UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCE

ANTI-DIABETIC AND PROBIOTIC EFFECT OF KOMBUCHA ON ALLOXAN-INDUCED DIABETIC RATS

BY

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILLMENT FOR THE REQUIREMENT OF THE AWARD OF DEGREE OF MASTER OF PHILOSOPHY IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

DEPARTMENT OF BIOCHEMISTRY, CEL AND MOLECULAR BIOLOGY

JULY, 2019

DECLARATION

I, Emmanuel Edem Adade, do hereby declare that except for the references to work of other people mentioned in this document, which has been duly acknowledged, this research was conducted by me in the Department of Biochemistry, Cell and Molecular Biology and the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP) of the University of Ghana, Legon. The work was carried out under the supervision of Dr. Elmer Ametefe, Prof. Laud K. N. Okine and Rev. Dr. W.S.K Gbewonyo.

……..... 5th February, 2020 ……

Emmanuel Edem Adade — Date

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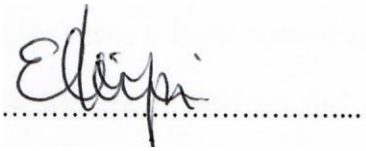

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Prof. Laud K. N. Okine — Date

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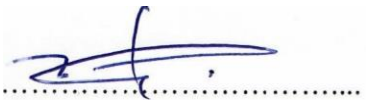

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Rev. Dr. W. S. K. Gbewonyo — Date

(Supervisor)

ABSTRACT

Diabetes mellitus, is a metabolic disorder caused by the inability of the beta pancreatic cells to adequately produce insulin or due to insulin resistance of cells. As a result of the increasingly high incidence of diabetes globally, the World Health Organization (WHO) has set timelines and guidelines for the reduction of the risk of mortalities and morbidities associated with non-communicable diseases including diabetes, by the year 2030. However, this agenda is hinged on the availability of affordable, safe and effective alternatives for the management and treatment of these diseases. Hence, there is a need to explore other alternatives to the conventional oral anti-hyperglycemic agents driven by factors such as patient's preference, demand among others.

Kombucha is tea fermented by a symbiotic culture of bacteria and yeasts (SCOBY). Consumers of Kombucha have reported several anecdotal evidences of its medicinal potential. This study seeks to investigate its anti-diabetic and probiotic effect on alloxan-induced diabetic rats. It was hypothesized that Kombucha, being a complex matrix of microorganisms and nutraceuticals, would play an essential role in the management of diabetes.

Molecular characterization of the microbiome of Kombucha using shotgun metagenomics (Oxford Nanopore MINION sequencing technology) showed *Brettanomyces bruxellensis CBS 2499* as the most abundant species within the microbial community accounting for about 51 % of all reads. *Brettanomyces anomalus, Komagataeibacter xylinus NBRC 15237, Bacillus nealsonii AAU1, Zygosaccharomyces bailii CLIB 213, Acetobacter, Gluconobacter* and over 300 other genera and species of microorganisms including archaea and viruses were also detected using a combination of REFSEQ and One Codex data bases (OXCDB). *In-vivo* experiment was used to evaluate the anti-diabetic property, safety and gut microbiome changes of Kombucha. Kombucha was found to

perform better than the conventional antidiabetic drugs, metformin and glibenclamide in lowering the fasting blood glucose (FBG) of the diabetic rats. Daily administration of 25 mg/kg and 100 mg/kg of freeze-dried Kombucha tea demonstrated a 5 fold reduction in FBG ($p<0.05$) and 40% and 50% respective increases in body weight of the alloxan-induced diabetic rats compared to the diabetic control (DC). Histological analysis, shows Kombucha enhances pancreas regeneration and hence the concomitant increase in insulin secretion as demonstrated in the study. Serum lipid profiling showed 100mg/kg Kombucha treatment increases the levels of total cholesterol (16%), high density lipoproteins (HDL) (13%) and low-density lipoproteins (LDL) (10%) but conversely reduces triglyceride level (17%) compared to the DC ($p>0.05$).

Further analyses demonstrated that Kombucha decreases the relative organ (liver and kidney) to body weight ratio in treated animals. In addition, Kombucha was able to reduce significantly the elevated levels of liver enzymes such as Alkaline phosphatase (ALP), Alanine transaminase (ALT) and Aspartate Aminotransferase (AST) as well as renal toxicity indices, creatinine and urea in treated animals. Histology of the kidney and liver also showed that Kombucha has no adverse effect on the morphology and cellular integrity of these organs suggesting its hepatoprotective and renal protective potentials. Urinanalysis also showed reduction of glucose in urine for the 100 mg/kg Kombucha-treated animals. Additionally, Kombucha protects the gut microbiome, most significantly by enhancing the *Lactobaccillaceae* family of bacteria within the gut and reduces the possibilities of colonization of the gut by other opportunistic bacterial species.

The study demonstrated that Kombucha is enriched with diverse microbial population with probiotic value and daily intake of Kombucha may be potentially helpful in the management of diabetes, protection against renal and liver toxicity and offer gut microbiome protection.

DEDICATION

This work is dedicated to my family for the immeasurable support they have given to me. Through it all, they always believed in me.

ACKNOWLEGDEMENT

I would want to express my profound gratitude to my supervisors Rev. Dr. W.S.K Gbewonyo, Dr. Elmer Ametefe and Prof. Laud K. N. Okine for their guidance and support throughout this project work. They have been more than just supervisors but also a mentors and life coaches. They supported me with their time, resources, energy and with abundance of love which inspired me daily to work hard to achieve this success. I am also grateful to my Dr. Olga Quasie of the Centre for Plant Medicine Research who was part of my Supervisory committee for helping me through my experiment. I thank Prof, Peter Gathumbi of the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi, Kenya who collaborated with my research team by helping with the histological analysis.

I acknowledge the West African Centre for Cell biology of Infectious Pathogens (WACCBIP) for their sponsorship (research, tuition and stipend) which supported throughout the entire training period and enabled me to undertake my project successfully. I would also want to thank the lecturers and non-teaching staffs of the Department of Biochemistry Cell and Molecular Biology for giving me the enabling academic environment for me pursuit of my graduate training program.

I am extremely thankful to Mr. Stephen Antwi, Collins Awaiga, members of the animal house and pharmacology department at CPMR, Jovis Williams of Immunology department, NMIMR, Jacob Donkor, Staff of Electron Microscopy department, NMIMR, all of the natural product lab team, Collins Misita Morang'a, Samuel Osabutey and Joyce Ngoi of WACCBIP NGS facility for their technical support during my bench work and my write-up. I am very grateful to Naa Nuerki Adumuah for her tremendous support throughout my Masters training. I want to specially thank my profound gratitude to my family for their constant prayers and support.

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LIST OF ABBREVIATIONS

DC	Diabetic Control
REFSEQ	Reference Sequence Database
OCXDB	One Codex Database
DM	Diabetes Mellitus
T2D	Type 2 Diabetes
SCOBY	Symbiotic Culture of Bacteria and Yeast
DPP-4	Dipeptidyl peptidase - 4
WHO	World Health Organization
FBG	Fasting Blood Glucose
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
TC	Total Cholesterol
TG	Triglycerides
DC	Diabetic Control
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CPMR	Center for Plant Medicine Research
NMIMR	Nugochi Memorial Institute for Medical Research
H&E	Haematoxylin and Eosin
rRNA	Ribosomal Ribonucleic Acid
ITS	Internal Transcribed Spacer

DKA	Diabetic Ketoacidosis
DNA	Deoxyribonucleic Acid
GLUT 2	Glucose Transporter 2
GLUT 5	Glucose Transporter 5
PBS	Phosphate Buffer Saline
KOMB	Kombucha
GLIB	Glibenclamide
MET	Metformin
WACCBIP NGS	West African Centre for Cell Biology of Infectious Pathogens Next Generation Sequencing
IBD	Inflammatory Bowel Disease
LCAT	Lecithin Cholesterol Acyl Transferase

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 BACKGROUND

Diabetes mellitus (DM) is a metabolic disorder that is associated with inadequate insulin production by the pancreatic beta cells and insulin resistance (Roglic *et al*., 2016). It is a chronic disease characterized by an abnormal elevation in the blood glucose level. Broadly, there are three types of DM. These are type 1, type 2 and gestational diabetes. Type 2 DM is the most prevalent type. Some clinical features of diabetes include hyperglycemia, polydipsia and poor wound healing (McDevitt, 2005)

In 2012, approximately 1.5 million deaths were recorded globally among patients with diabetes mellitus disease condition. Diabetes mellitus related complications such as cardiovascular diseases have been implicated to cause about 2.2 million deaths globally. The prevalence of diabetes globally in 2014 was recorded as 422 million people with the critical age group between 45 and 64 in developing countries (World Health Organization, 2018). These numbers have however been on the rise over the last decade in both developed and developing (middle – low income) countries (Roglic *et.al.*, 2016).

Current treatment therapies for controlling the abnormal blood glucose concentration associated with diabetes include the administration of insulin peptides (long lasting insulin analogues, short-acting insulin and the regular human insulin) traditionally via intravenous injection. Some patients are treated with sub-cuticular administered Mixtard insulin and other insulin analogs depending on the individual drug responses. In addition, these existing interventions are expensive to access costing about $ 16,752 yearly for each patient diagnosed with diabetes and patients would have to be on this treatment for their entire lifetime (“Cost of Treating Diabetes– Insurance, Insulin Prices

and Complications", 2018). The use of oral anti-hyperglycemic agents such as metformin and sulfonylureas are considered as choice drugs for the management of diabetes (T1D or T2D). However, the adverse side effects of these drugs are inevitable (Li, 2011). For example, phenformin was withdrawn from the market due to its side-effect of causing lactic acidosis in patients with renal insufficiency (Ritz *et al*., 2011). Metformin is a more acceptable drug for treatment of all diabetes. It is effective in its anti-hyperglycemic properties by controlling the production of glucose from the liver and enhancing insulin sensitivity by cells, however, it also has the risk of causing lactic acidosis (Ali *et al*., 2017).

There is therefore the need for research into the development of cheap, effective and safe interventions for the management and treatment of diabetes. In tandem, to drive the World Health Organizations agenda of reducing diabetes by 2025, research into the exploration of other alternative treatment therapies are very critical.

Kombucha is a fermented tea, which is made from the infusion of the Kombucha culture with sweetened tea broth (black or green tea) (Dufresne and Farnworth, 2000). The Kombucha culture is referred to as SCOBY, an acronym for *symbiotic culture of bacteria and yeasts*. The SCOBY is made of beneficial micro-organisms which carry out the fermentation processes. Kombucha is also a source of nutraceuticals such as flavonoids, saponins and polyphenolics.

Several health claims have been attributed to Kombucha, including antidiabetic property (Jayabalan *et al*., 2014). Studies have also demonstrated that the therapeutic components contained in Kombucha including phytochemicals and probiotics account for its ability to prevent cardiovascular diseases, promote digestive functions, enhance resistance to cancer, boost the immune system and reduce inflammation in the gut (Jayabalan *et al*., 2014). However, such claims on the potency of Kombucha remain essentially anecdotal. This gap in scientific evidence has

accentuated the need for further studies to understand the reported therapeutic effects of Kombucha (Greenwalt *et al*., 2000; Murugesan, 2009).

Kombucha-derived microorganisms produce a cocktail of diverse metabolites which are rich in fibers, organic acids, amino acids and antioxidants. The microbial constituent of Kombucha may also have an impact on the gut microbiome, however, the exact mechanism is not fully understood. The gut microbiome constitution has been demonstrated to be a key player in the incidence of obesity, a risk factor for type 2 diabetes (Chen *et al*., 2014). Furthermore, inflammation in the gut has also been shown to cause the destruction of pancreatic cells which will affect the overall production of insulin and result in dysregulation of free blood glucose levels (Wen and Duffy, 2017)

Studies have demonstrated that diabetogenic agents such as alloxan and streptozotocin are effective at inducing diabetes and applicable in anti-diabetic research in animal models (Radenković, *et al*., 2016). Alloxan is a toxic glucose analog which has a cytotoxic effect on the beta pancreatic cells. It accumulates in the cell to cause a build-up of free radicals that result in cellular stress and cell destruction (Argueda *et al*., 2009). This diabetes animal model has been reported as a good model to be good for testing the anti-diabetic effects of new therapeutics (Van den Bergh *et al*., 2009).

This study will also interrogate the anti-diabetic and probiotic effect of Kombucha in a murine model for diabetes.

1.2 RATIONALE

The World Health Organization (WHO) has projected that the global incidence of diabetes would be approximately 800 million cases globally by 2025 (Roglic *et al*., 2016). This among other factors have driven global efforts to reduce mortality associated with diabetes and its associated complications by making cheap, safe and effective medicines available to replace or augment the current expensive insulin-based therapies which need to be taken throughout the patient's lifetime as oral anti-hyperglycemic agents.

The discovery and repurposing of cost-effective alternative or complementary therapies as well as therapeutic agents are keys to attain this reduced diabetes morbidity status.

Claims about the potential ability of Kombucha to control blood glucose concentration by consumers are anecdotal. Although the beneficial effects of Kombucha have not been entirely determined scientifically by experiments, the positive desirable effects are not in doubt, considering the enormous probiotic and nutraceutical composition of this beverage. There is therefore the need to experimentally determine using murine model, the scientific basis for Kombucha usage as an alternative prophylactic or therapeutic anti-hyperglycemic agent.

1.3 HYPOTHESIS

Kombucha, being a complex matrix of microorganisms and nutraceuticals, plays an essential role in the management of diabetes.

1.4 AIM

To investigate the anti-diabetic and probiotic effect of Kombucha on alloxan-induced diabetic rats.

1.5 SPECIFIC OBJECTIVES

1. To determine the microbial ecology of black tea Kombucha.
2. To determine the anti-diabetic effect of Kombucha on alloxan-induced diabetic rats.
3. To evaluate the safety of Kombucha.
4. To assess the effect of different Kombucha treatments on the gut microbiome.

1.6 SIGNIFICANCE OF STUDY

Data generated from this research would provide the information on the therapeutic potency of Kombucha as an anti-diabetic product and evidence for further studies to better understand the mode of action by which the constituents of Kombucha confer their anti-diabetic properties and the possible identification of novel probiotic strains in Kombucha for future therapies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 DIABETES MELLITUS

Diabetes mellitus is a metabolic disorder that is greatly characterized by unusually high blood glucose levels due to inadequate insulin production as well as insulin resistance. This disease condition is mostly chronic and causes an abnormal rise in blood glucose, a medical condition known as hyperglycemia. The reference diagnosis range of fasting blood glucose was reported by WHO consultation as ≤ 7 0 mmol/l (Alberti and Zimmet, 1998). There are three main types of diabetes namely type 1, type 2 and gestational diabetes. The most common is the type 2 diabetes. The inadequate insulin production is usually due to the malfunction of the pancreas as seen in classical type 1 diabetic conditions (Diabetes Association, 2018) These uncontrolled raise in blood glucose levels usually results in other disease complications that affect several other organs such as the kidneys, eyes, blood vessels, heart and the nerves (McDevitt, 2005) Some common symptoms associated with diabetes mellitus include hyperglycemia, frequent urination, inability of wound to heal, fatigue, blurred vision, weight loss or gain (in some cases) and irritability. In most instances, when diabetes is not properly managed, it further develops into other complications that are more life threatening.

2.1.1 Types 1 Diabetes

Type 1 diabetes which was also known as insulin-dependent or juvenile diabetes is characterized by inability of the body's beta pancreatic cells to produce insulin. Patients suffering from this type of diabetes require daily administration of insulin to enable them to control their blood glucose. The exact cause of type 1 diabetes is currently not fully understood (Boitard *et al.*, 2005). However,

studies have associated the disruption of the pancreas due to inflammation within the gastrointestinal tract with probable cause of the disease. Some symptoms of this condition are weight loss, constant hunger, fatigue, frequent urination and blurred vision (McDevitt, 2005; Roglic *et al.*, 2016)

2.1.2 Type 2 Diabetes

Type 2 which was formerly known as non-insulin dependent diabetes or adult-onset diabetes is caused by the body's ineffectiveness to utilize insulin for glucose metabolism and control of blood glucose levels. This form of diabetes present with symptoms similar to that of type 1 diabetic condition but in most cases, symptoms are not dominant resulting in late diagnosis and undiagnosed cases. Conventionally, type 2 diabetes has been known to be a disease condition of the elderly but in recent time cases have been reported in children (Boitard *et al.*, 2005) Reports have shown an association between obesity and diabetes however not much of this is explicitly understood (Rössner, 2001)The gut microbiome has also been associated with onset of obesity, which is tied up with diabetes hence further study needs to be done to demonstrate the exact mechanism of this phenomenon.

2.1.3 Gestational Diabetes

This form is usually seen in pregnant women mostly a temporary condition but when not properly managed can lead to the risk of type 2 diabetes (Feig, 2012). The condition arises when the blood glucose levels exceed the normal range of diagnosis but is also relatively lower than the diabetic reference range. Gestational diabetes tends to cause a lot of adverse complications during pregnancy and delivery of the newborn child. The most appropriate way to diagnose this medical condition is through prenatal screening instead of awaiting symptoms.

2.2 SOME COMPLICATIONS OF DIABETES

The overall mortality associated with diabetes annually is significantly linked with these complications. For example, abnormally high blood glucose levels can trigger diabetic ketoacidosis (DKA) for both patients suffering from either type 1 or type 2 diabetes (Roglic *et al*., 2016). Conversely, low blood glucose (hypoglycemia) that arise from over exercise or untimely eating in diabetes patients can result in loss of consciousness and seizures. Other advance stage complications associated with diabetes are, neuropathy-nerve damage which can cause ulcers in feet as well as diabetic retinopathy which causes blindness resulting from damage of the small blood vessels within the retina of the eye. In cases of pregnancy, improper management of diabetes can result in several childbirth complications such as stillbirths, perinatal death and congenital malformation.

2.3 GLOBAL BURDEN OF DIABETES

The increasing incidence of diabetes globally has affected the economic cost of treatment and management of the disease (Diabetes Association, 2018). This report observed that due to the unprecedented rise in diabetes cases in the United States of America the economic cost has increased by 26 % from 2012 to 2017. This report is undoubtedly consistent with the expected economic cost involved in disease management in Africa and more specifically Ghana. According to the data published by WHO regarding the global burden of diabetes, 43 % of all mortality associated with hyperglycemia occur in people less than 70 years with most incidence in lower middle-income countries. In 2014, approximately 422 million of the adult population globally had diabetes. These numbers are estimated to increase drastically by 2030 if attention is not drawn to it. In tandem with this, diabetes is one of the four main non-communicable diseases being tackled by WHO worldwide. Generally, more mortality caused by hyperglycemia has been reported in

low-income and lower middle-income countries as compared to what is recorded in high-income economies, and more males than females (Figure 2.1). This could possibly be due to the high cost of treatment and management of the disease and the overall accessibility to affordable and essential medicine, as well as the introduction of personalized therapies which further increases the cost of effective diagnosis in developing countries. It is therefore necessary to develop effective and safe alternatives for management and hence reduction of the global burden and mortalities associated with diabetes and its complications (Figure 2.2).

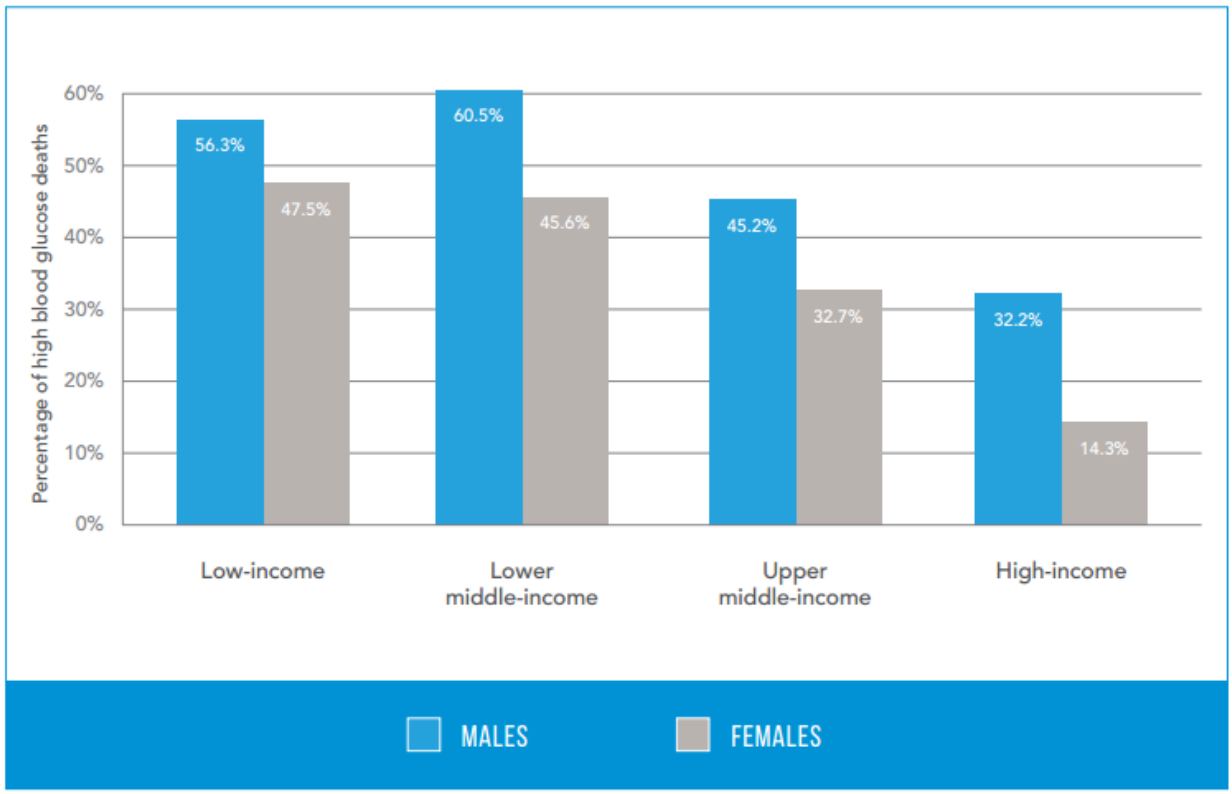

Figure 2.1: Percentage mortality due to hyperglycemia based on income of individuals aged 20-69 years. *[Adapted from Roglic et al., 2016].*

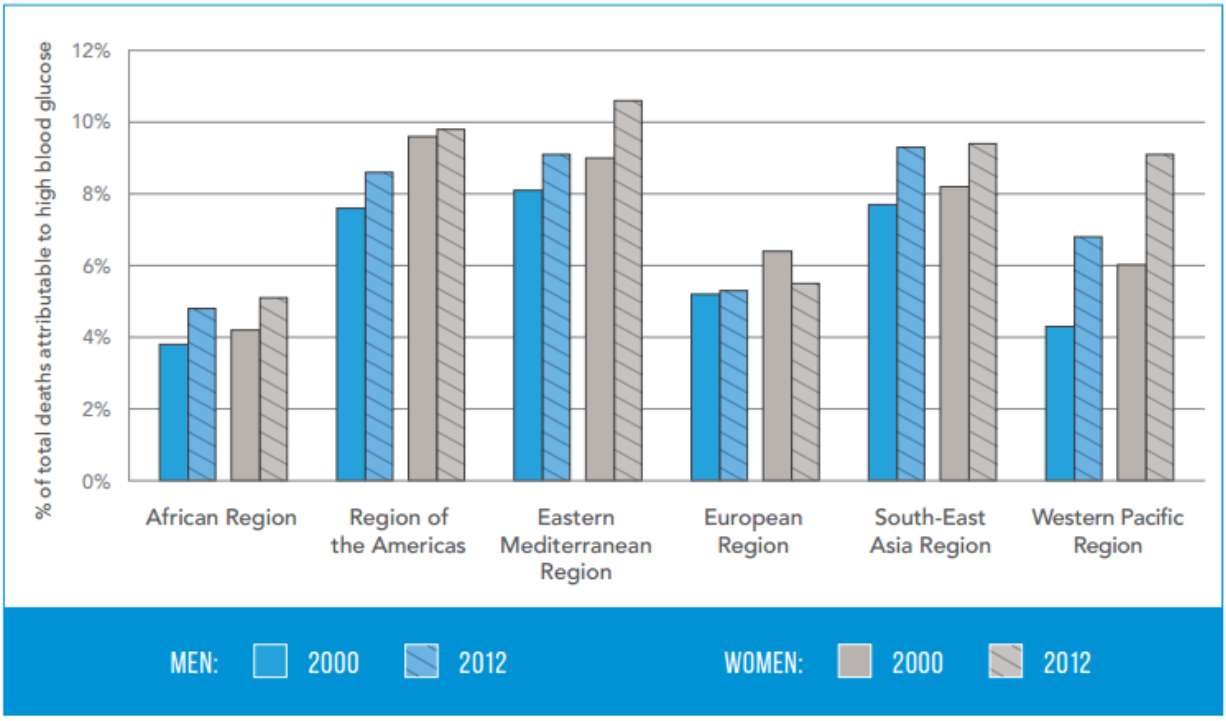

Figure 2.2: Percentage distribution of the all mortality associated with high blood glucose of adults within the ages of 20-69 by WHO geographical regions and sex for 2000 and 2012. *[Adapted from Roglic et al., 2016].*

2.4 GUT MICROBIOME AND OUTCOME OF DIABETES AND OBESITY

Emerging research into understanding the microbiome and its relevance in health and disease in recent times has been able to provide some information on the role of the gut microbiome and its implication in disease. Several studies have tried to characterize the gut microbial community to be able to define its microbial structure as well as the functions of these microbes within us.

