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ORIGINAL ARTICLE

Gut Microbiota Hypolipidemic Modulating Role in Diabetic Rats Fed with Fermented *Parkia biglobosa* (Fabaceae) Seeds

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ABSTRACT

Background: Modulation and balancing of host gut microbiota by probiotics has been documented by several literature. Prebiotic diets such as locust beans have been known to encourage the occurrence of these beneficial microorganisms in the host gut.

Objectives: To study the modulating role of gut microbiota in the hypolipidemic effect of fermented locust beans on diabetic Albino Wister rats as animal models.

Methodology: Albino rats (Wistar strain), averagely weighing 125g were successfully induced with alloxan. There after this induction, anti-diabetic treatment was carried out on various groups of rats by feeding them ad libitum with a diet of milled fermented and unfermented *Parkia biglobosa* seeds, respectively.

Results: After three weeks of treatment, it was observed that fermented locust beans caused a significant reduction ($p \leq 0.05$) in glucose, total triglycerides, total cholesterol and LDL, while the HDL levels were significantly elevated ($p \leq 0.05$). Results of fecal analysis showed that the fermented locust beans modulated the gut microbiota through the occurrence of probiotic bacteria, *Bacillus subtilis* in the gut and faeces of the rats.

Conclusion: This study support that fermented locust beans is a prebiotic diet that encourages the growth of *Bacillus subtilis* in the gut of animals and is associated with hypolipidemic activities which alleviate diabetes as portrayed in these rat models.

Keywords	*Address of Correspondence	Article info.
Gut, Microbiota, Probiotics, Hypolipidemic, Bacillus subtilis, Diabetes.	*olayinka.awoyinka@eksu.edu.ng*	*Received: September 10, 2020* *Accepted: December 22, 2020*

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INTRODUCTION

In recent times, the future of disease and infection management has been directed towards the development and use of natural products, combined with medical methods as well as diet and nutrition, genetic engineering, phytopharmacy etc. Diabetes mellitus is one of the common metabolic diseases that have plagued man over centuries and has become more prevalent across the world's population. It is sometimes described as "hyperglycemia", a condition of elevated blood sugar beyond normal[1-5].

Diabetes is a major threat to global public health with increasing prevalence and mortality. Studies have shown that worldwide, diabetes affects over 170 million people and an exponential increase in the prevalence over the next few decades have been predicted[2,3,6]. Hyperglycemia is plaguing the world and causing individuals, communities, governments, health organization and the world at large to invest millions in its treatment and management[7-8]. The management of this disease is dependent on four fundamental factors: Patient education about the disease, physical exercise, diet, and the use of hypoglycemic agents[9-10]. The most popular hypoglycemic agent used clinically to control diabetes is insulin. Although there has been successful advancement with the management and treatment of diabetes, the increase in prevalence of the disease and its associated complications remains unchanged[11]. Asides the associated side effects of most orthodox antidiabetic agents, this mode of treatment cannot reach the rural populace and poor urban dwellers because of the cost of the drug and other factors like easy availability. Hence, the needs for alternative therapies such as medicinal plants, without adverse reactions, have been experienced with the use of synthetic antidiabetic drugs[12-13]. There have been many studies on medicinal plants with antidiabetic properties[14-16]. In most low and middle income countries such as Nigeria, many people still rely on medicinal plants in their natural environment for the treatment of diabetes[11,14].

Parkia biglobosa belongs to the family Fabaceae[17]. They are widely cultivated in various habitats in Africa, and the parts of the tree are routinely used as food additives and for medicinal purposes[18-19]. It is generally grown throughout the West African savannah where it is commonly referred to as African locust bean[20]. The seeds are well known for their use in the production of local condiment due to its outstanding protein quality[21]. Although frequently consumed by the locals, *Parkia biglobosa* is overlooked as a gem in disease management[22-24]. However, its use in folkloric medicine for the treatment of diabetes mellitus has been reported in Senegal and South Western part of Nigeria[25-26]. Therefore, providing information on a more affordable and effective treatment methods such as diet on fermented African locust bean seeds will go a long way in combating hyperglycemia and diabetes.

