	2	96	4.9	1910	41.5	90	282	14	0	0	2	2
54.1	151	23.73	2	36.2	23.1	84.2	51.4	39.4	18.1	108.7	40	174	154.7	2.3	98	4.9	1905	44.5	82	277	13	1	0	3	3
55.4	161	21.37	2	34.1	19.2	84	34	61	25.5	92.8	40	173.9	148.8	3.2	92	5	2410	63.5	87	355	14	0	0	2	2
63.2	155	26.31	2	30.1	22.4	90	56.6	62.1	23.7	116.9	35.9	174	157.9	2.4	95	5.7	2400	64.5	93	370	12	0	0	2	2
59.4	154	25.05	2.4	30	22.3	88	46	52.3	24.2	112.1	44.1	182.4	155.1	1.9	96	4.7	2230.1	62.5	87	330	13.8	1	0	2	2
68.4	154	28.84	2.7	29.3	22.3	87.2	48	60	22.1	110.1	40.1	174.5	148.1	2.3	95	4.8	2409	63.5	92	360	13	0	0	2	2
59.3	158	23.75	2	30	18	88.2	47	39.1	18.7	111.1	36.1	180	154.1	2.1	111	5.1	2971.1	70	93	460	14.6	1	0	3	3
68.2	156	28.02	2.4	35	20.1	88.1	37	34.7	18.1	108.9	37.9	182.9	153.9	2.5	102	4.5	2869	76.5	89	470	15	0	0	2	2
49.4	139	25.57	3.1	29.2	16.5	83.4	44.4	48.9	19.9	94	34	163.3	141	2.7	99.4	6	2517	46.5	81	300	15	0	0	1	2

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