Studies have linked a few phyla of bacteria namely Actinobacteria, Bacteriodetes, Firmicutes, Proteobacteria and Verrucomicrobia as common members of the gut microbiome (Turnbaugh *et al*., 2006; Koren *et al*., 2012; Goodrich *et al*., 2014). Using advance sequencing technology such as NGS metagenome wide analysis, researchers have shown the significant correlation between

the gut microbiota, their metabolic processes and type 2 diabetes. Averagely the bacteria gene in the gut are about 159 times more than the human genome (Wen and Duffy, 2017)

Studies have shown the variations that exist within the microbiome of the disease and health population in diseases such as inflammatory bowel disease, obesity, diabetes and autism (Jovel *et al*., 2016). While some bacterial population seem to have emerged within their microbiome others have been lost in those study participants. One of the main questions faced by researchers in this field is the question of whether there is a core microbiome? Several studies with animal models have given insight into better understanding of factors that affect the gut microbiota ranging from genetics to feeding, medication especially anti-biotics and diet. Furthermore, certain gut microbiota signatures that drive intestinal inflammation have been linked to the development and risks of type 2 diabetes. Consumption of fermented foods containing probiotics, such as Kombucha and yogurt, have been reported to play keep roles in alleviating inflammation markers and weight loss as well as affecting the gut microbiome (Goodrich *et al*., 2014; Marco *et al*., 2017). Diet change is also one of the key modulators of the gut microbiome composition and has been shown to contribute to diabetes and obesity (Marsh *et al*., 2014). Approximately about 80 % of type 2 diabetes patients in the Western world are overweight; similarly, there is an increased surge in diabetes cases in Sub-Saharan Africa. An association has been established between obesity and type 2 diabetes with altered gut microbiome, gut barrier disruption and inflammation.

Germ-free mice models have also been shown to possess an abnormal immune system such as underdevelopment of lymph nodes and gut associated lymphoid tissues, hence affects the host immune system. (Turnbaugh *et al*., 2006; Goodrich *et al*., 2014).

2.5 IMPACT OF DIABETES ON BODY WEIGHT

Several organs within the body are affected in patients with diabetes. Interestingly, adverse effect of diabetes on the sexual function and fertility have also been reported in women with diabetes. However, minimal attention has been given to this particular occurrence in diabetic women (Attia *et al*., 2009). People with obesity have the most incidence of T2D confirming the strong correlation between T2D and increased body weight. This observation is highly prevalence in the western countries but alarmingly increasing in Africa. Furthermore, cardiovascular diseases incidence is also higher in obese patients (Daousi, 2006; Jonsson *et al*., 2002). It is very important to also note that not all patients with type 2 diabetes are obese. Normal weight people with T2D have a stronger genetic disposition to developing diabetes (Franco *et al*.,2013; Carnethon *et al.*, 2012). Genetic markers have been linked with obesity and other metabolic disorder (Perry *et al*., 2012).

2.6 TREATMENT AND REMEDIES FOR DIABETES MANAGEMENT

Administration of Thiazolidinediones such as metformin is one of the recommendations for T2D patients. The mode of action of metformin involves the reduction of blood glucose production and enhancing insulin sensitivity by the cells of the body. Even though the use of metformin is recommended for clinical applications, several side effects such as nausea, heart failures and diarrhea have been associated with its usage (Li, 2011). Other Sulfonylureas such as glyburide, glipizide and glimepiride help in the secretion of more insulin by the pancreatic cells of the body. Some of these drugs also result in weight gain. Insulin secretion stimulators stimulate the pancreas to produce more insulin. This sometimes causes patients to become hypoglycemic since there can be over production of insulin (WHO, 2018). Dipeptidyl peptidase -4 (DPP-4) inhibitors have been employed in recent times to reduce blood glucose levels. This approach bears little side effect compared with the conventional approaches. They may cause joint pain and predispose patients to

pancreatitis (Guillausseau, 2011). Insulin peptide injections are also alternatives for management of high blood glucose (Dolinar, 2009). These suggested treatment approaches are very expensive and life-long. There is therefore need for cheaper, safe and efficacious alternatives for DM management.

2.7 KOMBUCHA

Kombucha health beverage is a fermented drink made by the fusion of tea fungus and sugared tea. The medicinal usage of tea products dates from several years ago. However, fermented tea "Kombucha" usage started from China around 220 BC (Roche, 2018). In 414, Dr. Kombu introduced this fermented tea drink for the first time in Japan in the treatment of digestive troubles of the Emperor. Over several decades, Kombucha was been used in several parts of the world including, Germany, France, Russia and Italy. It was noted for its sweet-sour apple cider-like taste and was greatly produced and consumed domestically. Kombucha is locally prepared in the Department of Biochemistry, Cell and Molecular Biology (Figure 2.3)

Figure 2.3: Kombucha prepared at the Department of Biochemistry Cell and Molecular Biology using Rev. Dr. W.S.K. Gbewonyo's protocol. *[Picture taken from the lab].*

2.7.1 Chemical Properties/Phytochemical Constituents in Kombucha

The action of microorganisms within the SCOBY on the available substrate results in the production of several metabolites and intermediates that act either independently or synergistically to enhance the overall medicinal effect of Kombucha. Nutraceuticals associated with tea fermentations such as polyphenols including catechins and other fermentation products of Kombucha including organic acids such as pyruvate, lactic acid, acetic acid and glucoronic acids are some of the major constituents of Kombucha (Figure 2.4).Catechins, Vitamins B1, B2, B6, B12, minerals ions, fructose, glucose and sucrose have also been associated with Kombucha (Jayabalan *et al.*, 2014) (Figure 2.4).

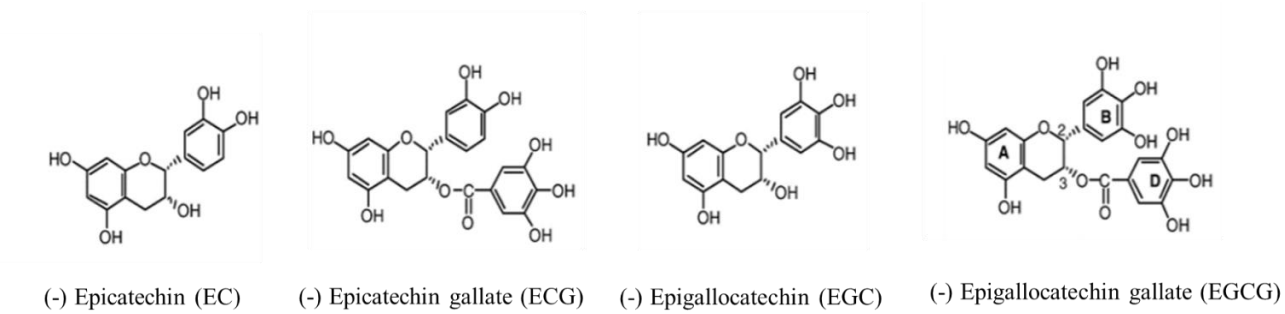

Figure 2.4: Some polyphenols found within Kombucha. *[Adapted from Jayabalan et al., 2014].*

2.7.2 Benefits and Medicinal Claims of Kombucha

Kombucha is well noted for its health benefits. Testimonies from regular consumers from various part of the world show that Kombucha helps in reducing the risk of development of cancer, cardiovascular diseases, facilitates digestion, boosts the immune system, reduces frequency of malarial attack and detoxification of the body among many others (Dufresne and Farnworth, 2000). It could also serve as a food supplement and probiotic.

However, aside all these relevant testimonies, there is little scientific research findings to buttress these acclaimed facts. It is therefore very crucial and demanding of scientists to deploy all available

resources to study and exploit Kombucha use as a prophylactic or therapeutic agent (Jayabalan *et al*., 2014).

2.7.3 SCOBY Composition of Kombucha: Probiotics within Kombucha

One of the most important components of Kombucha is the SCOBY. This is the acronym for Symbiotic Culture of Bacteria and Yeasts Kombucha SCOBY is made up of useful and beneficial microorganisms that ensure that the fermentation of the tea broth occurs. Several species of yeast and bacteria have been found to be associated with this SCOBY. Some of these bacteria include: *Acetobacter sp., Gluconobacter sp.*, Cellulose producing organism and some other nitrogen fixing bacteria. Some of the yeast species are *Saccharomyces, Saccharomycodes, Schizosaccharomyces, Zygosaccharomyces, Brettanomyces/Dekkera, Candida, Torulospora, Koleckera, Pichia, Mycotorula,* and *Mycoderma* (Reva *et al.*, 2015; Jayabalan *et al*., 2014). Usually, these organisms produce large mass of cellulose pellicle called the mat to which they attach themselves at the surface of the broth.

According to Abass (2016) molecular characterization of microbes present within black tea Kombucha using 16S targetted polymenrase chain reaction (PCR) showed the presence of the following bacteria strains: *Lactobacillus amylolyticus, Corynebacterium glutamic, Bacillus licheniforms, Paenibacillus lactis* and *Paenibacillus cineris.* However, the exact microbial composition of this complex community needs to be characterized.

2.7.4 Advantages of Probiotics in Modern Treatment Therapies

Modern therapeutic approaches have tried to present compelling arguments on the use of probiotic bacteria to enrich the gut microbiome. Ranging from the administration of live single strains of probiotics and other biotherapeutics, to the whole non-defined consortium of organisms in feacal transplants, have been some strategic approaches used to clinically diagnose patients with disease

resulting from dysbiosis. Probiotics can be classified based on their sources and applications (Figure 2.6). The success of probiotics to modulate the gut microbiome has been shown substantially in literature. Most strains have also been shown to have good survival abilities within the gastrointestinal tracts and remain metabolically active. However, literature has also reported short persistence of some strains in the gut with minimal effect on resistant microbiota. In all these approaches, feacal Microbiota Transplant (FMT) seems to be a more successful approach to engraft the microbiota (Mills *et al*., 2013; Zuo *et al*., 2017; Sanders *et al.*, 2018; Walter *et al*., 2018). The future of anti-diabetic therapies could be greatly hinged on the availability of safe, cheap and accessible alternative medicine. This allows for more research to be able to develop novel therapeutic interventions for this purpose. Probiotics and probiotic supplemented functional foods that have been shown to possess anti-diabetic potentials could be further exploited as anti-diabetic agents (Green *et al*., 2017; Sanders *et al*., 2018; Walter *et al*., 2018). Probiotic are able to colonize their respective niches within the gut by several mechanisms (Figure 2.5).

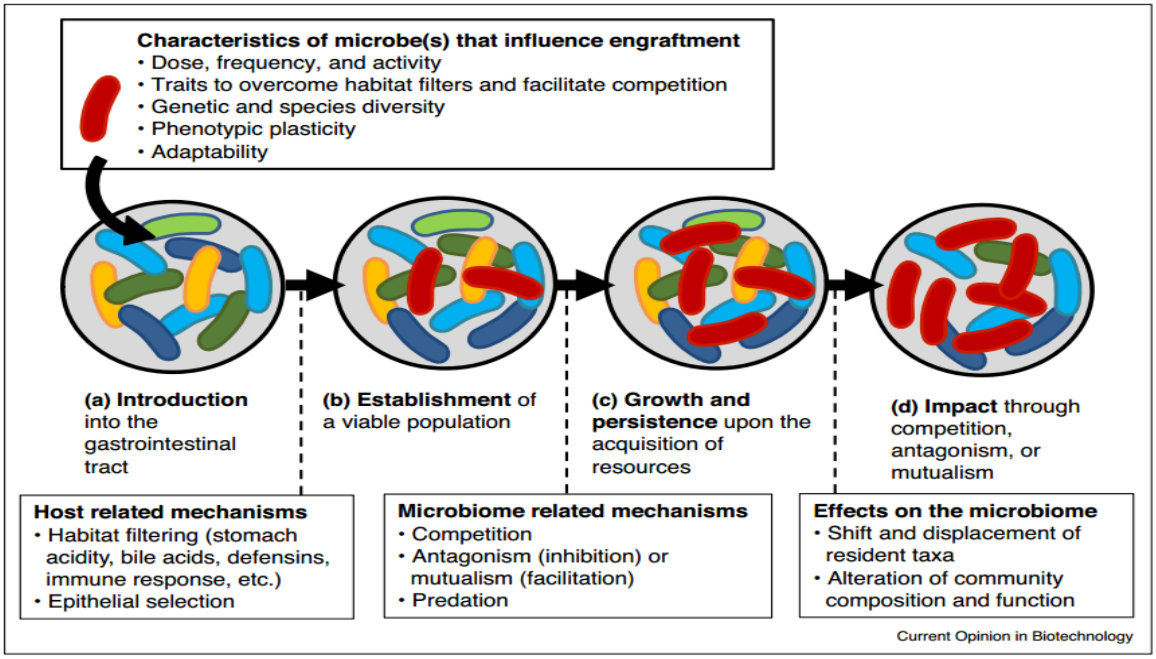

Figure 2.5: Four stages to demonstrate the successful invasion and colonization of the gut microbiome with introduced probiotic or biotherapeutic agent (a - d). *The effect of the introduction of this organism in the microbial composition and cellular processes. [Adapted from Mallon et al., 2015].*

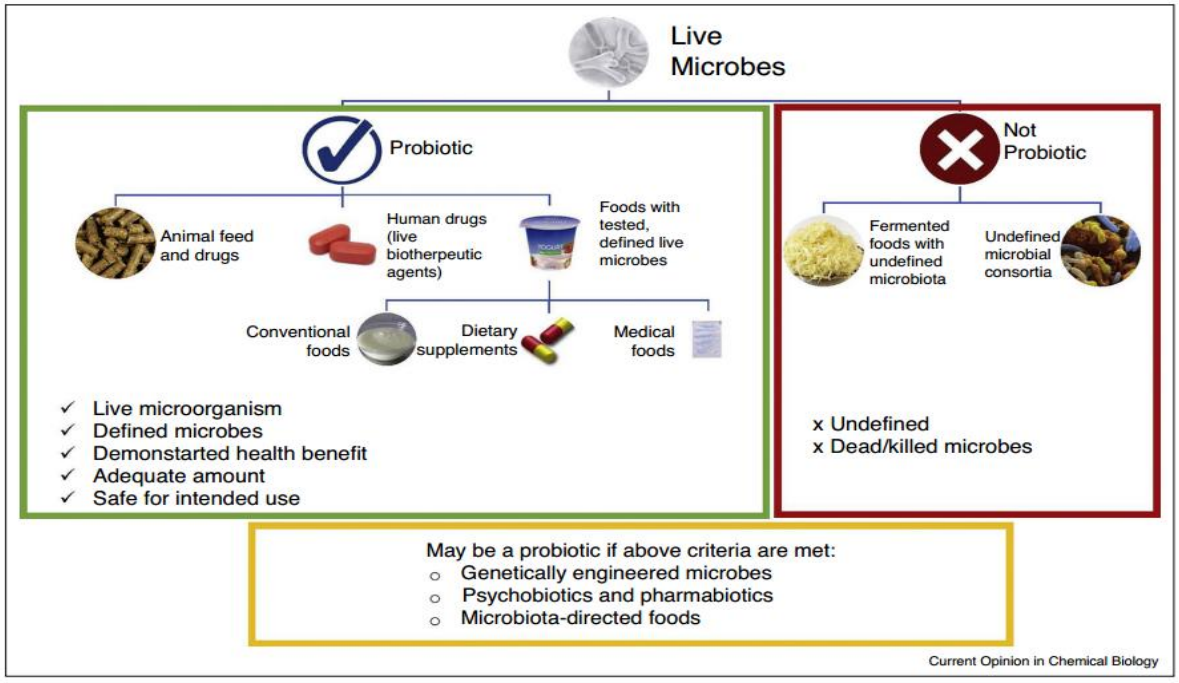

Figure 2.6: Characteristics and classification of probiotics. *[Adapted from Green et al., 2017; Marco et al., 2017; O'Toole et al., 2017].*

2.8 METAGENOMICS FOR IDENTIFICATION OF MICROBIAL COMMUNITIES

The advances in next generation sequences have provided the platform for understanding and the study of complex microbial community structure as well as the community functions of the organisms. Metagenomics is a high throughput genomic approach that provides a platform where all the members ranging from bacteria, fungal, protists, archaea and viruses within a microbial community could be analyzed in a single run of sequencing. Next generation sequencing has made room for the possibilities of generating big bioinformatics data or analysis but requires a good computational capacity to process data. Next generation sequencing has overcome the persistent challenge of the older sequencing technologies which were limited by a shortage of reference genomes (Caporaso *et al*., 2010; Scholz *et al*., 2012).

Shotgun whole genome metagenomics approach for identifying microbial community and function does not require use of primers to target a specific marker gene, making it possibly to directly find the function of the microorganism. However, it is more expensive and requires more reads to be amplified. It also requires high bioinformatics computation to identify the metagenome taxa structure. A major limitation to this technology as with all DNA based technologies, is that the presence of the bacteria does not necessarily determine whether the organism is active in the culture or not. One of the major strengths of metagenomics analysis is that it is able to comprehensively tell the plausible microbiome diversity within the population by overcoming the limitation of culturing to identify the microbial communities. Bioinformatics software and programs such as MEGAN, QIIME, MG-RAST and KRAKEN are very useful in identifying the microbial community profiling (Miller *et al*., 2010). I6S rRNA gene and ITS region targeted metagenomics approaches are also employed to be able to enrich microbial communities from host DNA that could impede the success of the sequencing (Meyer *et al*., 2008). Although this approach is very easy to employ, lost information is not recovered to be able to tell the function of the genes

in the microbial communities. Targeted metagenomics is cost effective and requires fewer reads to be able to identify the microbial taxa (Jovel *et al*., 2016).

2.9 ANIMAL MODELS OF DIABETES

The use of medicinal plants for management of diabetes dates back to several decades. About 1000 plants have been claimed to be efficacious in the treatment and management of diabetes mellitus. However, only a few of the plants have been scientifically and experimentally reviewed and studied for this purpose (Serafín *et al*., 2009)

In vivo experiments are critical elements of any drug discovery process. In ethnopharmacology, the use of animal models such as Wistar rats and IC mice have been very essential in demonstration of the medicinal properties as well as safety and toxicity of crude extracts. Animal testing simulates to a large extent what will possibly happen in human when such drugs/herbal preparations are taken by patients. To evaluate the mode of action, efficacy and side effect medicinal plant extracts with anti-diabetic properties, animal models have been extremely useful. Due to the diversity and variations within human populations, no single animal model experiment is sufficient to represent the human diabetic condition but rather serve as a critical point to begin the search for safe and efficacious drugs and drug-like (Van den Bergh *et al*., 2009).

Thus, many different animal models have been used, each displaying a different set of features seen in human diabetic states (Xiao *et al*., 2010). Normal nondiabetic animals and animals with impaired glucose tolerance and insulin resistance have been used to demonstrate hypoglycemic activity and to investigate the mode of action of anti-diabetic plant materials.

2.10 MECHANISM OF ACTION OF ALLOXAN IN INDUCTION OF DIABETES

Alloxan (2,4,5,6-tetraoxypyrimidine;5,6-dioxyuracil) is a chemical agent which is used to induce diabetes in animal models during experiments. Alloxan is very hydrophilic and this property allows it to easily cross the lipid bilayer whereas the glucose-like structure enables it to gain access to the beta pancreatic cells via the GLUT 2 transporter (Weaver *et al*., 1979; Gorus *et al*., 1982 Lenzen and Munday, 1999; Elsner *et al*., 2000; Lenzen 2008). Alloxan selectively destroys the beta pancreatic cells which causes an impairment in the insulin secretion by the pancreatic cells. This ultimately cause the unprecedented hyperglycemia in the animal and sequentially leading to diabetes. This acute toxicity to the pancreatic cells results in necrosis of the cells causing the pathological and metabolic disorder observed. As a result, there is heighten levels of plasma and blood glucose levels. For an effective action of alloxan in inducing diabetes, the experimental animal is fasted for 24 hours to reduce the plasma glucose levels since the alloxan has a similar structure as glucose, there would be competitive inhibition on the alloxan uptake resulting in the failure of the experiment (Radenkovic *et al*., 2013).Alloxan injection has also been reported to be toxic to the liver and kidneys by the production of lots of free radicals (Evan *et al*., 1984). Alloxan could be used to induce both TD1 and TD2 (Rees and Alcolado, 2005).

CHAPTER THREE

3.0 METHODOLOGY

3.1. KOMBUCHA PREPARATION

Black tea Kombucha was prepared by the standard protocol developed by Rev. Dr. W. S. K. Gbewonyo of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana. Harvested Kombucha was freeze-dried using the LABCONCO freeze dryer (LABCONCO Corporation, Catalog No. 7670530, Kansas City, Missouri) for further analyses.

3.2 MOLECULAR CHARACTERIZATION OF BACTERIA IN KOMBUCHA

3.2.1 Kombucha Preparation for DNA Extraction

About 100 milliliters of the fermented Kombucha culture was centrifuged at 14,000 x g for 10 minutes at 41^{o}C. DNA was isolated from pelleted cells. Bacterial DNA isolation from the pelleted cells was carried out as described by the manufacturer (Quick-DNA^{TM} Fungal/Bacterial Miniprep Kit, Cat No. D6005, Zymo Research).

3.2.2. DNA Extraction Using the Quick-DNA^{TM} Fungal/Bacterial Miniprep Kits (Catalog No. D6005)

The pelleted cells from the Kombucha, as described earlier, were resuspended in 200 µl of PBS. The suspension was then transferred to the ZR BashingBead^{TM} Lysis tube (0.1 mm & 0.5 mm) and 750 µl of BashingBead^{TM} buffer added. The tubes were subjected to vigorous shaking using the Genie Disruptor ^{TM} (Scientific Industries Inc, USA, Serial No. D 58-1173) for 20 minutes and centrifuged at 10,000 g for 1 minute. About 400 µl of the supernatant were transferred onto the

Zymo-Spin™ III-F Filter in a new collection tube and centrifuged at 8,000 g for 1 minute. and the filtrate (1.2 ml) of the genomic lysis buffer was added. The resulting mixture was transferred onto the Zymo-Spin™ IIC column[3] and span at 10,000 g for 1 minute and the flow through was discarded. Two hundred microlitres (200 µl) of DNA prep wash buffer was added to the Zymo-Spin™ IIC column[3] and centrifuged at 10,000 g for 1 minute. Subsequently, 500 µl of g-DNA wash buffer were added on to the Zymo-Spin™ IIC column[3] and span at 10,000 g for 1 minute. The final elution of the DNA was done by addition of 100 µl of the DNA elution buffer to the Zymo-Spin™ IIC column[3] and centrifuged at 10,000 g for 1 minute. The DNA obtained was quantified using Qubit4™ as well as nanodrop (Thermo Fisher Scientific, Serial No. AZY1601732). DNA was prepared for further downstream analysis.

3.2.3 Shotgun Metagenomics Sequencing Using the Oxford Nanopore Sequencer

The DNA obtained from the unfiltered Kombucha was prepared for shotgun whole genome metagenomics analysis.

3.2.3.1 Clean-up of extracted genomic DNA from Kombucha using the AmpureXP beads

Extracted DNA samples were vortexed and equal volumes of AmpureXp beads were added to the DNA. The mixture was pulse-centrifuged and incubated at 25 ^{o}C for 2 minutes. The mixture was placed on a magnetic rack and incubated for 2 minutes until the bead pelleted against the magnet and the solution became completely transparent. The supernatant was discarded. The pellet was pre-washed by addition of 200 µl of 80 % ethanol at room temperature several times and placed back on the magnetic rack. The supernatant was discarded, and the pellet allowed to dry for 1 minute. The pellet was re-suspended in 30 µl of EB buffer for 5 minutes. The DNA was quantified using Qubit4™ following the dsDNA protocol (Protocol by Artic Network).

3.2.3.2 Barcoding and adaptor ligation: One-pot protocol (End prep reaction set-up)

The reaction master mix (35 µl) was prepared using 25 µl of 100 ng of genomic DNA extracted from the Kombucha, 7 µl of the Ultra II end prep buffer and 1.5 µl of Ultra II end prep enzyme mix. The mixture was incubated at room temperature for 5 minutes, quickly transferred to 65 ^{o}C for 10 minutes and quickly placed on ice for 30 seconds. NBXX Barcode (2.5 µl), 32.5 µl of Ultra II ligation master mix and Ultra II ligation enhancer were directly added unto the previously prepared master mix and then incubated at room temperature for 30 minutes. 10 µl of 0.1 % SDS solution was added and incubated at 70 ^{o}C for 5 minutes and quickly placed on ice. The samples were then pooled together and cleaned-up using the Ampure XP beads (Protocol described earlier). To 19 µl of the cleaned-up barcode fragment, 20 µl of BAM 1D adaptor, 25 µl of LNB and 10 µl of Quick t4 DNA ligase were added and mixed gently. The mixture was incubated again at room temperature for 30 minutes. The sample was cleaned-up using Ampure XP beads. However, the wash step was done with SFB and not ethanol. The DNA was also quantified and again using the Qubit^{TM}. The prepare libraries were then sequenced using the Oxford nanopore sequencer (MINION flow cell). The flow cell was primed, and the samples loaded onto the spotON flow cell.

3.2.3.3 Metagenomics data analysis

The sequence data was generated by using the Oxford nanopore sequencer (Minion). The output was based called using the flip-flop base caller. The FASTQ data were analyzed using ONECODEX software.

3.3 ANTI-DIABETIC ACTIVITY AND SAFETY EVALUATION OF KOMBUCHA

3.3.1 Ethical Clearance and Animal Care

Appropriate ethical clearance to conduct animal model research was sought from the Center for Plant Medicine Research (CPMR). Adult Wistar rats were obtained from the Animal housing facility of the Center for Plant Medicine Research (CPMR). All animals were kept in metabolic cages under standard temperature of 25 ^{o}C, relative humidity of 60-70% and a 12-hour light-dark cycle. The care and handling of animals were done according to Foundation for Biomedical Research rules and methods on the application of animals (Foundation Research on the use of animals in research, 1987)

3.3.2 Animals and Treatments

Adult Wistar rats were used for this study. Stringent protocol and procedures for animal experimentation were followed. About 70 male adult Wistar rats aged 4 weeks, weighing 120 ± 20 g, were obtained from the Center for Plant Medicine Research (Mampong, Ghana). These rats were housed in an environmentally controlled breeding facility in metabolic cages. The animals were fed with purified water and pelleted meals. Prior to the induction, animals were tested for their diabetic status by measurement of fasting blood glucose levels using a Glucometer (Model No: BG01).