Recently, many researches have been focused on improving health through cheaper and more effective alternatives such as diet and natural resources. Researchers like Mduduzi *et al*.[60] and Aderiye B *et al*.[61] in their review stated that, diverse African traditional fermented foods and beverages produced using different types of fermentation have been used since antiquity because of their numerous nutritional values. In their reports, fermented product such as *Iru*, from *Parkia biglobosa* containing Bacillus and Staphylococcuss spp. as the major microorganism implicated as probiotics. The answer to a multitude of health problems lies in the ability to utilize and fully exploit environmental resources. These include the modulation of gut microbiota by probiotics (from a prebiotic-diet) to treat metabolic disorders such as diabetes. African locust beans seeds (*Parkia biglobosa*) have been associated with the treatment of diabetes by various studies[62-63]. However, there is dearth of information on the relationship between fermentation of the seeds, the gut microbiota and the lipid profile of the host as a trialogue of potential importance in the treatment of diabetes. Thus, this project would be a contribution to such innovations. Hence, we analyzed the hypoglycemic ability of fermented *Parkia biglobosa* seeds on Albino Wistar rats in relation to its function as prebiotic.

MATERIAL AND METHODS

Collection and Preparation of Materials

Unfermented seeds of *Parkia biglobosa* were procured at a local community market in Ado Ekiti. These were identified and authenticated by the Chief Botanist of Plant Science at our institution and deposited in the University Herbarium (UHAE 2020063). The method of Aderibigbe *et al*.[27] was adopted for the fermentation process. Briefly, the dried seeds were hand-picked to remove dirt and boiled under pressure for 3hrs; thereafter, the testa was removed by dehulling and thorough washing. Following re-boiling of the cotyledons for about 1hr, 300g of the boiled substrate each was weighed separately into twenty sterile baking pans. Then 2ml of suitably dialyzed starter cultures were used to inoculate each of the baking pans containing the substrate. The inoculated substrate were mixed using flamed spatula and incubated at 35°C for 36hrs.

Preparation of Starter Culture

For this, 6.25g of nutrient broth was weighed and dissolved in 250L of distilled water in a sterilized 500L conical flask. It was allowed to homogenize completely for 40-45min in a water bath; a clear yellow solution was formed. The homogenized clear solution was sterilized in an autoclave for 15min at 121°C to eliminate all the microorganisms present. A pure previously isolated *Bacillus subtilis* was obtained in an inactive slant form and was fed in the homogenized solution. This was then put in an incubator for 24hrs at 37°C, and turned turbid confirming activation. Subsequently, 10ml of each sample was centrifuged at 3000g. The cells were then rinsed off the broth and later homogenized with 5ml of water and set aside for fermentation.

Fermentation Process

Twenty (20) fermenting cans with suitable covers were purchased from market dealers. The cans were sterilized by swabbing with cotton wool soaked in ethanol; 500g of the prepared locust beans were weighed and kept in the fermenting cans. Then, 2ml of the Bacillus cells were sprinkled with the aid of a syringe on the locust beans and mixed with a spatula. The cans were covered and kept in an incubator at 37ºC for 2 days. At the end of fermentation, a whitish substance was formed on the *Parkia biglobosa* indicating a successful fermentation. The fermenting cans were put in a freezer to stop fermentation before microbiota analysis.

Microbiota Analysis

This was investigated using dilution streak plate method as described by Satish[28]. 1000mg of the fecal sample from each group was measured and kept in sterile test tubes. This was followed by the addition of 10ml of sterile water and the feces allowed to dissolute. Thereafter, 1ml of the suspension was pipetted into clean test tubes with 9ml of distilled water and shaken. This was repeated until a dilution of 10^1 obtained. Aseptically, already prepared nutrient agar was decanted in duplicates in petri dishes and labeled correctly. A loopful from each of dilution (10^{-3}) was streaked on the already prepared nutrient agar and then incubated at a temperature of 37ºC for 24hrs. The morphological characteristics and number of the colonies was observed and then sub-cultured on new plates containing nutrient agar for pure isolation of microorganisms. The pure colonies were transferred to a slant for further identification. Various biochemical tests were conducted on the fecal samples to detect and identify microorganisms. The tests include: gram staining, motility test, catalase, indole, coagulase, citrase, oxidase, urease and test for various sugars.