3.3.3 Induction of Diabetes using Alloxan

Diabetes was induced in the test rats by the administration of a single intra-peritoneal injection of a freshly prepared alloxan monohydrate (Sigma-Aldrich, USA) solution in normal saline at a specified dose of 150 mg/kg body weight. In order to prevent the provocation of the possible fatal hypoglycemia in rats due to the excessive release of pancreatic insulin, the rats were given about

5-10 ml of 10 % glucose solution orally after 6 h. The rats were given free access to 5 % glucose solution for the next 24 h to reduce the incidence of severe hypoglycemia. Two weeks later, the rats with moderate diabetes having glycosuria and hyperglycemia (i.e. with blood glucose levels ≥ 11 mmol/l) were selected for the experiments.

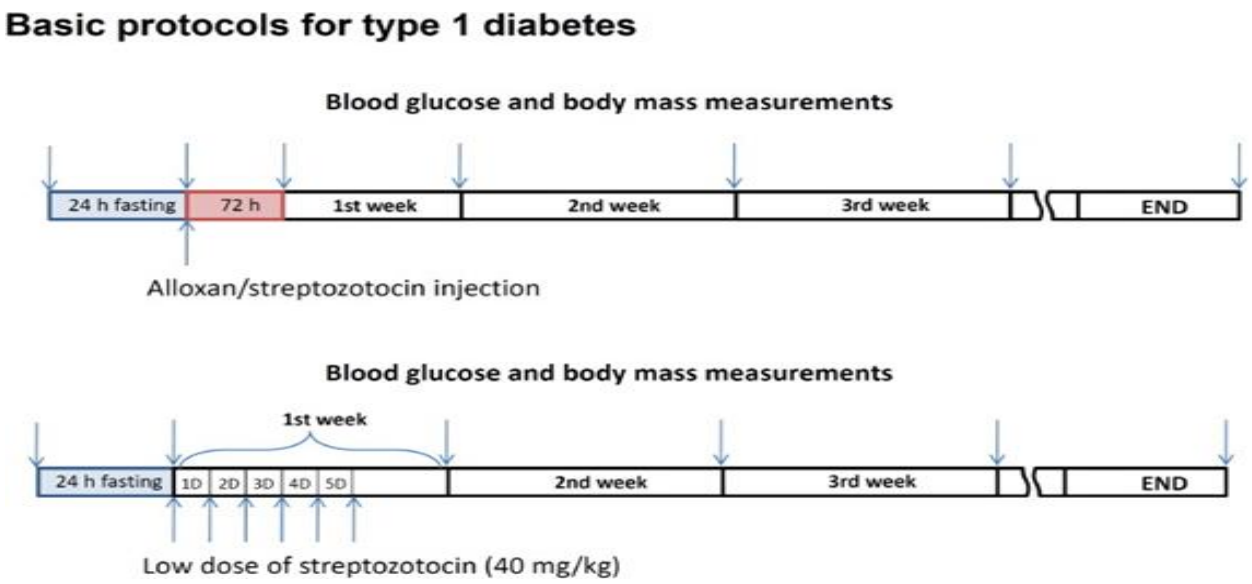

Figure 3.1: Induction of diabetes using alloxan or stretozotocin (STZ). *[Adapted from Randekovic et al., 2016].*

3.3.4 Animal Treatment Groupings

The rats were divided into six experimental groups of six animals each. Each group was subjected to a specific treatment. Table 3.1 is a summary of the treatment groups used for the anti-diabetic and safety studies.

Table 3.1: Treatment category of animals and respective treatment doses

Group	Treatment
Normal	Water
Diabetic Control	Water
Diabetic + Kombucha treatment	Sterile filtered (5 mg, 25 mg, 100 mg)/kg
Diabetic + Glibenclamide	Standard drug (5 mg/kg)
Diabetic + Metformin	Standard drug (10 mg/kg)

The normal control and diabetic rats were fed with normal diet and drinking water *ad libitum*. Diabetic rats were treated with freeze-dried sterile Kombucha by oral gavage (5, 25 and 100 mg/kg of body weight) once daily. Other groups of diabetic rats were given 10 mg/kg metformin and 5 mg/kg glibenclamide via oral gavage once daily. Animals were treated for a period of 28 days.

3.3.5 Mean Weekly Body Weights and Organ/Body Weight Ratios of Rats at Termination

The mean body weights of each group of animals were determined weekly and at termination of treatments. Animals were sacrificed at termination of treatments by cervical dislocation and selected organs (liver, kidney and pancreas) excised cleaned and weighed. The mean organ/body weight ratios of each group of animals were calculated and expressed as a percentage.

3.3.6 Blood Sampling and Serum Preparation

Animals were fasted overnight prior to collection of blood samples. Blood was obtained from the tail vein weekly (1 ml) and by cardiac puncture at termination (5 ml). A small portion of the 1 ml blood was used for fasting blood glucose (FBG) determination and a larger portion was used in the preparation of serum for insulin determination. Serum was prepared from the 5 ml of blood obtained at termination for other biochemical analyses. Briefly, the sera were prepared as follows;

blood collected into serum collection tubes were span at 15,000 rpm (MicroCL 17 Centrifuge, ThermoFIsher Scientific, Serial Number 42323893, Germany for 10 minutes. The serum obtained was stored at -80 °C for further analysis.

3.3.7 Determination of Fasting Blood Glucose (FBG) Concentrations

Some of the blood samples obtained from the tail vein were loaded onto the glucose test strip of the URIT G26 glucometer (Model No: BG01) in duplicate, and glucose concentrations read. This quantitative determination of glucose concentrations is based the glucose oxidase and peroxidase method described by Beach and Turner (1958).

3.3.8 Serum Biochemical Analyses

Serum insulin levels were determined using the Mercodia Rat Insulin ELISA kit in accordance with specified protocol (Catalog No. 10-1250-01, Mercodia AB, Sylveniusgatan 8A, Sweden). Exactly 10 µl each of the samples were pipetted into appropriate wells as well as the calibrators. A hundred microlitres (100 µl) of the enzyme conjugate 1X solution was added to each well and incubated on a plate shaker at 700 rpm (MicroCL 17 Centrifuge, ThermoFIsher Scientific, Serial Number 42323893, Germany) for 2 hours at room temperature (25 ^{o}C). About 6 repeated steps of washing was carried out with 700 µl of wash buffer 1X solution per well. 200 µl of substrate TMB were added into each well and incubated for 15 minutes at room temperature. The reaction was stopped with 50 µl of stop solution, shaken on the plate shaker for about 5 seconds for effective mixing and absorbance read at 450 nm.

The *in vitro* quantification of AST, ALT, ALP, creatinine, cholesterol and urea were carried out on the serum samples. These analyses to quantitatively determine the levels of theses biochemical indices were carried out using the stipulated protocol obtained from the HORIBA Medical

(Montpellier-France) and manufacturer guidelines of the fully automated blood chemistry analyzer Pentra C200 (HORIBA ABX SAS, Japan).

Whole blood was obtained in a EDTA tube and used for direct analysis of lipid biochemical profiles—high density lipoprotein (HDL), low density lipoprotein (LDL), total cholesterol and triglycerides. The analyses were carried out using the lipidoprofile analyzer.

These assays helped to investigate the effects of these treatments on the renal function, liver function as well as pancreatic function of the various treatments.

3.3.9 Urinalysis

About 4 ml of urine were collected from every animal weekly and subjected to a qualitative analysis of some urine biochemical and physicochemical parameters such as glucose level, ketone, protein levels, specific gravity and pH of the urine using the URIT urine test strips.

3.3.10 Histology of Selected Organs

Pieces of excised pancreas, liver, kidney and heart were fixed in a 10 % buffered formalin solution for about 2 months, processed (washing, dehydration, clearing and infiltration) and then embedded in wax. Sections of 5 µm thickness of embedded tissues were placed on glass slides and then stained with hematoxylin-eosin and examined by light microscopy.

3.3.10.1 Tissue preparation for histology

Formalin fixed tissues were cut into pieces and placed in cassettes with their respective labels. The cassettes were placed under running water for about 3 hours to wash away the fixative. The tissues were then dehydrated through a series of varying concentrations of ethanol (70 %, 80 %, 90 % and 2 changes of absolute ethanol) for 1 hour in each solution. Final dehydration of the tissues was

done in 3 changes of chloroform for a total of 2 hours 15 minutes. The tissues were then infiltrated in molten wax (3 changes) at 60 ^{o}C for 45 minutes each. The tissue was then embedded in molten wax and left to harden.

3.3.10.2 Sectioning of tissues

The blocks of tissue were sectioned using a Leica microtome (LEICA RM2235, Germany) set at 5-7 µm and a knife angle at about 4–5 degrees. Thin sections were fixed onto a glass slide and allowed to dry over a heating block before staining.

3.3.10.3 Hematoxylin-eosin (H&E) staining of tissues slides

The tissue slides tissues were de-waxed in 2 changes of xylene for a total of 4 minutes and moved into another 2 changes of absolute ethanol for 3 minutes each. The tissues were hydrated in two changes of 95 % ethanol and 1 change of 80 % ethanol for 3 minutes each. The slides were washed under running water for 5 minutes, stained in hematoxylin for 6 minutes and then rinsed under running water to remove excess hematoxylin. The tissue slide was then counter-stained with eosin for another 6 minutes followed by 3 changes of 95 % ethanol to dehydrate the tissue. The tissue was transferred into 2 changes of xylene for 2 minutes each after which the slides were mounted for light microscopy examination.

Morphological changes in tissues were examined with a camera-mounted Olympus microscope. The relative frequency, size, distribution and morphological features were recorded per tissue. Each tissue was examined under x40, 100 and 400 objective magnifications. The size of islets of Langerhans was measured in µm on camera-fitted Leica microscope and the image processed using computer-based Leica digital pathology software.

3.4 EFFECT OF KOMBUCHA ON RAT GUT MICROBIOME

3.4.1 Stool Sample Preparation for DNA Extraction

Stool samples were obtained from the rats before and after being subjected to the various kinds of treatments described previously (Table 1). The feacal samples were stored at -20 ^{o}C after collection.

3.4.2 DNA Extraction from Stool Samples

Total DNA was extracted from the samples using the Quick-DNA^{TM} Feacal/Soil Microbe Miniprep Kit (Catalog No. D6010) in accordance with the manufacturer's protocol.

About 150 mg of feacal sample were added to the ZR BashingBead^{TM} Lysis tube (0.1 mm & 0.5 mm) and 750 µl of BashingBead^{TM} buffer added. The tubes were subjected to vigorous shaking using the Genie Disruptor^{TM} (Scientific Industries Inc, USA, Serial No. D 58-1173) for 20 minutes and centrifuged at 10,000 g for 1 minute. About 400 µl of the supernatant were transferred on to the Zymo-Spin^{TM} III-F Filter in a new collection tube and centrifuged at 8,000 g for 1 minute. About 1.2 ml of the genomic lysis buffer were added to the filtrate. The resulting mixture was transferred onto the Zymo-Spin^{TM} IIC column^{3} and span at 10,000 g for 1 minute and the flow through was discarded. Two hundred microlitres (200 µl) of DNA prep wash buffer were added to the Zymo-Spin^{TM} IIC column^{3} and centrifuged at 10,000 g for 1 minute. Subsequently, 500 µl of g-DNA wash buffer were added on to the Zymo-Spin^{TM} IIC column^{3} and span at 10,000 g for 1 minute. The final elution of the DNA was done by addition of 100 µl of the DNA elution buffer to the Zymo-Spin^{TM} IIC column^{3} and centrifuged at 10,000 g for 1 minute. Six hundred microlitres (600 µl) of prep solution were added to another Zymo-Spin^{TM} III-HRC filter and span at 8,000 g for 3 minutes. The eluted DNA was then transferred onto the Zymo-Spin^{TM} III-HRC filter and

centrifuged at 13,300 g for 3 minutes to get the purified DNA for further analysis. The DNA obtained was quantified using Qubit4^{TM} as well as nanodrop (Thermo Fisher Scientific, Serial No. AZY1601732).

3.4.3 16S–Metagenomics Analysis of Rat Microbiome

Ion-torrent sequencing platform present at the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP NGS Facility) was used for the 16S–metagenomics analysis of the microbiome of the various animal treatment groups from stool DNA. Sequenced data was analyzed using the ThermoScientific Ion Reporters software and RStudios.

3.4.3.1 PCR to amplify 16S rRNA and ITS gene bacteria respectively and sequencing

Amplification of 16S rRNA gene was performed on genomic DNA of the isolates using universal 16S rRNA primers targeting the V_2_4_6 hypervariable region was carried out prior to the sequencing on the ion-torrent sequencer at the WACCBIP NGS facility. The PCR run step in the sequencing protocol was carried out by protocol of the 16 S rRNA ion torrent sequencing. The PCR amplicons were resolved on a 1.5 % ethidium bromide stained agarose gel and run at 100V. The resulting gel was visualized using the Gel Imager.

3.5 STATISTICAL ANALYSIS

Statistical analysis was performed using the Graphpad prism v8.0, RStudios and Microsoft excel 2016. ANOVA and T-test were used to compare the mean variations within the treatment groups at 95 % confidence interval. All graphs were plotted using the Graphpad prism and R.Studios.

CHAPTER FOUR

4.0 RESULTS

4.1 MICROBIAL ECOLOGY OF KOMBUCHA

4.1.1 Microbial Diversity in Kombucha using the OCXDB Reference Library

Analysis of the microbiome of the Kombucha from data obtained from the shotgun Oxford nanopore sequencing showed high degree of microbial diversity. *Brettanomyces bruxellensis CBS 2499, Brettanomyces bruxellensis* AWR1499, *Brettanomyces bruxellensis* were abundant species accounting for 42.22 %, 3.99 and 3.66 % respectively. *Brettanomyces anomalus* was also abundant making 6.98 % of all reads aligned to the taxa (Figure 4.1). *Gluconobacter sp.* SXCC-1 and *Kamagataeibacter rhaeticus. Kamagataeibacter nataicola, Kamagataeibacter hansenii, Bacillus nealsonii* and other bacteria (Figure 4.2) were also identified as bacteria present in the microbiome. Most of the strains that were identified had relatively low abundance within the microbial community. However, no archaea and viruses were detected using the OCXDB reference library. In all about 55 organisms were found in Kombucha using this OCXDB. (Supplementary data on the entire microbial ecology in Appendix 4)

4.1.2 Microbial Diversity in Kombucha using the REFSEQ Database

Further analysis of the sequence obtained using the REFSEQ database showed a relatively high abundance within the microbial community of Kombucha microbiome. Similarly, *Brettanomyces anomalus (4.97 %)* and *Brettanomyces bruxellensis CBS 2499 (51.79 %)* were the most abundant in the microbiome of Kombucha (Figure 4.1). *Janthinobacterium agaricidamnos, Cellulosilyticum lentobellum, Aequoriita sublithincola, Mucispirillum schaedleri, Serratia symbiotica, Photorhabdus luminenscens, Acetobacter tropicalis, Psychrobacteraquaticus, Komagataeibacter hansenii, Leptospira noguchii, Komagataeibacter medellinensis, Komagataeibacter xylinus* and

other bacteria were also present in the Kombucha microbiome (Figure 4.2). In addition, some archaea and viruses were also found in the microbial community, but they occurred in relatively lower abundance compared to the fungi population. *Menthsnsbrevibacter boviskoreani, menthanoshaera stadtmanae* and other archaea were found (Figure 4.3). In total, about 349 organisms were identified using this reference sequence database. (Supplementary data on the entire microbial ecology in Appendix.5)

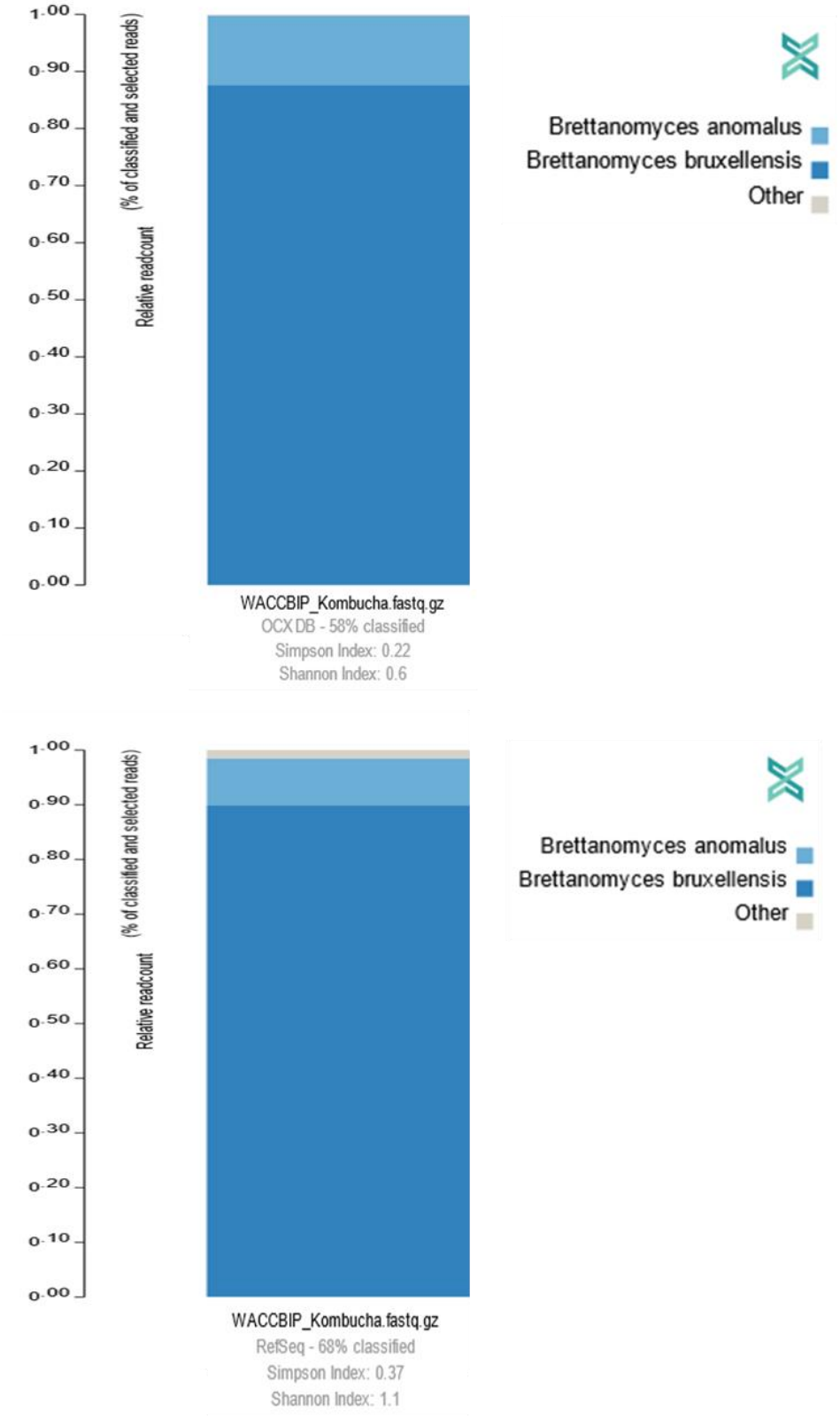

Figure 4.1: Comparison of the fungi community in Kombucha identified using the One Codex database (OCXDB) and the REFSEQ database. *This identification and frequencies were observed at the species level.*

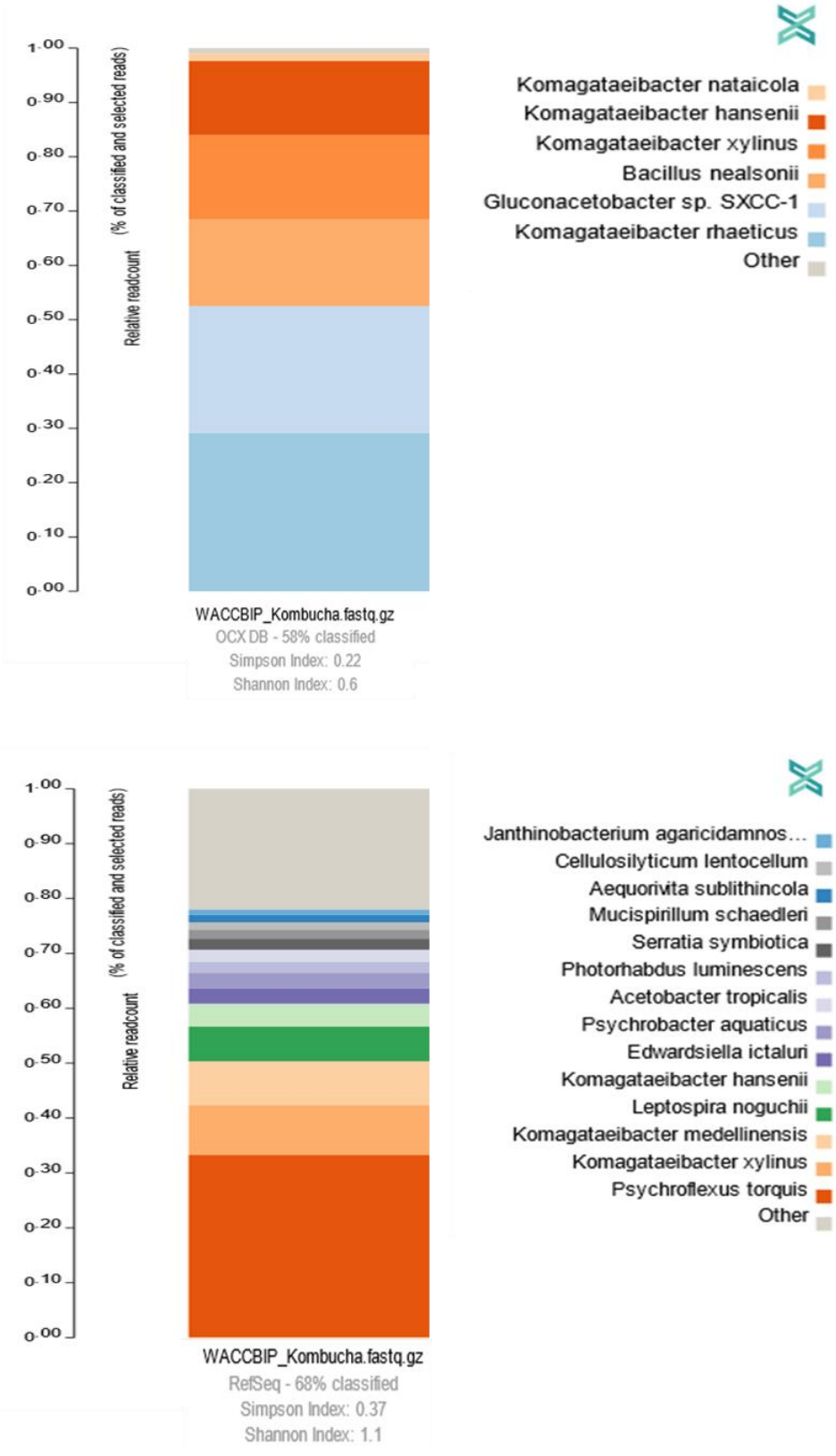

Figure 4.2: Comparison of the bacteria community identified in Kombucha using the One Codex database (OCXDB) and the REFSEQ database. *This identification and frequencies were observed at the species level.*

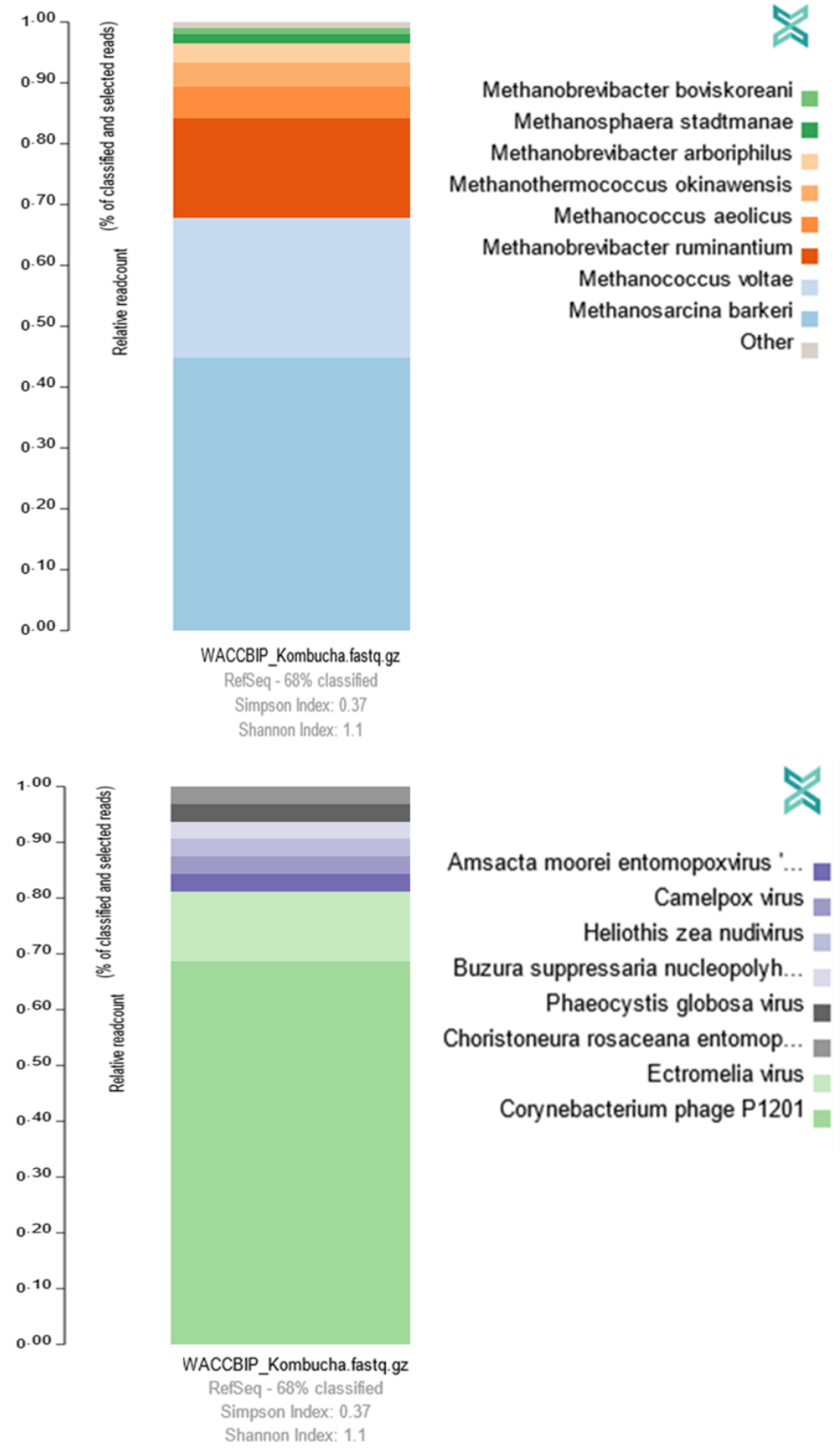

Figure 4.3: Archaea and viral community identified in Kombucha using the REFSEQ database. *This identification and frequencies were observed at the species level.*

4.2 ANTI-DIABETIC EFFECT OF KOMBUCHA IN ALLOXAN-INDUCED DIABETIC RATS

4.2.1 Alloxan-induced Diabetes in Rats

After the three days of administration with alloxan, fasting blood glucose levels were measured to select for animals that had been successfully induced into a diabetic state. Fasting blood glucose (FBG) levels above 10 mmol/l, were indications of successful diabetes induction resulting from hyperglycemia. Most animals were found to have FBG levels ranging between 20 mmol/l and 31 mmol/l after induction (Figure 4.4A). There was about a 3 to 5-fold increase in the mean blood FBG levels in alloxan-induced rats compared to control (6 mmol/l). This variation among the groups was statistically significant when compared using the paired sample t-test with a p value <0.0001 (α=0.05) (Figure 4.4). Furthermore, a significant reduction in the body weight was obtained comparing the mean weight of the animals before and after induction (Figures 4.4B). There was 30 % reduction in the body weight of the animals after successful induction with alloxan with a p-value<0.001 (α=0.05).

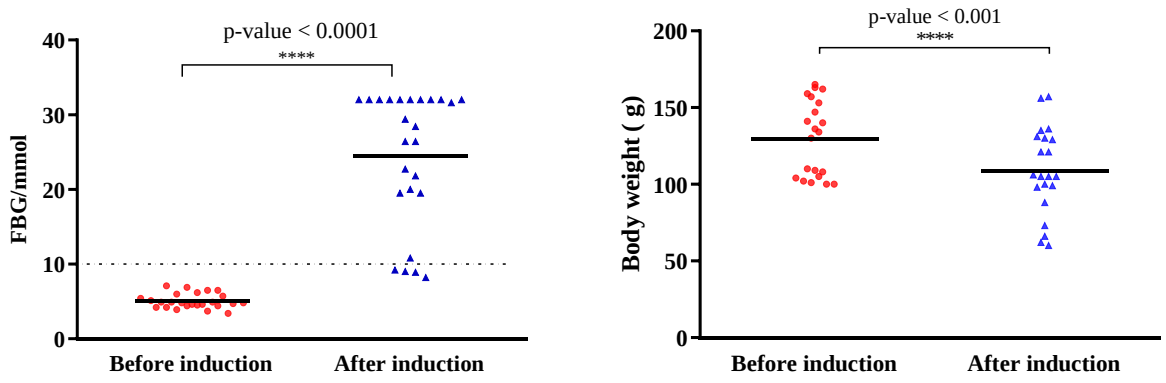

Figure 4.4: Indicators of successful alloxan induction of diabetes. *A) Change in fasting blood glucose level before and after induction B) Change in body weight before and after induction. Paired t-test was used to compare the variations within the groups using the Graphpad prism 8.0. P value < 0.05 denoted significant statistical variations between groups.*

4.2.2 Changes in Mean Animal Body Weights

Animals in the diabetic control group had significantly reduced body weight over the duration of treatment compared to the normal control group which had a relatively stable body weight throughout the period. Kombucha-treated groups at 25 mg/kg and 100 mg/kg had an upward trend in weight gain of about 50 % compared to the diabetic controls. The metformin, glibenclamide and the 5 mg/kg Kombucha-treated groups had relatively lower weight gains compared to those at 25 mg/kg and 100 mg/kg doses of Kombucha extract (Figure 4.5A). This is supported by the area under the curves of the various animal treatment groups (Figure 4.5B).

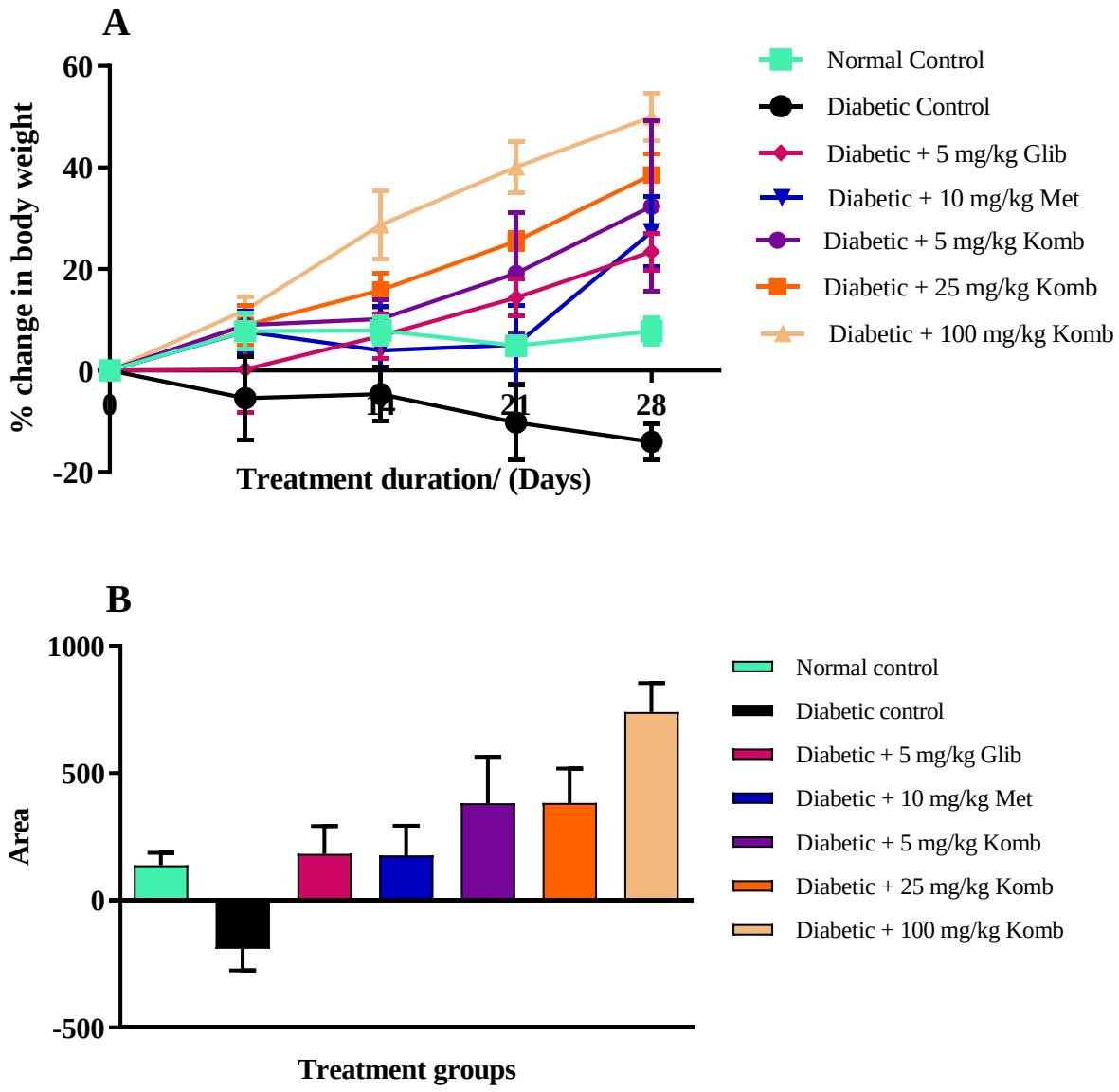

Figure 4.5: Effect of anti-diabetic treatments on body weight. *A) Relative changes in body weight during the treatment duration. B) Area under the curve for relative changes in body weight for the treatments. Graphpad prism 8.0 (Mean ± SEM (n=3) was used for the analysis). Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.*

4.2.3 Effects on Fasting Blood Glucose (FBG) Levels

The FBG of diabetic rats was about 5-fold that of normal control at baseline. There was a 5-fold significant reduction in the FBG levels to near normal control level at day 7 (from about 24 mmol/l to about 5 mmol/l) which remained relatively constant through day 28 in animals treated with 25 mg/kg and 100 mg/kg Kombucha. The normal control group had very minimal changes in basal FBG levels (4 mmol/l) over the study period whereas the diabetic control group had elevated levels in the range of 20-30 mmol/l. However, there were relatively significant reductions in FBG with metformin, glibenclamide and 5 mg/kg Kombucha-treated animals compared to diabetic control, albeit to a lesser degree (Figure 4.6A). This time-course data on FBG levels of the various animal treatment groups is supported by the areas under the curve (Figure 4.6B)

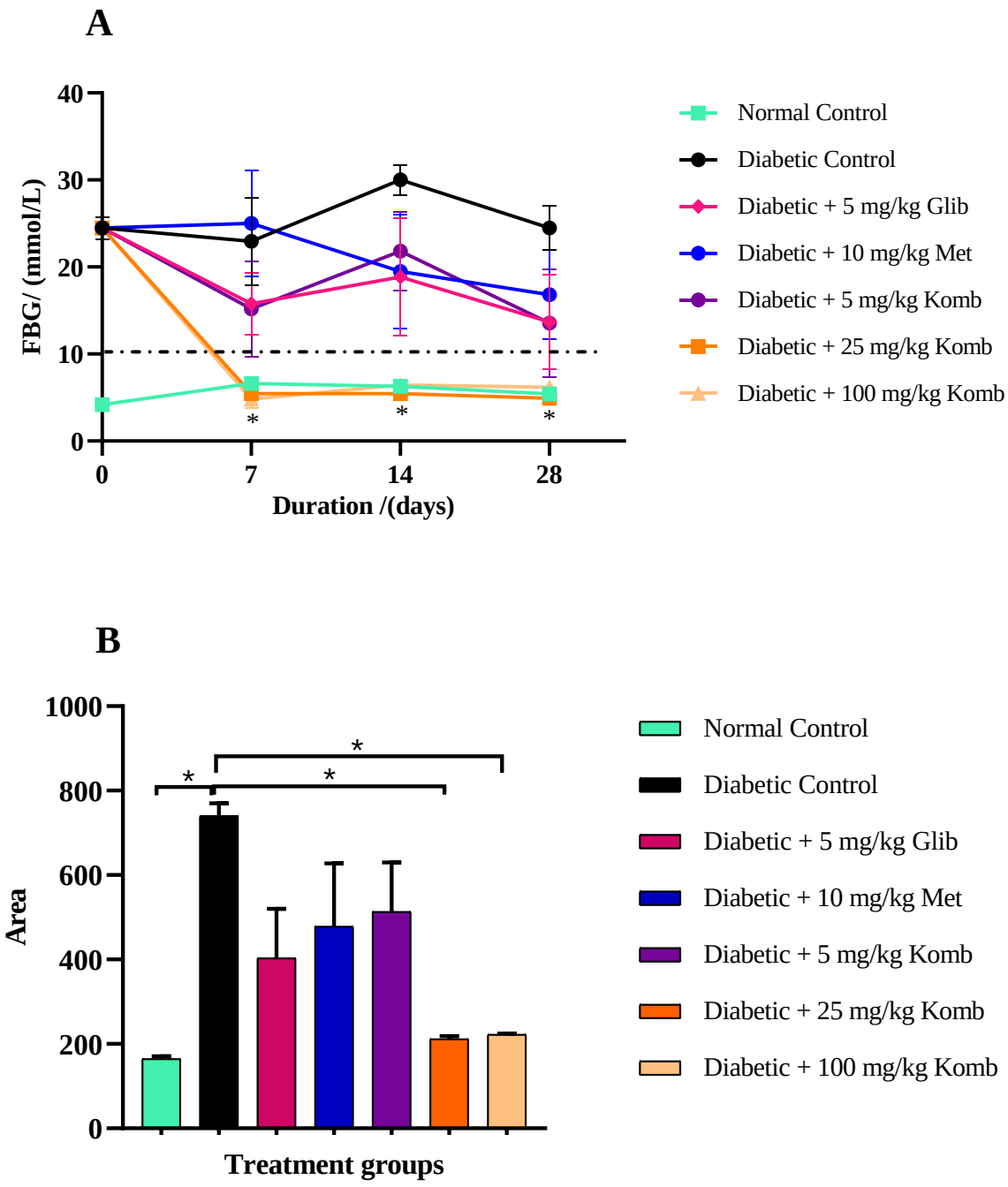

Figure 4.6: Effect of anti-diabetic treatments on fasting blood sugar levels. *A) Relative changes in fasting blood glucose levels during the treatment duration. B) Area under the curve showing relative changes in fasting blood glucose levels. ANOVA was used to compare the variations within the treatment groups using the Graphpad prism 8.0. (Mean ± SEM (n=3) was used for the analysis). Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.*

4.2.4 Effect on Serum Insulin Levels

Serum insulin levels in diabetic rats at day 7 were slightly lower than normal control level. However, beyond day 7 the serum insulin was significantly lower in diabetic rats than the normal control. Treatment of diabetic rats with metformin, glibenclamide and the 5 mg/kg Kombucha did not significantly change serum insulin levels over time except at day 14 where the serum insulin levels in the metformin-treated diabetic rats were significantly higher than diabetic control. However, diabetic animals treated with 25 and 100 mg/kg Kombucha showed gradual increase in serum insulin levels with time after day 7 (Figure 4.7A. These changes in serum insulin levels are reflected in the cumulative insulin secretion over time as represented by the area under the curve (Figure 4.7B).

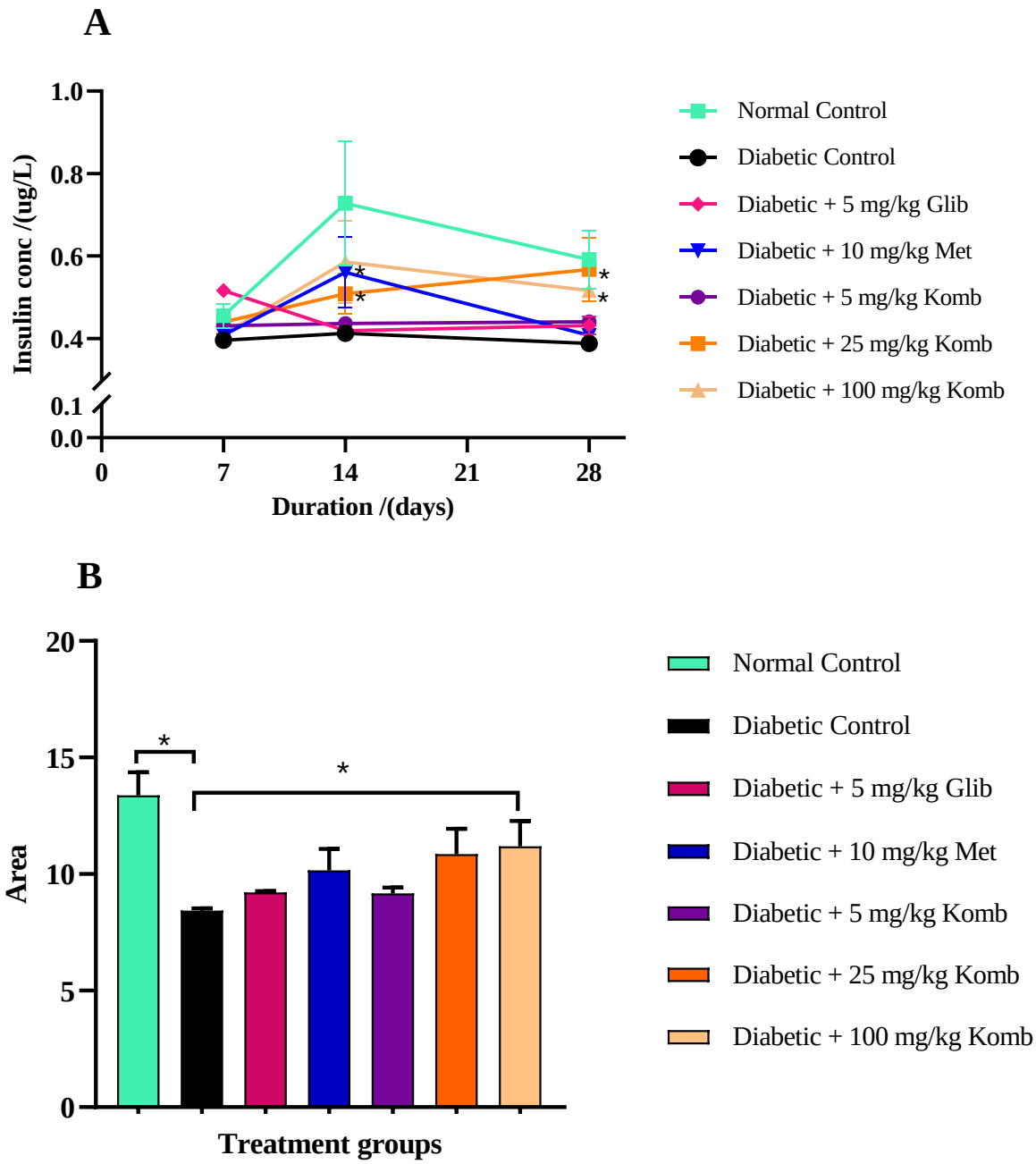

Figure 4.7: Effect of anti-diabetic treatments on serum insulin concentration. *A) Serum insulin concentrations for the treatments over 28 days duration. B) Area under the curve for serum insulin concentrations for the different treatments. ANOVA was used to compare the variations within the groups using the Graphpad prism 8.0. (Mean ± SEM (n=3)) was used for the analysis. Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.*

4.2.5 Effect on Morphology of Pancreas

Pancreas of normal control rat showed high cell density in several multifocal islet of Langerhans. Islets are present and conspicuous (having a large diameter) with no evidence of injury. The cells have hyperchromatic rounded to ovoid nucleus and eosinophilic cytoplasm (H&E x400) (Figure 4.8A). Pancreas of diabetic control demonstrated that islets of Langerhans were mostly not present and the cells of islets had been replaced by fibrous tissues (fibroblast) occurring in the foci that were previously occupied by islet. A few lobules of the pancreas had been invaded by fibrous tissue that replaced the exocrine pancreas. This lesion was initiated by pancreatic necrosis that triggers fibrosis to replace the necrotized tissue as seen in diabetic controls (Figure 4.8B). For the metformin-treated rats, the islet of Langerhans had fewer cells when compared with normal control and the cells occurred within a matrix of hyalinized fibrous connective tissue. The cells are smaller (atrophied) than in normal control rat group (Figure 4.8C). The pancreas of the glibenclamide-treated rats had islets of Langerhans which were small and not frequently visualized. Islets were scattered far and between and have low cell density and occur in a hyalinized connective tissue matrix. The islets were different compared to the normal control. Also, the cells within the islets of the glibenclamide-treated rats had small hyperchromatic nucleus and scanty eosinophilic cytoplasm than in rats of normal control (Figure 4.8D). The islets of the pancreas of the 5 mg/kg Kombucha-treated rats are prominent, numerous and had a high cell density. The histological changes are morphologically comparable to those in 25mg/kg Kombucha-treated rats (Figure 4.8E). The pancreas of rats treated with 25 mg/kg of Kombucha showed islets of Langerhans that were large, numerous and frequently observed. Furthermore, these histopathological changes were closely comparable to those in the pancreas of normal rats (Figure 4.8F). The pancreas of 100 mg/kg Kombucha-treated rats had islets of Langerhans that are frequent but much smaller than in normal control rats. The cells were viable with round to ovoid hyperchromatic nucleus surrounded

by eosinophilic cytoplasm. The cell morphology was similar, but cell density was slightly less than for the normal control (Figure 4.8G). The summary of the histological examination of the pancreas of various animal treatment groups is reported below (Table 4.1).

Table 4.1: Summary for histological examination of the pancreas

Treatment Group	Largest Dimension / µm	Shortest Dimension / µm	Remarks
Normal control	76.67	70.68	Many prominent islets that have histologically normal viable cells
Diabetic control	33.28	17.79	No islet seen, only one site that is a remnant of islet. No islet cells present. They have been replaced by fibrous connective tissue cells (fibroblasts). The measurements do not represent an islet but the fibrous tissue laden lesion
Diabetic + 10 mg/kg Metformin	45.18	33.66	Islets are few and not prominent, they occur as small islands located far and between. The cells are viable.
Diabetic + 5 mg/kg Glibenclamide	34.18	28.93	Islets are small and few, cells are viable
Diabetic + 5 mg/kg Kombucha	80.00	49.54	Many, prominent (large) islets of varying sizes.
Diabetic + 25 mg/kg Kombucha	97.79	59.52	Many prominent islets that have viable cells
Diabetic + 100 mg/kg Kombucha	29.06	27.90	Small scattered islets that have viable cells

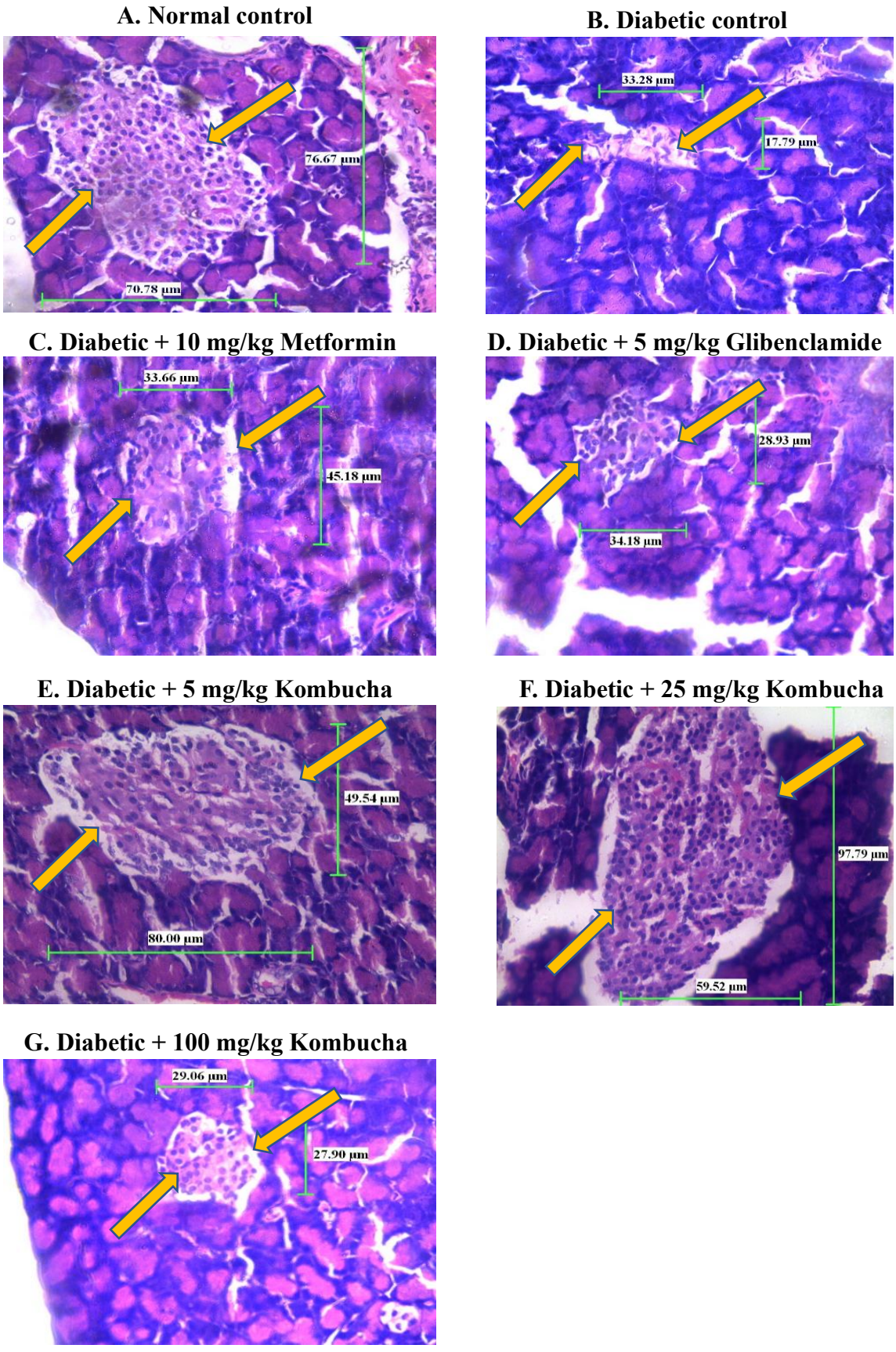

Figure 4.8: Hematoxylin-eosin (H&E) staining of the pancreas. *The arrows point to the islets of Langerhans and the cells within the islets. Images were taken at a magnification of 400x.*

4.2.6 Effects on Serum Lipid Profiles

Diabetic rats treated with Kombucha, glibenclamide and metformin caused insignificant increases (10-16 %) in serum total cholesterol, HDL and LDL levels (Figures 4.9 A-C). However, only glibenclamide caused a significant reduction (17 %) in serum triglyceride level when compared to the diabetic control (Figure 4.9 D), which represented a return to normal control level.