Animals and Alloxan Administration

Following ethical approval from the Experimental Animal Research Ethics Committee, Ekiti State University (ORD/ETHICS/AD/043); 20 male Albino Wistar rats averagely weighing 125g were used. They were kept under standard environmental conditions and fed rat pellets and water ad libitum. After one week acclimatization, they were divided into four groups. Diabetes mellitus was induced in all the rats by administration of freshly prepared Alloxan (120 mg/kg) (British Drug house, London, UK) solution intraperitoneally using 0.9% w/v NaCl as the vehicle. Diabetes was confirmed with fasting blood glucose above 80mg/dl. Group 1 was the diabetic control group (no treatment). Groups 2 and 3 were treated with a diet of milled fermented and unfermented *Parkia biglobosa* seeds (60g mixed with 40g per cage of grower's mash) respectively, while Group 4 was treated with oral administration of a reference anti-diabetic drug Glibenclamide (0.01mg/150g body weight). All the treatments lasted for 3weeks.

Determination of Fasting Blood Sugar

As described by Airaodion *et al*.[29], following a 12hr overnight fast, the blood was collected from the tails of the rats and sugar was estimated with a digital glucometer (Sinocare, China).

Animal Sacrifice and Collection of Serum

On day 22, rats in each study group were sacrificed under ketamine anesthesia following an overnight fast as described by Alese *et al*.[21]. Thereafter, venous blood was collected from each rat and transferred into sterile bottles before centrifuging at 3000rpm for 5min. The supernatants were decanted and stored at 4ºC until use.

Lipid Profile Analysis

The aliquot samples were used for determination of lipid profile according to manufacturer's instruction (Randox, USA). The HDL-C was determined using the enzymatic spectrophotometric method; the samples were precipitated by the addition of phosphotungstic acid and magnesium chloride. After centrifugation at 3000g for 10min at 25ºC, the clear supernatant contained the HDL fraction, which was assayed for cholesterol concentration using a Randox kit while LDL calculated using the formula of Friedwald *et al*.[30].

Statistical Analysis

All the data were subjected to t-test and one-way analysis of variance with the use of statistical Graph Pad-prism software[31]. Statistical significance was set at $p \leq 0.05$.

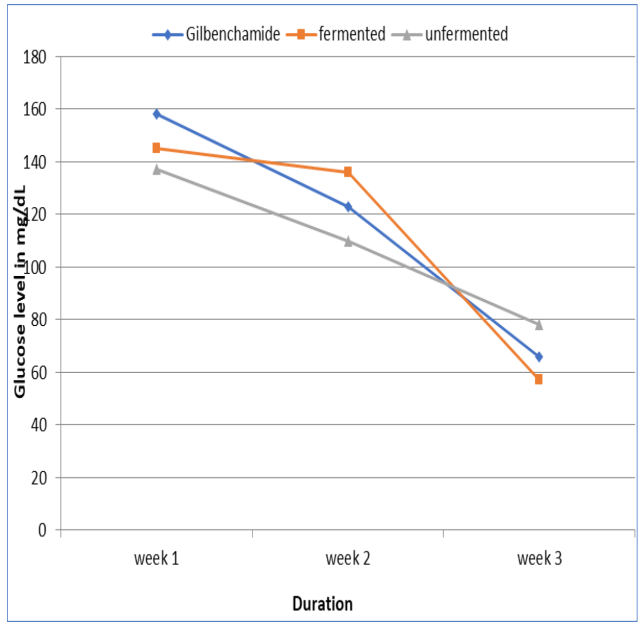

Fig 1. Fasting blood sugar level through the period of the experiment.

Table 1. Identification of Isolated Organisms from Fecal Sample of Rats.