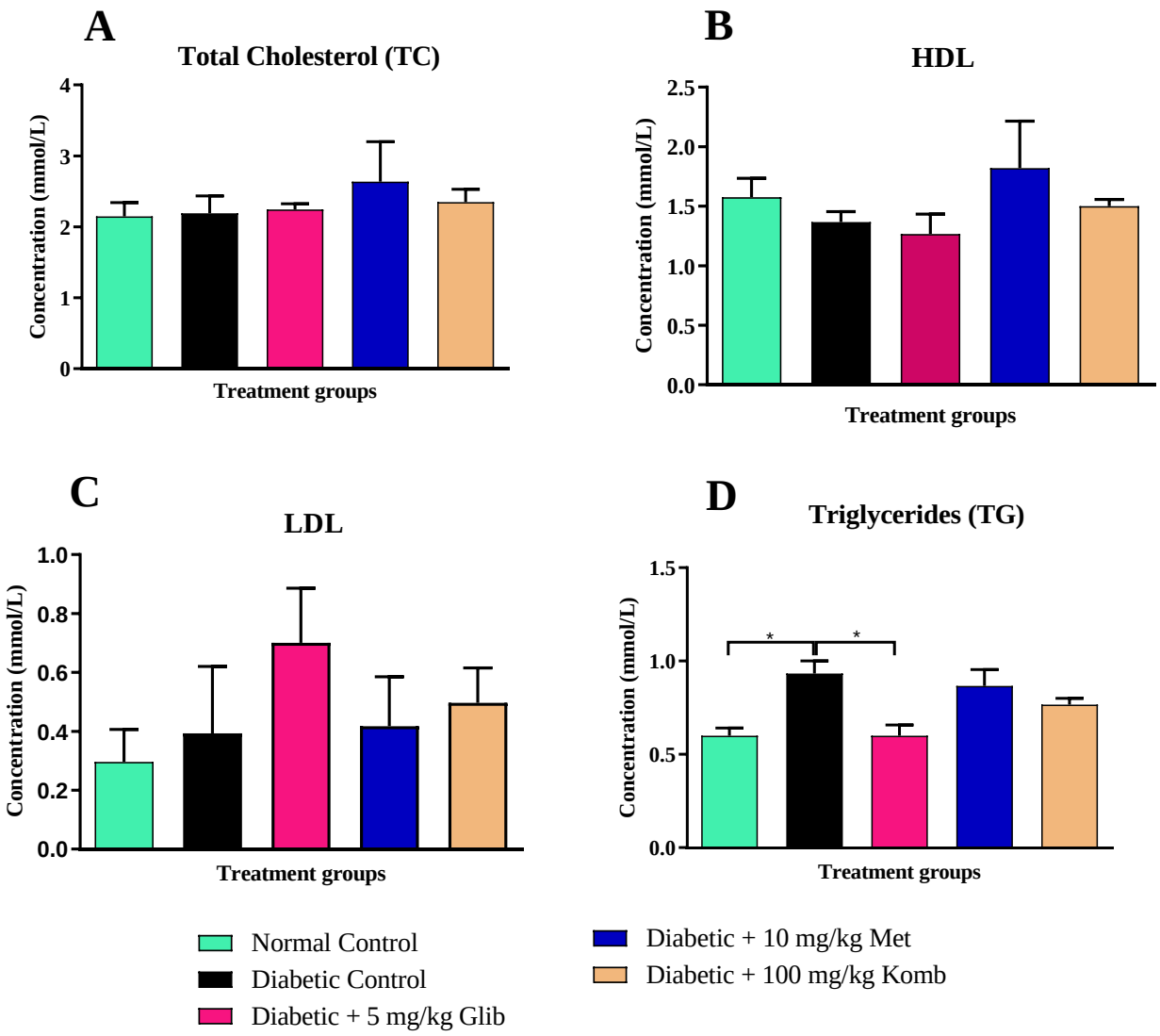

Figure 4.9: Serum lipid profile. *A) Total cholesterol (TC), B) High density lipoprotein (HDL), C) Low density lipoprotein (LDL) D) Triglycerides (TG). ANOVA was used to compare the variations within the groups using the Graphpad prism 8. (Mean ± SEM (n=3) was used for the analysis). Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.*

4.3 SAFETY EVALUATION OF KOMBUCHA IN DIABETIC RATS

4.3.1 Mean Organ /Body Weight Ratio

Induction of the diabetic state in rats treated with alloxan caused significant ($p < 0.05$) increases in mean organ weight/body weight ratios of the liver and kidney, expressed as a percentage, which was reversed to near normal control value by treatment with glibenclamide, metformin and 100 mg/kg Kombucha (Figure 4.10 A & B). However, the induction of the diabetic state did not affect the mean organ weight/body weight ratios of the pancreas and diaphragm, which was also unaffected by treatment with glibenclamide, metformin and Kombucha (Figure 4.10 C & D).

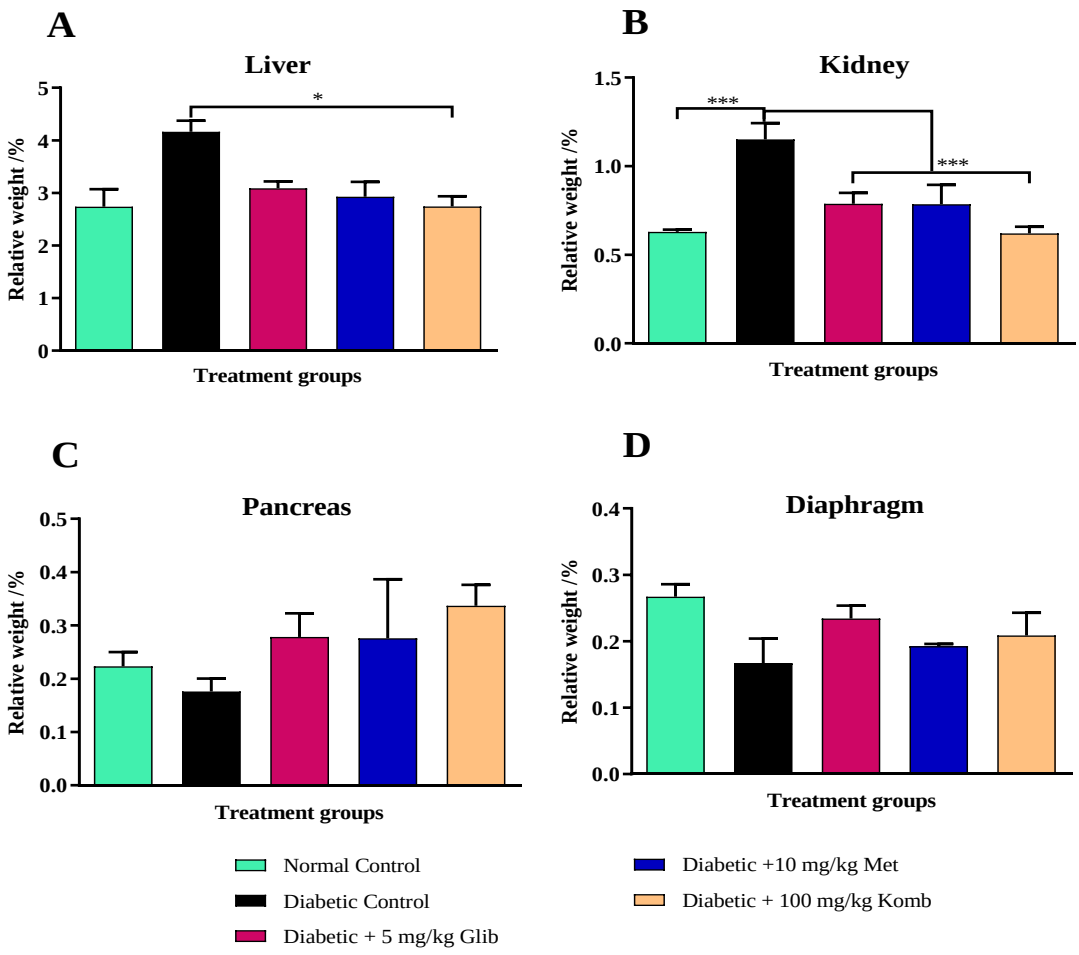

Figure 4.10: Relative organ weight to body weight ratio. *ANOVA was used for statistical comparison within groups using the Graphpad prism 8. (Mean ± SEM (n=3) for the analysis). Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.*

4.3.2 Serum Indices of Hepatotoxicity

Serum indices of liver toxicity (AST, ALP and ALT) in alloxan-induced diabetic rats were significantly ($p < 0.05$) higher than the normal control (figure 4.11 A-C). These indices were significantly reduced to near normal control levels by treatment of the diabetic rats with 100 mg/kg Kombucha, metformin and glibenclamide.

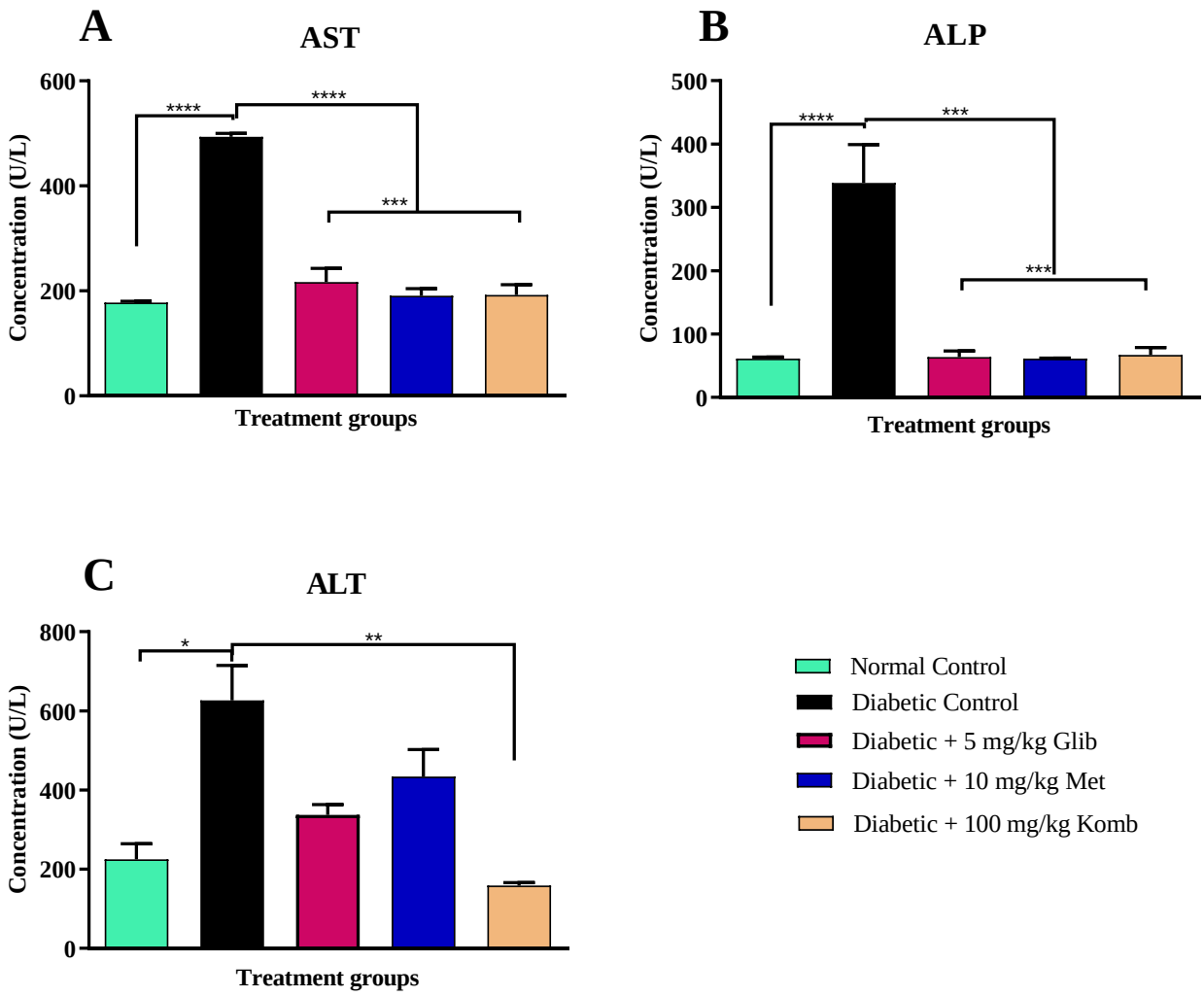

Figure 4.11: Liver function test. *A) AST, B) ALP, C) ALT. ANOVA was used to compare the variations within the groups using the Graphpad prism 6. (Mean ± SEM (n=3) was used for the analysis. Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.*

4.3.3 Morphological Changes in Liver

Liver of normal rat showed normal columns of hepatocytes around a central vein of hepatic lobule. The organ architecture and cellular morphology of the hepatocytes were not pathologically affected (Figure 4.12 A). Similar observations were made for diabetic rats treated with Kombucha, metformin and glibenclamide (Figure 4.12 A, C-E). However, the diabetic control rats showed sharp demarcations of necrotic zones from viable hepatocytes and a slightly pathologically affected tissue (Figure 4.12 B).

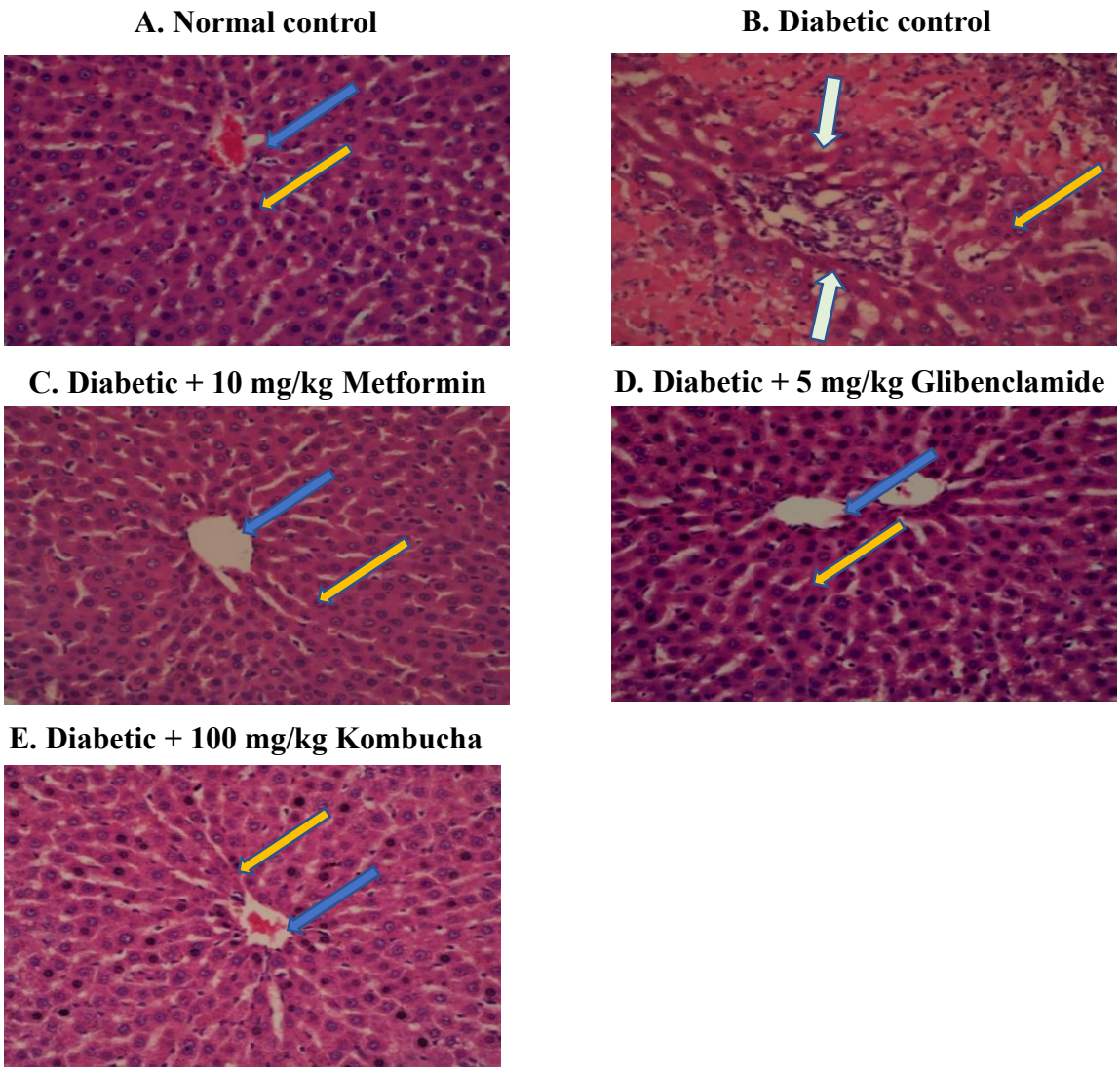

Figure 4.12: Hematoxylin-eosin (H&E) staining of the pancreas. *Blue arrows point towards the central vein, yellow arrows point towards the hepatocytes and gray arrows point towards necrotic zones. Pictures were taken at a magnification of 400x.*

4.3.4 Serum Indices of Renal Toxicity

There was a significant elevation of serum urea concentration in diabetic animals compared to normal control. This was significantly reduced by treatment with Kombucha and glibenclamide treatment ($p<0.05$). However, treatment of diabetic rats with metformin insignificantly reduced serum urea levels (Figure 4.13 A).

Serum creatinine level in diabetic animals was not statistically different from the normal control ($p>0.05$) and treatment of diabetic rats with glibenclamide, metformin and Kombucha did not significantly change this outlook (Figure 4.13 B).

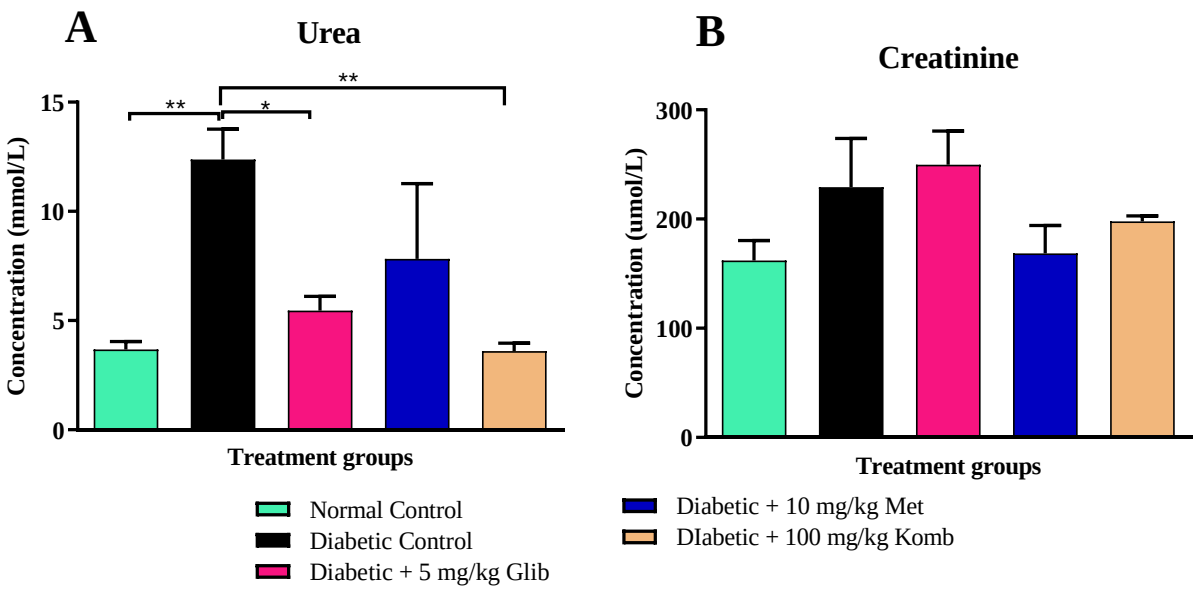

Figure 4.13: Renal function tests for levels of serum. *A) Creatinine B) Urea. ANOVA was used to compare the variations within the groups using the Graphpad prism 8. (Mean ± SEM (n=3) was used for analysis) Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.*

4.3.5 Morphological Changes in the Kidney

Kidney of normal control rats showed no pathological changes to indicate an injury to the organ evidenced by normal architecture of kidney tubules lined by normal cuboidal epithelium, interstitial tissues and glomeruli. Similar observations were made for kidneys obtained from untreated and treated diabetic animals (Figure 4.14).

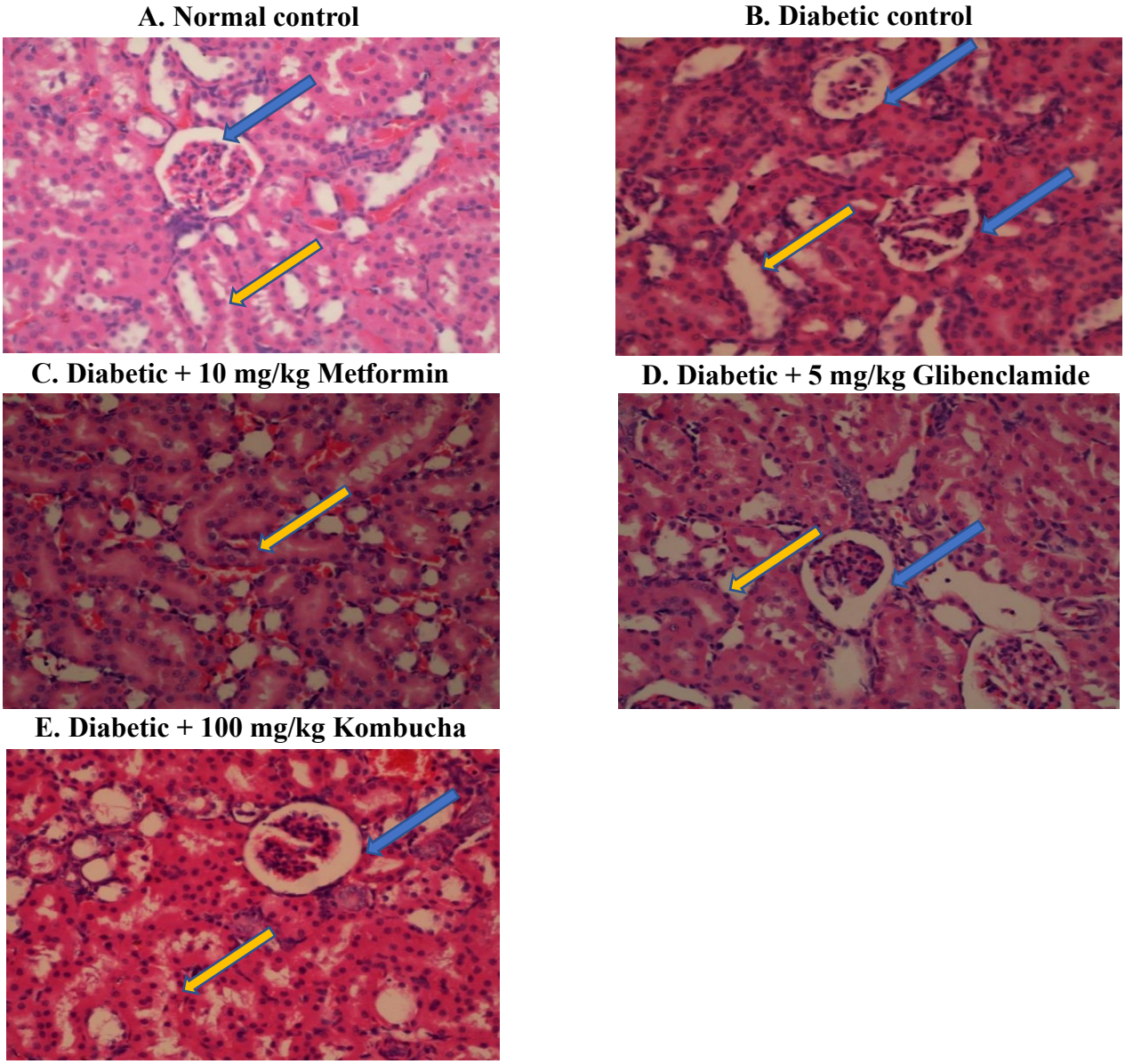

Figure 4.14: Hematoxylin-eosin (H&E) staining of the kidney. *Blue arrows point towards the glomeruli, yellow arrows point towards the cuboidal epithelium lining. Pictures were taken at a magnification of 400x.*

4.3.6 Urinalysis

Marked reductions in glucose level in urine were observed for the diabetic rats treated with 25 mg/kg and 100 mg/kg Kombucha. Glucose recovered from urine was in trace amounts compared to the high levels recorded in the diabetic control. Metformin and glibenclamide were not effective at reducing blood glucose levels in urine. Also, there was a 3-fold significant reduction ($p < 0.05$) in overnight urine volume (from about 35 to 12 ml) in 25 mg/kg and 100 mg/kg Kombucha-treated rats to near normal control level (10 ml) (Table 4.4).

Table 4.2: Urinalysis of samples from animal treatment groups

Treatment groups	Glucose	Protein	Specific Gravity	Ketone	Ave. Urine Vol /ml[a]
Normal Control	-	++++	1.025	-	10 ± 3*
Diabetic Control	++++	-	1.020	-	35 ± 5*
Diabetic + 5 mg/kg Glib	++++	+++	1.025	-	27 ± 4**
Diabetic + 10 mg/kg Met	+++	+++	1.020	-	24 ± 2.5**
Diabetic + 5 mg/kg Komb	++++	± (trace)	1.015	-	25 ± 4**
Diabetic + 25 mg/kg Komb	± (trace)	+++	1.015	-	14 ± 2**
Diabetic + 100 mg/kg Komb	-	++++	1.015	-	12 ± 3**

[a]Results are Means ± SEM (n=3). Glib = Glibenclamide; Met = Metformin; Komb = Kombucha.
**Value significantly different from normal control; $p < 0.05$.*
***Value significantly different from diabetic control; $p < 0.05$.*

4.5 16S ION TORRENT METAGENOMIC ANALYSIS OF MICROBIOME CHANGES IN DIABETIC ANIMALS

4.5.1 16S rRNA Gene Amplification from Stool DNA

Using primers targeting the hypervariable V2_4_6 regions of the 16 S rRNA gene of bacteria. The amplification was successful for all samples used except for the negative control and PBS representing the extraction control. The expected molecular weight of approximately 275 bp was obtained (Figure 4.15).

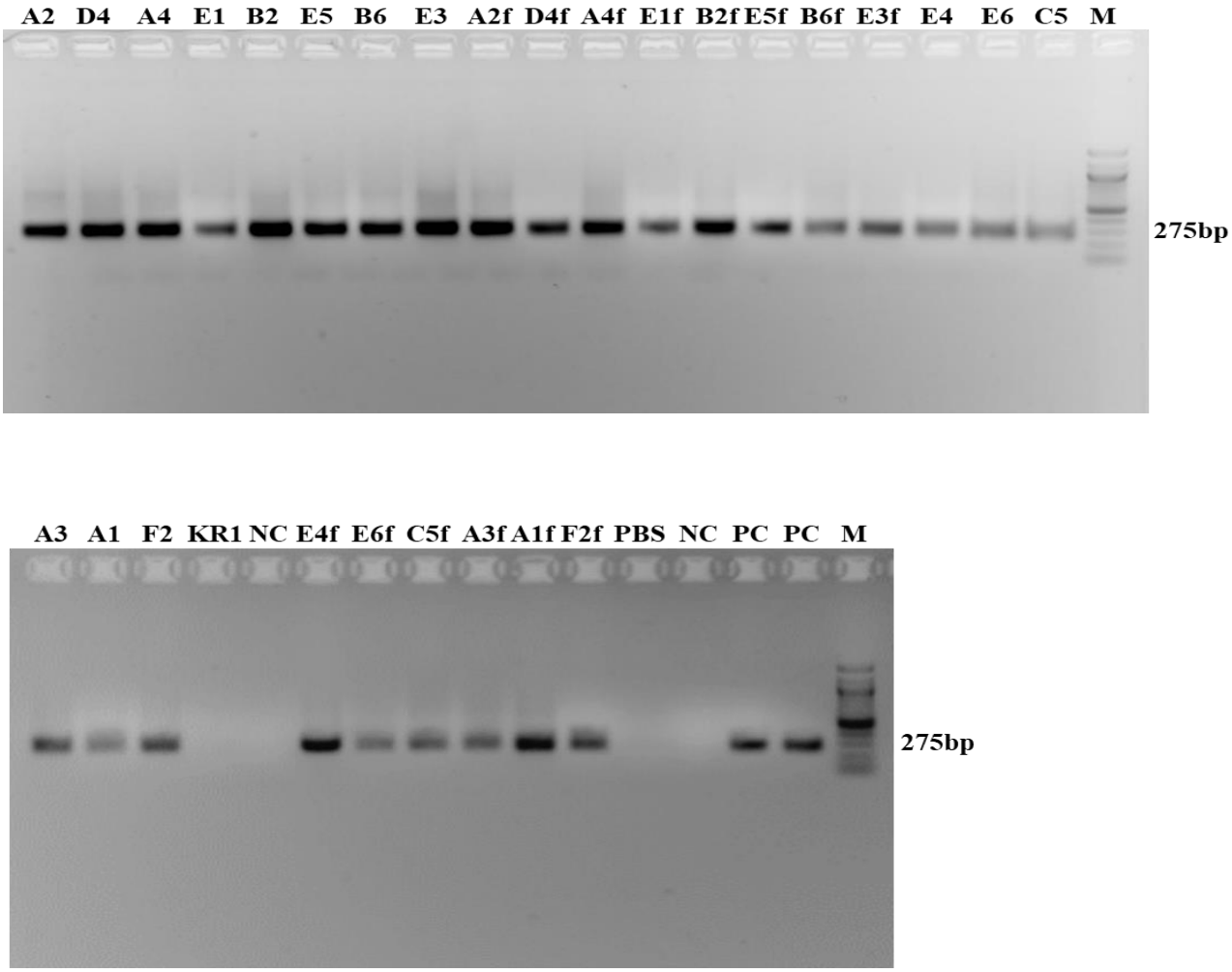

Figure 4.15: Amplification of 16S rRNA gene of bacteria targeting the V2_4_6 hypervariable region for species identification. *PC denotes positive control, NC denotes negative control, M demotes molecular weight marker and all other viable denotes samples.*

4.5.2 Changes in Rat Gut Microbiome Composition

4.5.2.1 Phyla level variation in the gut microbiome before and after treatment

The most predominant bacterial phyla in all treatment groups including the diabetic and normal control groups are the *Firmicutes*. There was a reduction in the microbial population of the *Firmicutes* in the diabetic control group over the treatment period. However, after the treatment period with Kombucha, there was an enrichment in the level of *Firmicutes* population. *Actinobacteria* were also observed to have increased in diabetic animals and the metformin-treated group and almost lost in the 100 mg/kg Kombucha-treated diabetic group. Phyla *Bacteriodetes* were represented throughout the treatment period. Furthermore, high levels of unclassified bacteria phyla were seen in the diabetic groups compared to the other groups. *Verrucomicrobia*, *Chlamydiae*, *Spirochaetes*, *Tenericutes* and *Chloroflexi* were other phyla that were present in the gut microbial composition of the rats (Figure 4.16).

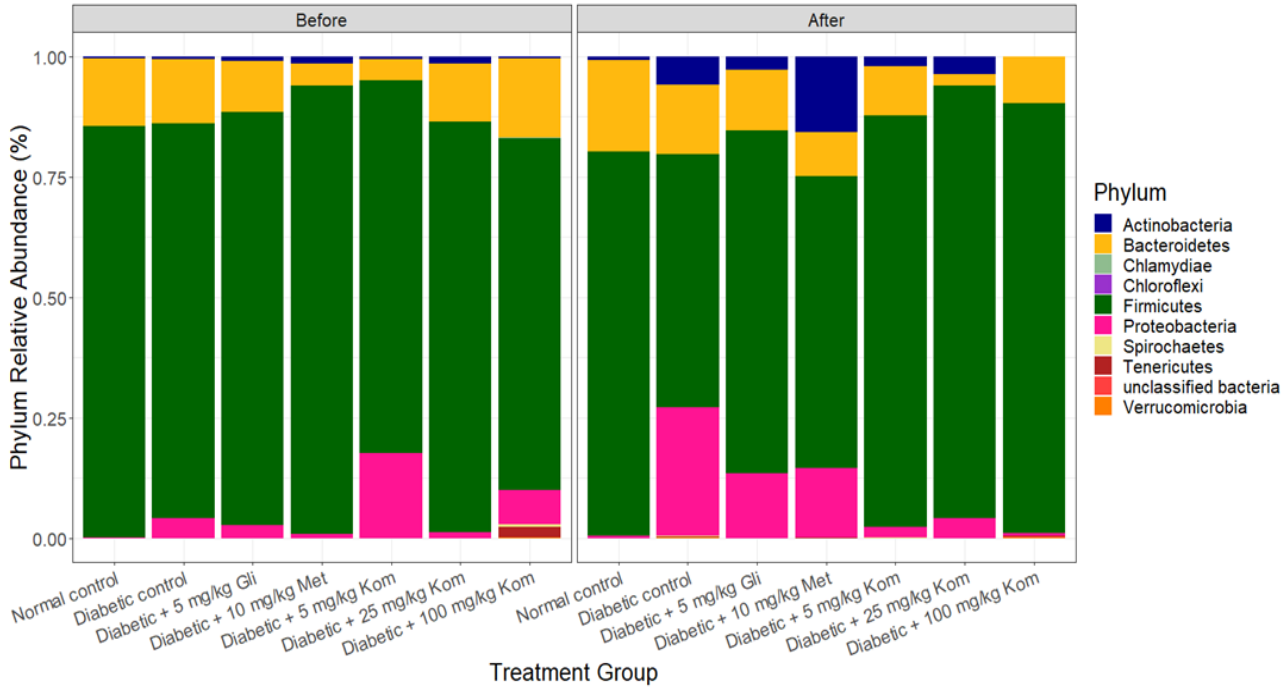

Figure 4.16: Phyla level changes in gut microbiome structure of diabetic rats before and after receiving different anti-diabetic treatments.

4.5.2.2 Family level variation in the gut microbiome before and after treatment

Lactobacillaceae were the most abundant family within the gut microbial community. Their composition however, declined drastically in the diabetic control after the treatment duration of the experiment with a concomitant increase in the number of *Enterobacrteriaceae* thus enhancing the diversity of the microbial community in the gut. However, as seen in the normal control and the Kombucha treated, *Lactobacillaceae* population were enhanced and sustained after treatment. *Syntrophaceae* was present in all diabetic animals after treatment but in higher abundance in diabetic control compared to the other treatment groups. Microbial diversity was reduced in 100 mg/kg Kombucha-treated rats after treatment with subsequent enrichment of *Lactobaccillaceae* comparable with the normal health controls. *Moraxellaceae* were also found to be present in only diabetic control and metformin-treated groups after treatment but not before treatment (Figure 4.17).

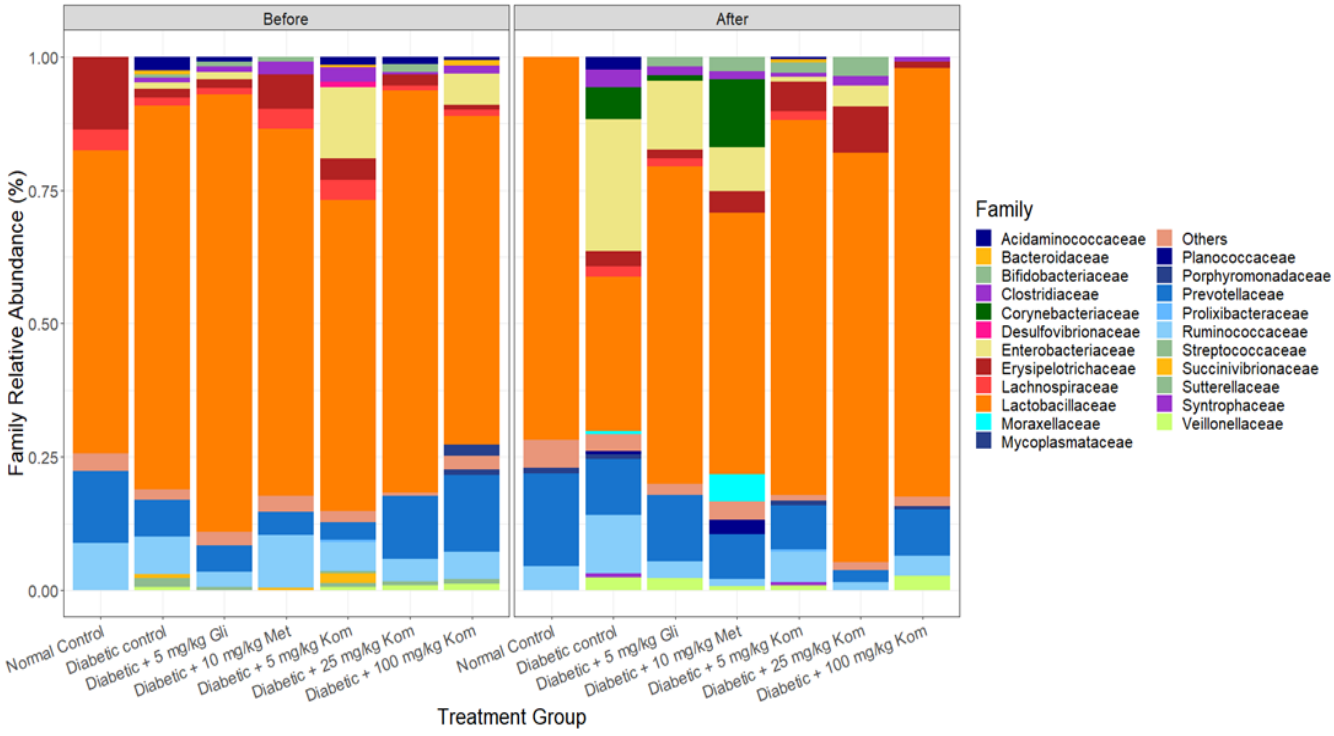

Figure 4.17: Family level changes in gut microbiome structure of diabetic rats before and after receiving different anti-diabetic treatments.

4.5.3 Gut Microbiome Diversity Before and after Treatment

4.5.3.1 Chao 1 index for microbial diversity analysis

Estimation of the number of species in the microbial community of the gut with the Choa1 index showed no statistical variation over the treatment period for normal controls, diabetic controls, and diabetic animals treated with metformin and 100 mg/kg Kombucha ($p>0.05$). A significant reduction in the number of species found within the microbiome composition of the glibenclamide and 5 mg/kg Kombucha treatment groups ($p<0.05$) were comparable before and after treatment. Twenty-five milligram per kilogram (25 mg/kg) Kombucha treatment showed a relative increase in the species population dynamics before and after treatment (Figure 4.18).

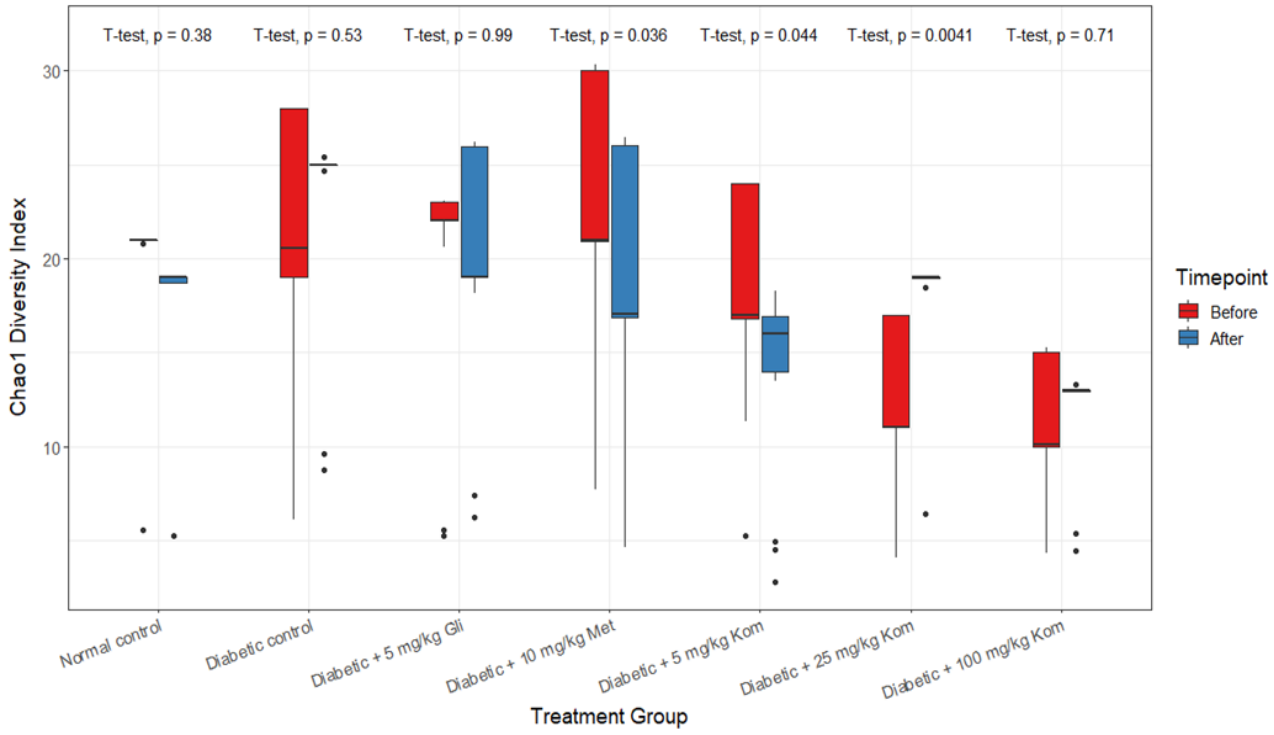

Figure 4.18: Chao 1 index for comparison of the gut microbial diversity observed before and after various anti-diabetic treatments. *T-test was used to compare the variations within the before and after treatment groups at 95 % confidence interval.*

4.5.3.2 Observed species richness for microbial diversity analysis

Similar trends were observed for the relative richness in species found within the gut microbiome of the diabetic and healthy rats. However, there was a significant reduction in the richness of the microbiome in the control group. Even though there was a rise in the richness seen in the diabetic control this was not statistically significant over the treatment duration (Figure 4.19).

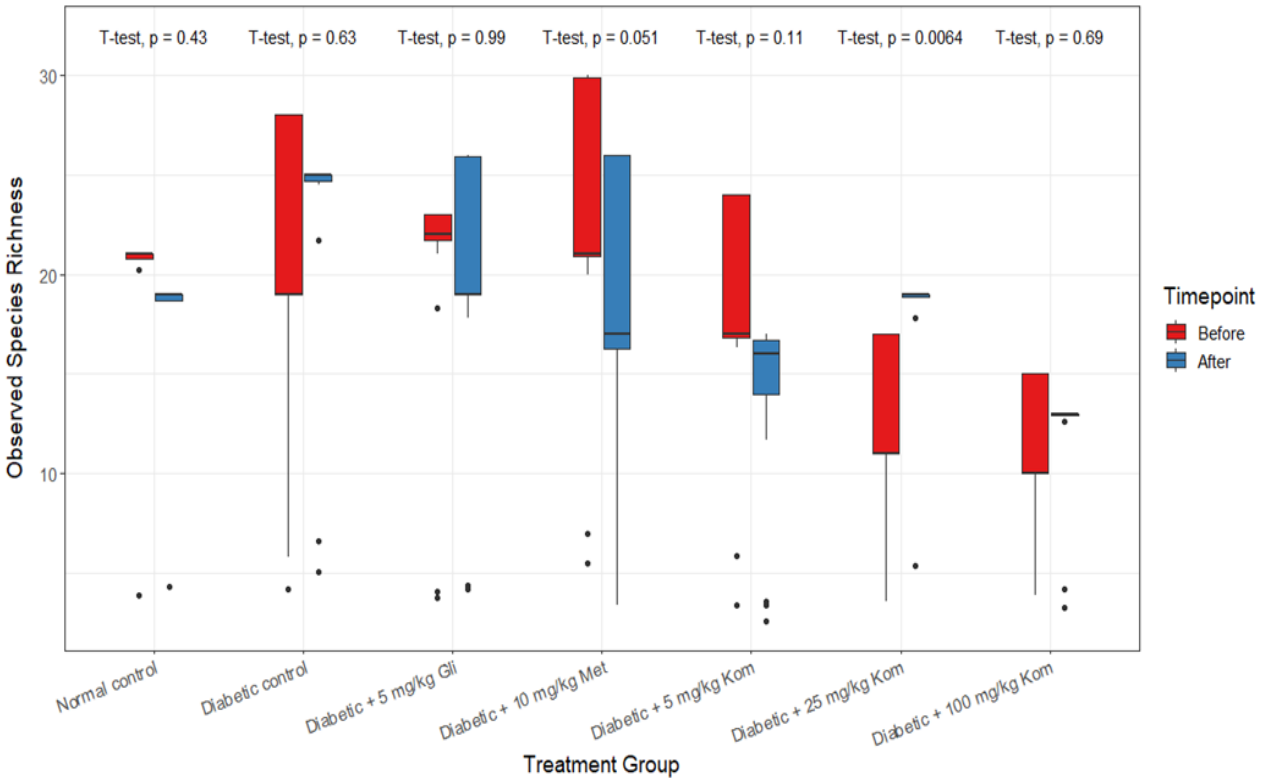

Figure 4.19: Observed species richness comparison of the gut microbial diversity observed before and after various anti-diabetic treatments. *T-test was used to compare the variations within the before and after treatment groups at 95 % confidence interval.*

4.5.4 Alpha Diversity within the Gut Microbiome

4.5.4.1 Shannon diversity index for microbial diversity analysis

The gut microbiome diversity estimated by the Shannon diversity index showed that there were significant variations observed in the gut microbiota of all the various groups of animals before and after the treatment period except for the normal control and the glibeclamide-treated groups ($p>0.05$). Shannon diversity index was seen to be increased in the diabetic control as compared before and after the experimental duration. However, a decrease Shannon index was estimated for the metformin- and Kombucha-treated groups before and after treatment ($p<0.05$). A higher Shannon diversity index was observed after treatment for 25 mg/kg Kombucha than before treatment (Figure 4.20).

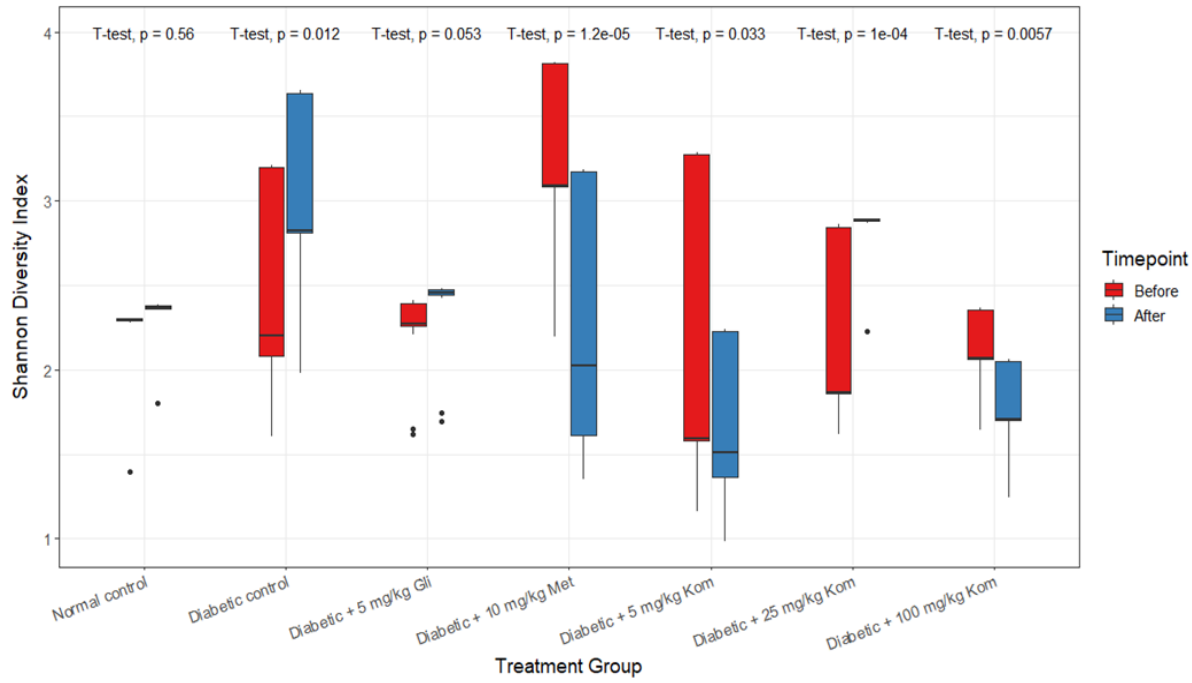

Figure 4.20: Comparison of the microbial diversity before and after treatment using the Shannon index. Shannon diversity index—estimator of species richness. T-test was used to compare the variations within the before and after treatment groups at 95 % confidence interval.

4.5.4.2 Simpson diversity index for microbial diversity analysis

Similar trends shown by the Shannon index was observed by the Simpson diversity index. A significant increase in evenness was shown by a high Simpson diversity index value after treatment for normal control, diabetic control, glibenclamide-treated and 25 mg/kg Kombucha-treated groups ($p<0.05$). Even though the Simpson diversity index was low in 5 mg/kg Kombucha group the variation was statistically significant (Figure 4.21).