S. No.	Group	No. of Colonies	Edge	Color	Shape	Size	Surface	Organism Detected
1	Fermented locust beans	35 ± 0.01	Serrated	Cream	Round/ Irregular	Small	Smooth/Rough	*Escherichia coli* *Bacillus subtilis*
2	Unfermented locust beans	7 ± 0.1	Serrated	Cream/ Yellow	Round	Small	Flat/ Elevated	*Proteus vulgaris* *Citrobacter freundii*
3	Glibenclamide	91 ± 0.1	Smooth	Cream/ Pink	Round	Small	Flat	*Citrobacter freundii* *Proteus vulgaris* *Enterobacter aerogenes*
4	Control group	20 ± 1.5	Smooth	Pink/ Cream	Round	Small	Flat	*Staphylococcus aureus* *Bacillus subtilis* *Enterobacter aerogenes*

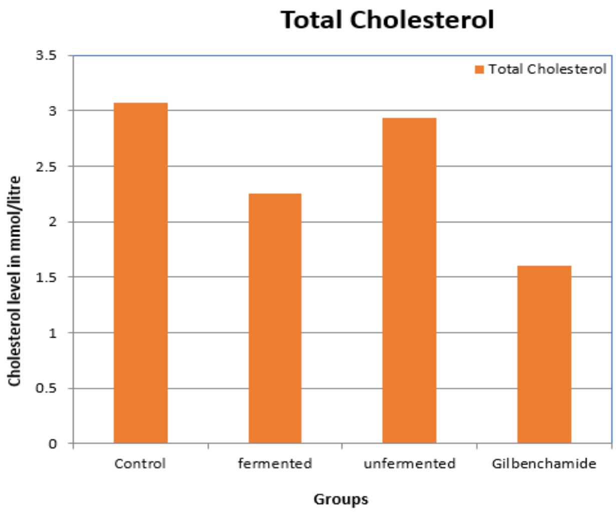

Fig 2. Effect of locust beans on total cholesterol of diabetic rats.

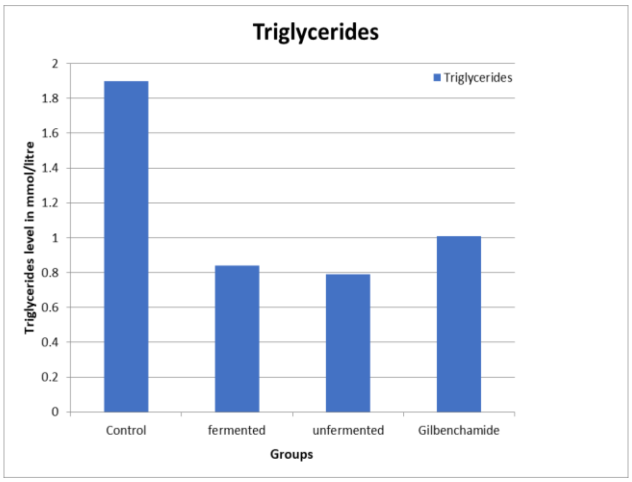

Fig 3. Effect of locust beans on total triglycerides of diabetic rats.

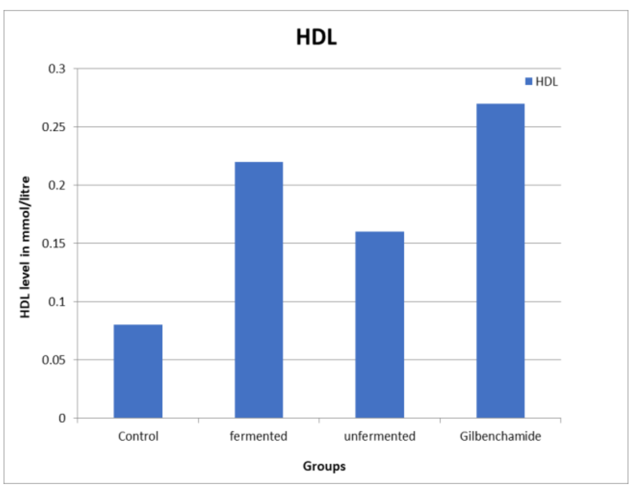

Fig 4. Effect of locust beans on high density lipoprotein of diabetic rats' serum.

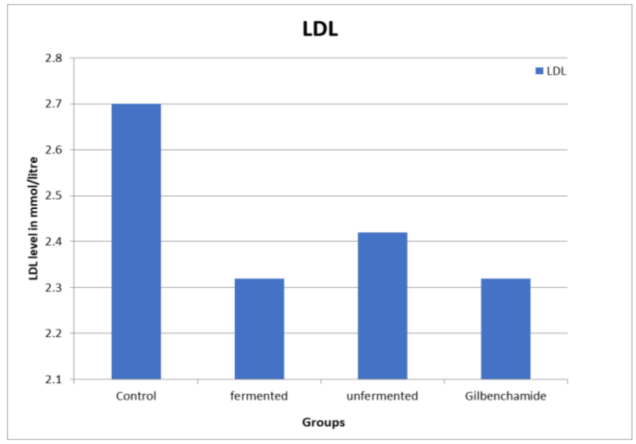

Fig 5. Effect of locust beans on low density lipoprotein of diabetic rats.