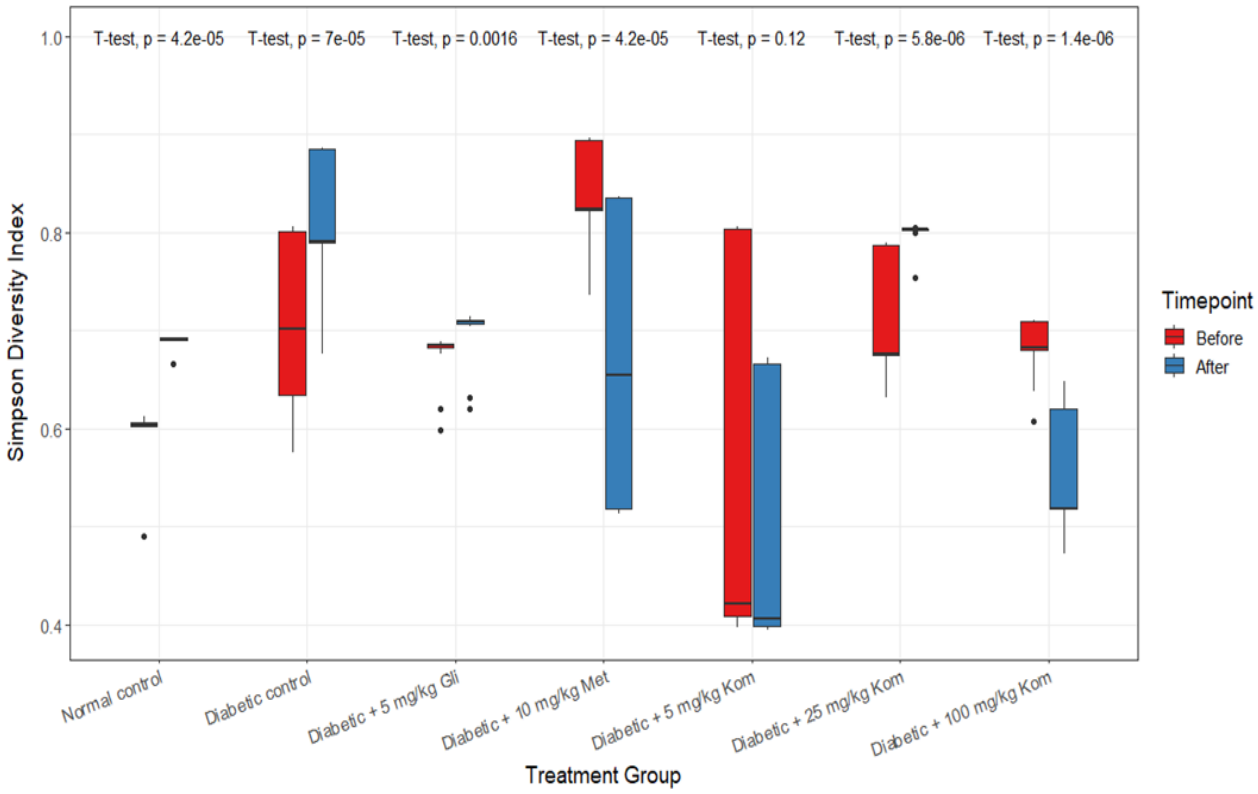

Figure 4.21: Comparison of the microbial diversity before and after treatment using the Simpson index. *Simpson diversity index—estimator of species evenness. T-test was used to compare the variations within the before and after treatment groups at 95 % confidence interval.*

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The global incidence of diabetes is increasing alarmingly and there is, therefore, an urgent need to explore novel and innovative intervention strategies (WHO, 2108). Current treatment therapies of diabetes have been successful in providing a solution to the hyperglycemic condition in diabetes patients. However, there have been some reported limitations to treatment such as costs, systemic acidosis and weight loss (Li, 2011). Several studies have sought to use animal models to validate the potential of newly discovered anti-diabetic agents for future therapies. Despite the success and suitability of diabetic animal models in the study of this disease, it does not holistically represent what happens in humans (Xiao et al., 2010). There is an urgent need to explore Kombucha which has been anecdotally reported to possess anti-diabetic properties experimentally to provide sufficient scientific evidence to support these claims. Kombucha has an enriched complexity of nutraceuticals and probiotics which might be very essential in its anti-diabetic potential, safety evaluation and its impact on the gut microbiome.

Probiotics found within fermented beverages play an essential role in conferring health benefits to the consumers. However, identifying the exact microbial probiotics species from a plethora of organisms within a probiotic beverage or product has been a challenge for application in therapies. Metabarcoding of Kombucha as reported by Reva *et al.* (2015), has shed some insights into the microbial community present within the Kombucha microenvironment. Understanding this poly-microbial diversity requires the use of advance sequencing technology that can overcome the challenges of culturing and obsolete sequencing technologies.

Recent focus on shotgun metagenomics has provided the ability to identify the roles and functions of microbial communities. In the present study, Kombucha's microbial community has been shown to be enriched with high abundance of yeast species specifically *Brettanomyces bruxellensis CBS 2499* and *Brettanomyces anomalus* (Figure 4.1). These yeasts species have been reported to play essential roles such as converting the sugar substrates to alcohols in the wine fermentation and processing (Oelofse *et al*., 2009). Also, several other species of yeast such as *Saccharomyces*, *Candida parapsilosis*, *Pichiae* and other sub-dominant taxa were also identified to be present in Kombucha fermentation (Appendix 4 and 5). These organisms have been reported to play critical role in the fermentation of cider and beer as they produce sub-levels of phenols that contribute to the flavor and odour of the final products. Yet not all organisms identified are key players in fermentation (Buron *et al.*, 2011, Buron *et al*., 2012; Reva *et al*., 2015; Smith and Divol, 2016).

Furthermore, the yeast present in the Kombucha seem to be very important fermentation agents and as such produce low levels of ethanol which are further converted to organic acids by the *Acetobacteraceae* family of bacteria present within the microbial community (Marsh *et al*., 2014). Natural fermentation processes are driven by indigenous microbial communities present within the microenvironment of most fermenting cultures which form biofilms under aerobic conditions. The communities are similar to those found in other fermented beverages but differ in microbial richness and diversity due to the differences in the production channel and methodologies (Coton *et al.*, 2006).

The bacterial diversity within Kombucha has been shown to be under-represented, however, their critical role in the fermentation process is observed in its ability to convert the ethanol produced to organic acids. Acetobacter family of bacteria involving *Gluconacetobacter spp* (refer to

Appendix, 6), *Komagataeibacter, spp*. *Serratia symbiiotica* and many others (Figure 4.2) were observed as was also demonstrated by Reva *et al.* (2016). Kombucha microbiome is a typical mutualistic metabolic cooperation of yeast and bacteria (Teoh *et al*., 2004; Marsh *et al*., 2014).

The REFSEQ database revealed more microbial diversity in Kombucha than the OCXDB. All these probiotics and organisms identified seem to play important roles in maintaining and sustaining the Kombucha microbial community as demonstrated by the fact that some organisms within the microbial community are not able to live all by themselves because they require the presence of others within the environment to survive. However, one limitation to this DNA based techniques is the fact that not all organisms observed are alive and active in the culture. Further analysis on the functional roles of these organisms is required to be able to explicitly define the role they play in the community. Next generation sequencing has provided researchers an extensive platform to study complex microbial communities and to gain insight into the functions and roles of these microorganisms in their environment.

In recent times, global focus has shifted from the energy providing roles of food and beverages to the exploration and re-purposing as nutraceuticals with several clinical applications. Kombucha can be very critical in this respect with its potential anti-diabetic characteristics as well as playing an essential role in ensuring that gut health is maintained and enriched due to the combinatorial effects of the probiotics and nutraceuticals present in it. Kombucha could be used as an alternative substitute for people with lactose intolerance as a source of probiotics in place of diary based fermented foods which is very essential in maintainance of the gut health (Prado *et al*., 2008; Vasudha and Mishra, 2013; Walter *et al*., 2018) as well as in anti-diabetic management therapy.

Administration of 25 mg/kg and 100 mg/kg of Kombucha demonstrated about a 5 fold reduction in the fasting blood glucose (FBG) levels in the diabetic animals which was a better response

compared to the effect of metformin and glibenclamide (Figure 4.6). Metformin is an anti-hyperglycemic agent which works by reducing insulin resistance by a mechanism of enhancing the sensitivity of the liver cells and other essential peripheral tissues as the muscles to insulin without having any effect on stimulating insulin secretion and glucose uptake (Ali *et al.*, 2017). The use of alloxan to induce diabetes is targeted to destroy the beta cells of the pancreas (Radenkovic *et al.*, 2016), this resulted in little or no insulin production hence the ineffectiveness of the standard drugs used since metformin could not significantly reduce the blood glucose, which was similar to what was observed for glibenclamide (Figure 4.6).

Kombucha has been reported to be enriched with probiotics such as *Lactobacillaceae* and nutraceuticals such as polyphenolics, saponins, tannins, organic acids and other essential bioactive compounds. These compounds have been linked essentially to the medicinal claims and good antioxidant activity associated with Kombucha (Madhuri and Naik, 2017; Samarghandian *et al*., 2017). The rich nutraceutical and antioxidant compound in Kombucha could be responsible for the reduction in blood glucose levels by enhancing cellular glucose metabolism and uptake. Srihari *et al* (2013) have suggested based on a similar study that, the antihyperglycemic ability of Kombucha could be attributable to its ability to modulate the immune system with concomitant decrease in β-cell damage. The ability of Kombucha to stimulate insulin production (Figure 4.7) is very critical for the demonstration of this blood glucose lowering effect. The potential mechanism involved could be that Kombucha treatment of diabetic rats causes insulin secretion which in turn boosts the body's ability to effectively utilize glucose by affecting glycolysis, glycogenesis and lipogenesis. These insulin dependent mechanisms play key roles in glucose regulation within the body as well as regulating lipid profiles (Ali *et al.*, 2017). Also, anti-oxidants and probiotics found within Kombucha have been shown to inhibit glucose absorption in the small intestine thereby reducing glucose export via the glucose transporter 5 (GLUT 5), glucose

transporter 2 (GLUT 2) and sodium glucose transport protein 1 causing an overall lowering of glucose that ends up in the blood (Kwon *et al.*, 2007). In addition, probiotics in the gut microbiome have been reported to produce short chain fatty acids and other organic acids such as acetate which are important for the maintenance of colonocyte homeostasis. Dysbiosis in these microbial flora within the gut has been linked with obesity, Inflammatory Bowel Disease (IBD) as well as type 2 diabetes (Qin *et al*, 2012; Brestoff and Artis, 2013; Kostic *et al.*, 2014; Vital *et al.*, 2014). This suggests that Kombucha in its totality plays a functional role in diabetes management especially with regards to anti-hyperglycemic effect. However, it is unclear whether the effect observed is due solely to either the probiotic or the nutraceutical constituents within the broth or a combination of the two key elements. Kombucha treatment demonstrated anti-triglyceridemic effect in diabetic rats (Figure 4.9), which could be due to the fact that the phenolic compounds enhanced lipid metabolism through increased excretion of bile acid and decrease in cholesterol absorption by mopping them up as they move through the brush border membranes. Furthermore, it could also enhance the activity of lecithin cholesterol acyl transferase (LCAT), an enzyme which converts cholesterol to cholesterol esters— critical in lipid metabolism (Zern and Fernandez, 2005; Rahimi-Madiseh *et al*., 2017). The pancreas regeneration effects demonstrated by Kombucha (Figure 4.8) could be due to the anti-oxidant effect of Kombucha, which is responsible for the protection and repair of the pancreatic cells thus enhancing insulin secretion, it could also be due to the contribution of the probiotics in complementing the action of anti-oxidants by reducing gut inflammation. Studies by Ofori *et al.* (2014) reported the presence of phenolics and flavonoids which are good anti-oxidants in Kombucha. This entire mechanism is not fully understood hence future research could explore understanding of the exact mechanism (Zubaidah *et al*, 2019). The action of probiotics in stabilizing and enriching the gut microflora of diabetic rats undergoing treatment with Kombucha could also be a critical player in the observed pancreatic regeneration

as well as the protection of liver and kidney cells from damage (Figures 4.12 & 4.13). Metabolites from probiotic actions as well as the nutraceuticals within Kombucha may have been acting synergistically to enhance levels of insulin secretion and relief of oxidative stress within the body and offer these cellular level protections to these important organs.

Kombucha treatment demonstrated the ability to reverse the high levels of serum ALT, AST and ALP, indices of liver toxicity, and the high levels of serum creatinine and urea, as indices of renal toxicity, in the alloxan-induced diabetic rats and further confirms its protection of these two organs against cellular injury in the diabetic animals. Evidence from this study shows that signs of liver damage may be due to the elevated amount of the liver enzymes in serum. Toxic substances such as alloxan are known to induce a lot of free oxidative radical species that destroys the liver as well as the kidneys (Cruz *et al.*, 2005; Gupta and Misra, 2006). Toxicity to these organs may result in marked increases in serum ALT, ALP, AST, creatinine and urea levels (Figures 4.11 & 4.13). Histology of the liver in the diabetic control showed clear zones of necrosis which is indicative of liver damage (Figure 4.12). This damage could be associated with oxidative stress within the system resulting from excessive production of oxyradicals by alloxan (Feng *et al., 2010)*. The ability of Kombucha to protect the liver and the kidneys from this oxidative stress could be associated with its rich anti-oxidant constituents which mop up the reactive oxygen species. Substances that tend to possess hepatoprotective and renal protective abilities are known to be able to reverse the elevated levels of serum indices of liver and kidney toxicity by mechanisms described earlier (Shakil, 1995; Feng *et al.*, 2010) Kombucha was also able to reduce the organ to body weight ratio of the liver and kidney significantly indicating organ restoration and recovery (Figure 4.10). The renal protective effect of Kombucha could also be due to the fact that it enhances lipid peroxidation and levels of endogenous anti-oxidants (Yoshioka, 1979). Furthermore, probiotics have been found to be able to produce peptides and other compound that could scavenge

free radicals from the body. Generally, as reported by other studies Kombucha has been shown to be very useful in detoxification of the body (Sai Ram *et al*., 2000). Acetic acid and glucuronic acids in Kombucha have the ability to conjugate toxins and eliminate them from the body. The elaborate network of nutraceutical and probiotics within Kombucha makes it a potent anti-oxidant in relieving oxidative stress (Sai Ram *et al*., 2000; Gharib, 2010). By this study, Kombucha is seen to be a safe and protective beverage which could be used as a therapeutic agent. When the organ is damaged, there is usually edema resulting from impaired osmoregulation and thus increasing the organ to body weight ratio. It was also observed that Kombucha also demonstrated a good ability to reduce the urinary glucose levels in diabetic rats which is indicative of its hypoglycaemic actions. There was reduction in the overall volume of the overnight urine pass by diabetic animals, an indication that Kombucha was effective in ameliorating the frequent urination symptom associated with diabetes (Table 4.2).

The gut microbiota has been reported to be very significant in maintaining gut health as well as prevention of other forms of disease. The gut microbiome is largely predominated by a complex bacterial population within the intestines (Turnbaugh *et al*., 2006; Koren *et al*., 2012; Goodrich *et al*., 2014). Several factors such as anti-biotic consumption, inflammation, diet and gastrointestinal infections have been shown to affect the homeostasis within the gut resulting in dysbiosis. The consumption of probiotics is currently being explored as a means of reconstituting the gut microflora to maintain a healthy gut. The present study shows that the diversity within the gut changes significantly over a period in the diabetic state (Figures 4.18,4.19, 4.20 & 4.21). This disease state tends to enhance the richness within the gut over the entire treatment duration but causes decrease in the *Lactobacillacea*e family which are members of the *Firmicutes* phyla resulting in the colonization of the gut by other opportunistic infections. The *Lactobacillaceae* family have been reported to be a protective microbial population within the gut conferring

protection to the gut by out-competing other pathogenic organisms from colonizing the gut. Once the *Lactobacillaceae* family is compromised within the gut community in a diseased state, the gut becomes vulnerable to other gastrointestinal infections. Introduction of probiotics tends to maintain the gut flora by enriching the *Lactobacillaceae* communities and reducing the ability of other pathogenic infections from colonizing the gut (Figure 4.17). Probiotic organisms may not necessarily reside in the gut, however, their introduction into the gut plays a critical beneficial role in regulating and protecting the gut (Sanders *et al*, 2018; Walter *et al*, 2018;). Probiotics may also stimulate the immune system to mount immune responses against pathogens within the gut. Richness indices are used to explain the alpha diversity within the samples, focusing on the richness and evenness of the distribution of the microbial communities (Simpson, 1949; McCarthy and Maguraran, 2004; Chao, *et al*., 2006; Lemos *et al*., 2011). Generally, the diversity was low after the 28 days treatment period because *Lactobacillaceae* family was enhanced for protection of the gut, thus out-numbering the other lowly represented microbial populations (Figure 4.17). Increase in *Lactobacillaceae* in the gut may trigger the inhibition of lipopolysaccharides production and increased tight junction formation of the epithelial cells (Wen and Duffy, 2017). This also helps in reducing inflammation within the gut reducing the chances of pancreatic damage and insulin production. Enrichment of the gut with probiotics is also essential in diabetes management because these organisms produce essential short chain fatty acids such as butyrates, propionates for maintaining intestinal homeostasis (Qin *et al*, 2012; Flint, 2012; Broderick and Lemaitre, 2012). There is great variability that exist within the microbiome of various human and animal models, certain core phyla have been associated with the gut as potential core biome offering protection from disease, of which the *Firmicutes* are predominant, which is consistent with the results of this present study. One of the main reasons why the study of the gut microbiome is very significant in diabetes is that, most obese people tend to suffer from type 2 diabetes. Despite

the fact that dysbiosis is not absolutely linked to biome, type 2 diabetes, obesity, gut inflammation and barrier disruption are strongly linked to this phenomenon (Gómez-Ambrosi *et al*., 2011; Wander *et al*., 2012; Everard *et al*., 2013).

Diabetes is a multifactorial disorder, and as such several driving factors could be responsible for its manifestation. It is, therefore, imperative to exploit solutions across every possible mechanism in order to develop novel interventions to reduce global incidence of diabetes.

5.2 CONCLUSIONS

Kombucha is constituted by a highly diverse microbial community with probiotic value together with other nutraceutraticals including flavonoids and tannins. *Brettanomyces bruxellensis CBS2499* and *Brettanomyces anomalus* are the most abundant yeast species found within the microbial community. Even though the bacterial community are under-represented in the culture, the role of the *Acetobacteraceae* family is significant. Also, Kombucha helps to restore body weight in diabetic rats, a positive sign of recovery. The study also revealed that Kombucha had anti-hyperglycemic and anti-lipidemic effect and hence has the potential as alternative anti-diabetic agent. Kombucha enhances regeneration of the pancreas and hence stimulation of insulin secretion which is very critical in any anti-diabetic therapy. Additionally, Kombucha reverses elevated levels of liver and renal toxicity indices as such, daily intake of Kombucha may protect against liver and kidney damage demonstrating its safety in applications for prophylactic and/or therapeutic interventions in diabetes management. Kombucha also appears to play a key role in maintaining and sustaining a healthy gut microbiome in the diabetic state.

5.3 RECOMMENDATIONS

Based on this study, further study needs to be done to explore the exact roles that the probiotics and nutraceuticals may be playing in conferring this anti-diabetic effect. Thus, this study can be replicated using filtered Kombucha.

Additionally, the mechanism of action of Kombucha in demonstrating its anti-hyperglycemic effect maybe investigated with specific focus on key insulin dependent metabolic processes such as glycolysis and glycogenolysis.

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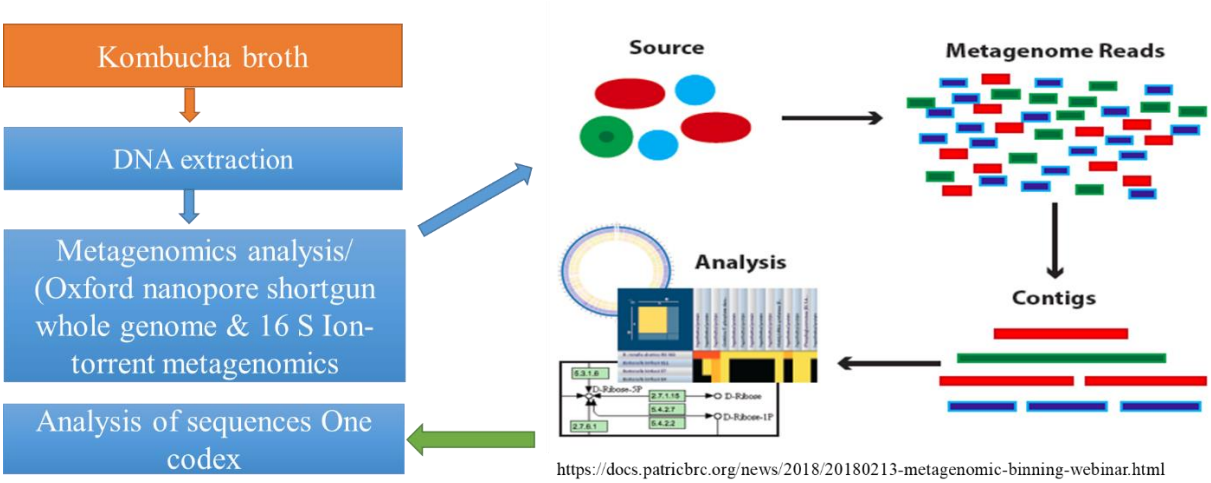
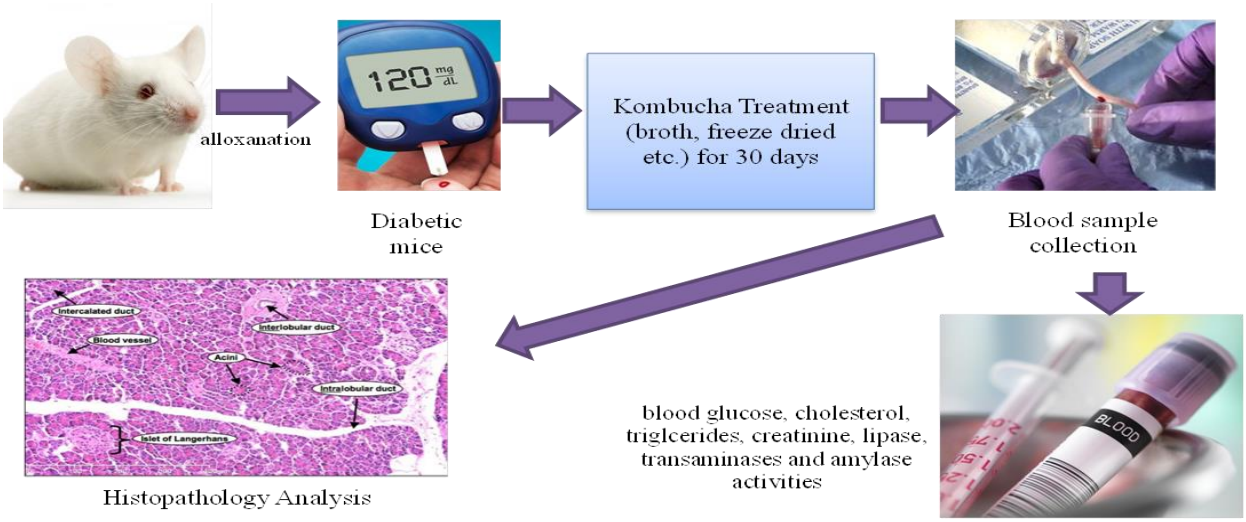
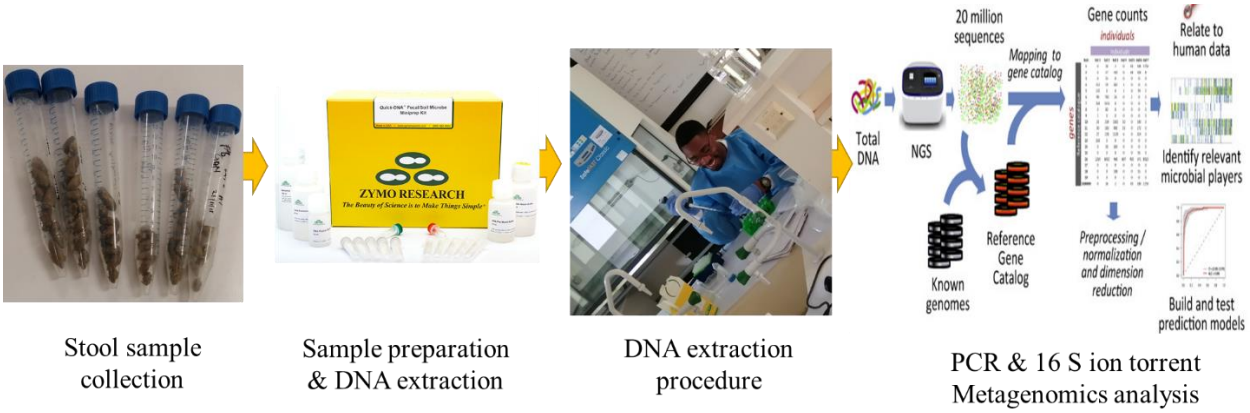
APPENDIX



Appendix 1: Flowchart of the sequence of experiments for Kombucha microbial ecology identification.



Appendix 2: Flowchart of the sequence of experiments for the *in vivo* testing of anti- diabetic activity and toxicity of Kombucha.



Appendix 3: Flowchart of the sequence of experiment for the gut modulatory impact of Kombucha.