RESULTS AND DISCUSSION

Diabetes mellitus is a metabolic condition characterized by hyperglycemia over a prolonged period with myriads of complications with associated morbidity and mortality[32-33]. Untreated or poorly treated diabetes can result in many complications including, neuropathy, retinopathy, nephropathy, cardiovascular complications, anemia, diabetic ketoacidosis, infection and inflammation, hyperosmolar hyperglycemic state and death[33]. Previous studies have demonstrated the hypoglycemic and insulin-stimulatory effects of various medicinal plants[8]. In Nigeria, *Parkia biglobosa* seeds are commonly used as ingredients in local dishes; besides this, it is used as alternative medicine for the management of diabetes. Hence, the need to verify its efficacy in mitigating diabetic complications and its effects on the patient[34-36].

In this study, it was observed that fermented *Parkia biglobosa* reduced the level of blood glucose and lipid profiles in the experimental rats. This reduction was reflected as progression or regression of the concentrations of these metabolites (glucose and lipids).

Following alloxan treatment, there was a significant gain ($p \leq 0.05$), in blood glucose levels of the rats (Fig. **1**). However, there was a significant lowering of blood sugar level following treatment with both unfermented ($p \leq 0.05$) and fermented ($p \leq 0.05$) locust beans when examined with the untreated group. Also, the observed reduction in the blood glucose level of the animals fed with fermented locust beans (mean value, 59mg/dl) tallied favorably with the hypoglycemic effect of glibenclamide (mean value, 62mg/dl). Likewise, fermented locust beans proved more effective than the unfermented seeds in lowering the glucose levels of the blood as there was a significant increase in the mean value ($p \leq 0.05$).

The possible role of the associated microbiota is of special involvement in this present project. Alterations in the make-up of the gut microbiota have been linked with an array of diseases, including cardiovascular diseases (CVD)[37-38]. The advancement of the growth of specific probiotics is believed to have preventative effects on heart complications due to the influence of these bacteria on human physiology, including the ability to reduce total serum low density lipoprotein-cholesterol and inflammation[39-40]. According to Mach[41], many diseases at present are as a result of lack of probiotic bacteria in the gut flora. Dietary substrates such as *Parkia biglobosa* pass largely un-metabolized in the upper gastro-intestinal tract where they are selectively utilized for the benefit of the host[42-43]. As shown in Table **1**, the organisms isolated from the fecal sample of the fermented-locust beans treated rats were *Escherichia coli* and *Bacillus subtilis*. *Proteus vulgaris* and *Citrobacter freundii* were found as fecal microbiota of the unfermented locust beans treated group. The fecal samples collected from the glibenchamide treated rats had *Citrobacter freundii*, *Proteus vulgaris, Enterobacter aerogenes* while *Staphylococcus aureus*, *Bacillus subtilis* and *Enterobacter aerogenes* were isolated from the control group. A common organism, *Bacillus subtilis* was observed following comparison of the microbiota between the fermented locust beans treated rats and the untreated control. Although, the source of this microorganism is unknown in the control group of rats; its presence in the gut of the fermented locust beans treated animals is adequately explainable. This is due to the fact that the fermentation of locust beans seeds was expedited with the action of *Bacillus subtilis* starter culture, hence, the microbial cells was consumed live with the milled seeds during treatment. A number of literatures have confirmed the persistence of *Bacillus subtilis* in the gut for as long as 18 days and more[44-45]. Members of the *Bacillus* spp have been associated with numerous probiotic properties such as production of extracellular enzymes, bile, salt and pH tolerance, bio-film formation, cellular aggregation and cell surface hydrophobicity[46] as well as sensitization of the immune system, synthesis of antimicrobials such as bacteriocins, regulation of the composition of gut microbiota and anti-inflammatory effects[40,47,48]. In a particular study, purified exopolysaccharide from *Bacillus subtilis* expressed therapeutic effects on cardiovascular diseases-related parameters such as reduction in blood sugar level, troponin, total cholesterol, LDL-C, and VLDL as well as suppression of ICAM and VCAM expression in streptozotocin induced diabetic rats[37, 40]. Also, Zouari *et al.*[49] previously explored the hypoglycemic and anti-lipidemic properties of biosurfactants produced by *Bacillus subtilis* SPB1 strain in alloxan-induced diabetic rats. In their study, the biosurfactant reduced the plasma alpha-amylase activity and rendered protection to pancreatic beta cells. Apart from the hyperglycemic effects, biosurfactants controlled lipid level by promoting HDL-cholesterol and inhibited the absorption of LDL-cholesterol and triglycerides. This corroborates our study result which confirms that the presence of *Bacillus subtilis* as part of the gut microbiota that may have contributed to lowering of blood glucose level of the rats; hence the general hypoglycemic effect of fermented locust beans.

Hyperglycemia remains a major clinical feature of poorly controlled diabetes which is associated with protein glycation (non-enzymatic glycosylation). A number of proteins including albumin, hemoglobin, collagen, and LDL undergo glycation in diabetes. The significant decrease in albumin levels in the fermented locust beans treated rats when compared to control indicates the hypoglycemic efficacy of fermented locust beans[33].

Asides regulating carbohydrate metabolism, insulin acts a crucial role in lipid metabolism. Similar to diabetes mellitus, insulin insufficiency is linked with hypercholesterolemia and hypertriglyceridemia. These conditions have been reported to occur in diabetic rats[29]. High level of cholesterol could cause a relative molecular ordering of the residual phospholipids, resulting in a decrease in membrane fluidity[50-51]. Also, elevation in the levels triglycerides is one of the leading risk factors in heart disease. Abnormalities in lipid profiles concentration have been shown to play a major role in the pathogenesis and progression of several disease conditions[52]. In this present work, the concentrations of total cholesterol (Fig. **2**) and triglycerides (Fig. **3**) significantly decreased ($p < 0.05$) when animals treated with fermented locust beans were examined with the untreated control group. This implies that fermented locust beans may be capable of preventing the progression of coronary heart diseases.

The two of the main groups of plasma lipoproteins that are involved in lipid metabolism and the exchange of cholesterol, cholesterol ester and triglycerides between tissues are high density lipoproteins (HDL) and low density lipoproteins (LDL)[29,53-54] Studies have shown that increased concentrations of total cholesterol and/or LDLs in the blood are important risk factors for coronary heart disease[55-56]. While HDL is usually termed 'good cholesterol', LDL is known as 'bad cholesterol'. In this study, treatment of the rats with fermented locust beans resulted in a significant increase in the serum level of HDL-cholesterol (Fig. **4**) when compared with the diabetic control animals while it significantly decreased the level of LDL-cholesterol (Fig. **5**). The increased in HDL/LDL ratio in the animals treated with fermented locust beans when compared with the control diabetic rats indicates that a diet of fermented locust beans can reduce the risk of developing heart diseases, because a high HDL/LDL ratio has been confirmed to be beneficial and cause lower risk of coronary heart diseases[34,54,57-59]. In this study, treatment with *Parkia biglobosa* seeds restored the derangements in diabetic rats. However, the fermented seeds showed the best efficacy in hypoglycemic and anti-diabetic activities. This performance could be possibly attributed to the prebiotic property of *Parkia biglobosa* as well as the probiotic activities of the starter culture Bacillus subtilis and the gut beneficial microbiota of the rats.

CONCLUSION

The findings of this study shows that African locust beans fermented with *Bacillus subtilis* is capable of producing modulation in the gut microbiome composition of Albino Wistar rats, thereby leading to reduction of lipid levels as well as amelioration of blood glucose levels and the subsequent alleviation of diabetic state in the rats. The positive results obtained from this study suggests that well controlled diets with use of preferably fermented locust bean as prebiotics can serve as a major non-pharmacologic option for the treatment of diabetes.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper and no part of this manuscript has been submitted for publication to another journal.

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LIST OF ABBREVIATIONS

HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
Tc	Total Cholesterol
TG	Triglyceride

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