Appendix 4: Analysis of Kombucha microbiome showed high diversity using One Codex Database (OCXDB)

Organism Name	Rank	Tax ID	% of All Reads
1. Brettanomyces bruxellensis***	Species	5007	3.660678
2. *Brettanomyces anomalus****	Species	37662	6.980109
3. *Komagataeibacter rhaeticus*	Species	215221	
4. *Gluconacetobacter sp. SXCC-1*	Species	1004836	0.009359
5. *Komagataeibacter hansenii*	Species	436	0.004867
6. *Zygosaccharomyces parabailii*	Species	1365886	0.011419
7. *Schizosaccharomyces pombe*	Species	4896	0.010483
8. *Komagataeibacter xylinus*	Species	28448	
9. *Komagataeibacter nataicola*	Species	265960	0.000562
10. *Metschnikowia cerradonensis*	Species	390697	0.000374
11. *Acetobacter senegalensis*	Species	446692	0.000374
12. *Campylobacter*	Genus	194	0.000187
13. *Pseudomonas*	Genus	286	0.00131
14. *Acetobacter*	Genus	434	0.002808
15. *Gluconobacter*	Genus	441	0.000187
16. *Escherichia*	Genus	561	0.000187
17. *Photobacterium*	Genus	657	0.000187
18. *Prevotella*	Genus	838	0.000187
19. *Prochlorococcus*	Genus	1218	0.000187
20. *Staphylococcus*	Genus	1279	0.000187

21. *Bacillus*	Genus	1386	0.000562
22. *Pichia*	Genus	4919	0.072442
23. *Saccharomyces*	Genus	4930	0.008236
24. *Saccharomycopsis*	Genus	4943	0.000187
25. *Zygosaccharomyces*	Genus	4953	0.006177
26. *Aspergillus*	Genus	5052	0.000187
27. *Fusarium*	Genus	5506	0.000562
28. *Brettanomyces****	Genus	13366	0.149564
29. *Metschnikowia*	Genus	27320	0.026955
30. *Geobacter*	Genus	28231	0.000187
31. *Microbotryum*	Genus	34416	0.000562
32. *Ralstonia*	Genus	48736	0.000187
33. *Kytococcus*	Genus	57499	0.000187
34. *Chryseobacterium*	Genus	59732	0.000187
35. *Starmerella*	Genus	75735	0.000374
36. *Sphingopyxis*	Genus	165697	0.000187
37. *Blastomyces*	Genus	229219	0.000187
38. *Komagataella*	Genus	460517	0.002621
39. *Ogataea*	Genus	461281	0.000562
40. *Nematocida*	Genus	586132	0.000187
41. *Komagataeibacter*	Genus	1434011	0.002059
42. *Sordariomyceta*	No Rank	715989	0.000187
43. *Saccharomycetales incertae sedis*	No Rank	241407	0.000749
44. *Saccharomyceta*	No Rank	716545	0.011419
45. *Leotiomyceta*	No Rank	716546	0.002621
46. *Brettanomyces bruxellensis CBS 2499****	No Rank	747657	42.21947
47. *Brettanomyces bruxellensis AWRI1499****	No Rank	1124627	3.986013
48. *Bacillus nealsonii AAU1*	No Rank	1202533	0.006364
49. *Komagataeibacter xylinus NBRC 15237*	No Rank	1220579	0.000562
50. *Komagataeibacter hansenii JCM 7643*	No Rank	1231352	0.000562
51. *Komagataeibacter xylinus E25*	No Rank	1296990	0.005616
52. *Mortierella elongata AG-77*	No Rank	1314771	0.000374
53. *Zygosaccharomyces bailii CLIB 213*	No Rank	1333698	0.001872
54. *Komagataeibacter rhaeticus AF1*	No Rank	1432055	0.011606
55. *Terrabacteria group*	No Rank	1783272	0.000374

Appendix 5. Analysis of Kombucha microbiome showed high diversity using REFSEQ database

	Organism Name	Rank	Tax ID	% of All Reads
1.	*Ogataea*	Genus	461281	0.052038681
2.	*Brettanomyces*	Genus	13366	0.021339603
3.	*Saccharomyces*	Genus	4930	0.005241306
4.	*Aspergillus*	Genus	5052	0.001871895
5.	*Methanobrevibacter*	Genus	2172	0.001310326
6.	*Wickerhamomyces*	Genus	599737	0.001310326
7.	*Metarhizium*	Genus	5529	0.000374379
8.	*Starmerella*	Genus	75735	0.000374379
9.	*Komagataeibacter*	Genus	1434011	0.000374379
10.	*Acetobacter*	Genus	434	0.000187189
11.	*Methanococcus*	Genus	2184	0.000187189
12.	*Neurospora*	Genus	5140	0.000187189
13.	*Ceratocystis*	Genus	5157	0.000187189
14.	*Asaia*	Genus	91914	0.000187189
15.	*Tunalikevirus*	Genus	187217	0.000187189
16.	*Nematocida*	Genus	586132	0.000187189
17.	*Brettanomyces bruxellensis CBS 2499****	No Rank	747657	51.78728534
18.	*Gyalolechia flavorubescens KoLRI002931*	No Rank	1353255	0.073191094
19.	*Saccharomyceta*	No Rank	716545	0.050915544
20.	*Ophiostoma novo-ulmi subsp. novo-ulmi H327*	No Rank	1224258	0.049792407
21.	*Psychroflexus torquis ATCC 700755*	No Rank	313595	0.047171754
22.	*Leotiomyceta*	No Rank	716546	0.04492548
23.	*Cyberlindnera jadinii NBRC 0988*	No Rank	1128122	0.034442868
24.	*Kluyveromyces wickerhamii UCD 54-210*	No Rank	861556	0.027704046
25.	*Colletotrichum orbiculare MAFF 240422*	No Rank	1213857	0.026393719
26.	*Schizosaccharomyces octosporus yFS286*	No Rank	483514	0.023585877
27.	*Saccharomyces paradoxus NRRL Y-17217*	No Rank	226125	0.020029276
28.	*Nosema apis BRL 01*	No Rank	1037528	0.019467708
29.	*Metarhizium brunneum ARSEF 3297*	No Rank	1276141	0.019093329
30.	*Nakaseomyces delphensis CBS 2170*	No Rank	1279113	0.01871895
31.	*Methanosarcina barkeri str. Fusaro*	No Rank	269797	0.016472676
32.	*Metarhizium album ARSEF 1941*	No Rank	1081103	0.015349539
33.	*Komagataeibacter xylinus E25*	No Rank	1296990	0.012728886
34.	*Botryobasidium botryosum FD-172 SS1*	No Rank	930990	0.012167317
35.	*Piloderma croceum F 1598*	No Rank	765440	0.011792938
36.	*Komagataeibacter medellinensis NBRC 3288*	No Rank	634177	0.011418559
37.	*Lachancea waltii NCYC 2644*	No Rank	262981	0.010108233

38. *Fusarium virguliforme Mont-1*	No Rank	994087	0.009733854
39. *Leptospira noguchii serovar Panama str. CZ214*	No Rank	1001595	0.008985096
40. *Hanseniaspora uvarum DSM 2768*	No Rank	1246595	0.008985096
41. *Kluyveromyces dobzhanskii CBS 2104*	No Rank	1427455	0.008610717
42. *Methanococcus voltae A3*	No Rank	456320	0.008423527
43. *Spathaspora arborariae UFMG-19.1A*	No Rank	1343157	0.008423527
44. *Candida parapsilosis CDC317*	No Rank	578454	0.007113201
45. *Kluyveromyces aestuarii ATCC 18862*	No Rank	854826	0.006551632
46. *Nosema bombycis CQ1*	No Rank	578461	0.005990064
47. *Methanobrevibacter ruminantium M1*	No Rank	634498	0.005990064
48. *Komagataeibacter hansenii ATCC 23769*	No Rank	714995	0.005802874
49. *Sporothrix schenckii ATCC 58251*	No Rank	1391915	0.005428495
50. *Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7*	No Rank	1095631	0.005054116
51. *Plicaturopsis crispa FD-325 SS-3*	No Rank	944288	0.004305358
52. *Edwardsiella ictaluri 93-146*	No Rank	634503	0.004118169
53. *Psychrobacter aquaticus CMS 56*	No Rank	1354303	0.003930979
54. *Amanita jacksonii TRTC168611*	No Rank	1417757	0.003930979
55. *Saccharomyces uvarum MCYC 623*	No Rank	226127	0.002995032
56. *Photorhabdus luminescens subsp. laumondii TTO1*	No Rank	243265	0.002995032
57. *Serratia symbiotica str. 'Cinara cedri'*	No Rank	568817	0.002807842
58. *Oidiodendron maius Zn*	No Rank	913774	0.002807842
59. *Sporothrix brasiliensis 5110*	No Rank	1398154	0.002807842
60. *Cladonia macilenta KoLRI003786*	No Rank	1353983	0.002620653
61. *Saccharomyces mikatae IFO 1815*	No Rank	226126	0.002433463
62. *Mucispirillum schaedleri ASF457*	No Rank	1379858	0.002433463
63. *Cladonia metacorallifera KoLRI002260*	No Rank	1400770	0.002433463
64. *Wickerhamomyces anomalus NRRL Y-366*	No Rank	885923	0.002246274
65. *Trichoderma hamatum GD12*	No Rank	1247866	0.002246274
66. *Anncaliia algerae PRA339*	No Rank	1288291	0.002246274
67. *Ascosphaera apis ARSEF 7405*	No Rank	392613	0.002059084
68. *Fusarium fujikuroi B14*	No Rank	1215603	0.002059084
69. *Emmonsia parva UAMH 139*	No Rank	1246674	0.002059084
70. *Taphrina wiesneri JCM 22204*	No Rank	1450757	0.002059084
71. *Methanococcus aeolicus Nankai-3*	No Rank	419665	0.001871895
72. *Clostridium lentocellum DSM 5427*	No Rank	642492	0.001871895
73. *Aequorivita sublithincola DSM 14238*	No Rank	746697	0.001871895
74. *Wolfiporia cocos MD-104 SS10*	No Rank	742152	0.001684705
75. *Venturia pyrina ICMP 11032*	No Rank	1437871	0.001684705
76. *Methanothermococcus okinawensis IH1*	No Rank	647113	0.001497516
77. *Sphaerobolus stellatus SS14*	No Rank	990650	0.001497516

78. *Edhazardia aedis USNM 41457*	No Rank	1003232	0.001497516
79. *Ascocoryne sarcoides NRRL 50072*	No Rank	1016881	0.001497516
80. *Saccharomyces pastorianus CBS 1513*	No Rank	1073566	0.001497516
81. *Janthinobacterium agaricidamnosum NBRC 102515 = DSM 9628*	No Rank	1349767	0.001497516
82. *Taphrina populina JCM 22190*	No Rank	1450760	0.001497516
83. *Fusarium oxysporum f. sp. lycopersici 4287*	No Rank	426428	0.001310326
84. *Aspergillus kawachii IFO 4308*	No Rank	1033177	0.001310326
85. *Basilea psittacipulmonis DSM 24701*	No Rank	1072685	0.001310326
86. *Trichoderma longibrachiatum SMF2*	No Rank	1234776	0.001310326
87. *Lichtheimia corymbifera JMRC:FSU:9682*	No Rank	1263082	0.001310326
88. *Delftia acidovorans SPH-1*	No Rank	398578	0.001123137
89. *Grosmannia clavigera kw1407*	No Rank	655863	0.001123137
90. *Fusarium circinata FSP 34*	No Rank	1076874	0.001123137
91. *Meyerozyma caribbica MG20W*	No Rank	1091232	0.001123137
92. *Passalora fulva CBS 131901*	No Rank	1156157	0.001123137
93. *Lichtheimia ramosa B5399*	No Rank	1274788	0.001123137
94. *Methanobrevibacter arboriphilus ANOR1*	No Rank	1401244	0.001123137
95. *Proteiniclasticum ruminis DSM 24773*	No Rank	1410668	0.001123137
96. *Agaricomycotina*	No Rank	5302	0.000935947
97. *Pseudogymnoascus destructans 20631-21*	No Rank	658429	0.000935947
98. *Ophiostoma piceae UAMH 11346*	No Rank	1262450	0.000935947
99. *Cryptococcus pinus CBS 10737*	No Rank	1296096	0.000935947
100. *Kuraishia capsulata CBS 1993*	No Rank	1382522	0.000935947
101. *Rasamsonia emersonii CBS 393.64*	No Rank	1408163	0.000935947
102. *Corynespora cassiicola UM 591*	No Rank	1451142	0.000935947
103. *Fusarium verticillioides 7600*	No Rank	334819	0.000748758
104. *Xenorhabdus bovienii SS-2004*	No Rank	406818	0.000748758
105. *Bipolaris maydis C5*	No Rank	701091	0.000748758
106. *Cyanobacterium aponinum PCC 10605*	No Rank	755178	0.000748758
107. *Piriformospora indica DSM 11827*	No Rank	1109443	0.000748758
108. *Alternaria arborescens EGS 39-128*	No Rank	1122867	0.000748758
109. *Hysterium pulicare CBS 123377*	No Rank	1136866	0.000748758
110. *Clostridium paraputrificum AGR2156*	No Rank	1280689	0.000748758
111. *Penicillium paxilli ATCC 26601*	No Rank	1292256	0.000748758
112. *Saccharomyces bayanus 623-6C*	No Rank	226231	0.000561568
113. *Gluconacetobacter diazotrophicus PA1 5*	No Rank	272568	0.000561568
114. *Methanosphaera stadtmanae DSM 3091*	No Rank	339860	0.000561568
115. *Borrelia valaisiana VS116*	No Rank	445987	0.000561568

116. *Spizellomyces punctatus DAOM BR117*	No Rank	645134	0.000561568
117. *Photobacterium damselae subsp. damselae CIP 102761*	No Rank	675817	0.000561568
118. *Mixia osmundae IAM 14324*	No Rank	764103	0.000561568
119. *Veillonella ratti ACS-216-V-Col6b*	No Rank	883156	0.000561568
120. *Ceriporiopsis subvermispora B*	No Rank	914234	0.000561568
121. *Ceratocystis fimbriata CBS 114723*	No Rank	1035309	0.000561568
122. *Hirsutella minnesotensis 3608*	No Rank	1043627	0.000561568
123. *Ganoderma lucidum G.260125-1*	No Rank	1077286	0.000561568
124. *Aquimarina latercula DSM 2041*	No Rank	1121006	0.000561568
125. *Moritella dasanensis ArB 0140*	No Rank	1201293	0.000561568
126. *Gluconobacter oxydans H24*	No Rank	1224746	0.000561568
127. *Piscirickettsia salmonis LF-89 = ATCC VR-1361*	No Rank	1227812	0.000561568
128. *Metarhizium majus ARSEF 297*	No Rank	1276143	0.000561568
129. *Borrelia parkeri SLO*	No Rank	1313294	0.000561568
130. *Byssochlamys spectabilis No. 5*	No Rank	1356009	0.000561568
131. *Spraguea lophii 42_110*	No Rank	1358809	0.000561568
132. *Aspergillus parasiticus SU-1*	No Rank	1403190	0.000561568
133. *Penicillium expansum T01*	No Rank	1407458	0.000561568
134. *Phellinus noxius OVT-YTM/97*	No Rank	1417759	0.000561568
135. *Fonsecaea pedrosoi CBS 271.37*	No Rank	1442368	0.000561568
136. *Psychromonas ingrahamii 37*	No Rank	357804	0.000374379
137. *Buchnera aphidicola BCc*	No Rank	372461	0.000374379
138. *Acetobacter pasteurianus IFO 3283-01*	No Rank	634452	0.000374379
139. *Bacteroides salanitronis DSM 18170*	No Rank	667015	0.000374379
140. *Galerina marginata CBS 339.88*	No Rank	685588	0.000374379
141. *Aspergillus sojae NBRC 4239*	No Rank	927772	0.000374379
142. *Bipolaris victoriae FI3*	No Rank	930091	0.000374379
143. *Suillus luteus UH-Slu-Lm8-n1*	No Rank	930992	0.000374379
144. *Zymoseptoria pseudotritici STIR04_4.3.1*	No Rank	985143	0.000374379
145. *Helicobacter bizzozeronii CIII-1*	No Rank	1002804	0.000374379
146. *Clostridium senegalense JC122*	No Rank	1033737	0.000374379
147. *Bacillus massiliosenegalensis JC6*	No Rank	1034347	0.000374379
148. *Aureobasidium pullulans EXF-150*	No Rank	1043002	0.000374379
149. *Acetobacter nitrogenifigens DSM 23921 = LMG 23498*	No Rank	1120919	0.000374379
150. *Blautia producta ATCC 27340 = DSM 2950*	No Rank	1121114	0.000374379

151.	*Glycomyces arizonensis DSM 44726*	No Rank	1121926	0.000374379
152.	*Fistulina hepatica ATCC 64428*	No Rank	1128425	0.000374379
153.	*Pleurotus ostreatus PC15*	No Rank	1137138	0.000374379
154.	*Nematocida sp. 1 ERTm6*	No Rank	1138374	0.000374379
155.	*Providencia burhodogranariea DSM 19968*	No Rank	1141662	0.000374379
156.	*Methanobrevibacter boviskoreani JH1*	No Rank	1214066	0.000374379
157.	*Curvularia lunata CX-3*	No Rank	1263492	0.000374379
158.	*Heterobasidion annosum 03012*	No Rank	1264691	0.000374379
159.	*Neurospora africana FGSC 1740*	No Rank	1266766	0.000374379
160.	*Syncephalastrum racemosum B6101*	No Rank	1274787	0.000374379
161.	*Metarhizium anisopliae ARSEF 549*	No Rank	1276135	0.000374379
162.	*Phaseolibacter flectens ATCC 12775*	No Rank	1336237	0.000374379
163.	*Alkaliphilus transvaalensis ATCC 700919*	No Rank	1408422	0.000374379
164.	*Mycoplasma cricetuli ATCC 35279*	No Rank	1415781	0.000374379
165.	*Rhinocladiella mackenziei CBS 650.93*	No Rank	1442369	0.000374379
166.	*Dactylococcopsis salina PCC 8305*	No Rank	13035	0.000187189
167.	*Salmonella enterica subsp. enterica serovar Typhimurium str. LT2*	No Rank	99287	0.000187189
168.	*Geminocystis herdmanii PCC 6308*	No Rank	113355	0.000187189
169.	*Pseudomonas fluorescens SBW25*	No Rank	216595	0.000187189
170.	*Hyphomonas neptunium ATCC 15444*	No Rank	228405	0.000187189
171.	*Burkholderia mallei ATCC 23344*	No Rank	243160	0.000187189
172.	*Phanerochaete chrysosporium RP-78*	No Rank	273507	0.000187189
173.	*Gluconobacter oxydans 621H*	No Rank	290633	0.000187189
174.	*Thiobacillus denitrificans ATCC 25259*	No Rank	292415	0.000187189
175.	*Aliivibrio salmonicida LFI1238*	No Rank	316275	0.000187189
176.	*Paracoccus denitrificans PD1222*	No Rank	318586	0.000187189
177.	*Sulfurimonas denitrificans DSM 1251*	No Rank	326298	0.000187189
178.	*Syntrophomonas wolfei subsp. wolfei str. Goettingen G311*	No Rank	335541	0.000187189
179.	*Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305*	No Rank	342451	0.000187189
180.	*Campylobacter hominis ATCC BAA-381*	No Rank	360107	0.000187189

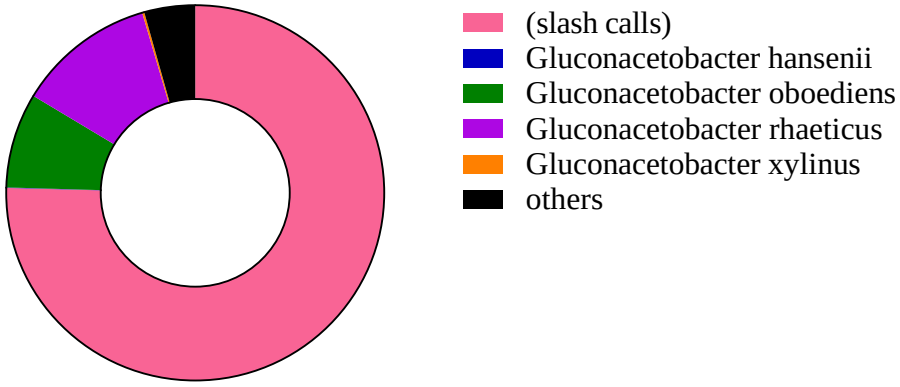
181.	*Verminephrobacter eiseniae EF01-2*	No Rank	391735	0.000187189
182.	*Pseudobacteroides cellulosolvens ATCC 35603 = DSM 2933*	No Rank	398512	0.000187189
183.	*Shewanella sediminis HAW-EB3*	No Rank	425104	0.000187189
184.	*Marinitoga piezophila KA3*	No Rank	443254	0.000187189
185.	*Sorangium cellulosum So ce56*	No Rank	448385	0.000187189
186.	*Streptomyces sviceus ATCC 29083*	No Rank	463191	0.000187189
187.	*Kytococcus sedentarius DSM 20547*	No Rank	478801	0.000187189
188.	*Moorea producens 3L*	No Rank	489825	0.000187189
189.	*Capnocytophaga ochracea DSM 7271*	No Rank	521097	0.000187189
190.	*Anaerococcus prevotii DSM 20548*	No Rank	525919	0.000187189
191.	*Capnocytophaga gingivalis ATCC 33624*	No Rank	553178	0.000187189
192.	*Saccharomyces cerevisiae S288c*	No Rank	559292	0.000187189
193.	*Asticcacaulis excentricus CB 48*	No Rank	573065	0.000187189
194.	*Vibrio tasmaniensis LGP32*	No Rank	575788	0.000187189
195.	*Anaerobaculum hydrogeniformans ATCC BAA-1850*	No Rank	592015	0.000187189
196.	*Alternaria brassicicola ATCC 96836*	No Rank	622360	0.000187189
197.	*Syntrophothermus lipocalidus DSM 12680*	No Rank	643648	0.000187189
198.	*Silicibacter lacuscaerulensis ITI-1157*	No Rank	644107	0.000187189
199.	*Schizosaccharomyces cryophilus OY26*	No Rank	653667	0.000187189
200.	*Acetobacter aceti ATCC 23746*	No Rank	663932	0.000187189
201.	*Microbotryum violaceum p1A1 Lamole*	No Rank	683840	0.000187189
202.	*Hebeloma cylindrosporum h7*	No Rank	686832	0.000187189
203.	*Leptotrichia goodfellowii DSM 19756*	No Rank	714315	0.000187189
204.	*Streptomyces bingchenggensis BCW-1*	No Rank	749414	0.000187189
205.	*Spiroplasma mirum ATCC 29335*	No Rank	838561	0.000187189
206.	*Streptococcus intermedius B196*	No Rank	862967	0.000187189
207.	*Methanomethylovorans hollandica DSM 15978*	No Rank	867904	0.000187189
208.	*Pisolithus tinctorius Marx 270*	No Rank	870435	0.000187189
209.	*Myroides odoratus DSM 2801*	No Rank	929704	0.000187189
210.	*Serendipita vermifera MAFF 305830*	No Rank	933852	0.000187189

211. *Clostridium botulinum B str. Eklund 17B (NRP)*	No Rank	935198	0.000187189
212. *Gymnopus luxurians FD-317 M1*	No Rank	944289	0.000187189
213. *Sporisorium reilianum SRZ2*	No Rank	999809	0.000187189
214. *Prevotella stercorea DSM 18206*	No Rank	1002367	0.000187189
215. *Photorhabdus temperata subsp. khanii NC19*	No Rank	1004151	0.000187189
216. *Gallibacterium anatis UMN179*	No Rank	1005058	0.000187189
217. *Rhizomucor miehei CAU432*	No Rank	1031333	0.000187189
218. *Periglandula ipomoeae IasaF13*	No Rank	1037531	0.000187189
219. *Drechslerella stenobrocha 248*	No Rank	1043628	0.000187189
220. *Bizionia argentinensis JUB59*	No Rank	1046627	0.000187189
221. *Tulasnella calospora MUT 4182*	No Rank	1051891	0.000187189
222. *Pochonia chlamydosporia 123*	No Rank	1052797	0.000187189
223. *Kazachstania naganishii CBS 8797*	No Rank	1071383	0.000187189
224. *Tolypocladium inflatum NRRL8044*	No Rank	1086729	0.000187189
225. *Gluconobacter morbifer G707*	No Rank	1088869	0.000187189
226. *Laccaria amethystina LaAM-08-1*	No Rank	1095629	0.000187189
227. *Claviceps purpurea 20.1*	No Rank	1111077	0.000187189
228. *Mycoplasma canis PG 14*	No Rank	1117644	0.000187189
229. *Acinetobacter tandoii DSM 14970 = CIP 107469*	No Rank	1120927	0.000187189
230. *Aequorivita capsosiphonis DSM 23843*	No Rank	1120951	0.000187189
231. *Clostridium akagii DSM 12554*	No Rank	1121293	0.000187189
232. *Terrisporobacter glycolicus ATCC 14880 = DSM 1288*	No Rank	1121315	0.000187189
233. *Caldibacillus debilis DSM 16016*	No Rank	1121917	0.000187189
234. *Psychrilyobacter atlanticus DSM 19335*	No Rank	1123032	0.000187189
235. *Rubritepida flocculans DSM 14296*	No Rank	1123072	0.000187189
236. *Salinimicrobium xinjiangense DSM 19287*	No Rank	1123235	0.000187189
237. *Omphalotus olearius VT 653.13*	No Rank	1128401	0.000187189
238. *Penicillium digitatum Pd1*	No Rank	1170230	0.000187189
239. *Leptospira borgpetersenii serovar Mini str. 200901116*	No Rank	1192864	0.000187189
240. *Moritella marina ATCC 15381*	No Rank	1202962	0.000187189
241. *Lysinibacillus massiliensis 4400831 = CIP 108448 = CCUG 49529*	No Rank	1211035	0.000187189
242. *Halorubrum kocurii JCM 14978*	No Rank	1230456	0.000187189
243. *Acetobacter okinawensis JCM 25146*	No Rank	1236501	0.000187189
244. *Bacillus wakoensis JCM 9140*	No Rank	1236970	0.000187189

245. *Clostridium pasteurianum DSM 525 = ATCC 6013*	No Rank	1262449	0.000187189
246. *Caldimonas manganoxidans ATCC BAA-369*	No Rank	1265502	0.000187189
247. *Listeria aquatica FSL S10-1188*	No Rank	1265818	0.000187189
248. *Neurospora sublineolata FGSC 5508*	No Rank	1266767	0.000187189
249. *Spiroplasma culicicola AES-1*	No Rank	1276246	0.000187189
250. *Stachybotrys chartarum IBT 40293*	No Rank	1280524	0.000187189
251. *Cryptococcus dejecticola CBS 10117*	No Rank	1296121	0.000187189
252. *Dokdonia donghaensis DSW-1*	No Rank	1300343	0.000187189
253. *Pyrenophora seminiperda CCB06*	No Rank	1302712	0.000187189
254. *Hirsutella thompsonii MTCC6686*	No Rank	1303645	0.000187189
255. *Pseudozyma hubeiensis SY62*	No Rank	1305764	0.000187189
256. *Laceyella sacchari 1-1*	No Rank	1341151	0.000187189
257. *Streptomyces rapamycinicus NRRL 5491*	No Rank	1343740	0.000187189
258. *Kitasatospora cheerisanensis KCTC 2395*	No Rank	1348663	0.000187189
259. *Penicillium roqueforti FM164*	No Rank	1365484	0.000187189
260. *Lignosus rhinocerotis TM02*	No Rank	1379160	0.000187189
261. *Bordetella trematum CCUG 13902*	No Rank	1392839	0.000187189
262. *Sporothrix schenckii 1099-18*	No Rank	1397361	0.000187189
263. *Mycoplasma anseris ATCC 49234*	No Rank	1408454	0.000187189
264. *Penicillium camemberti FM 013*	No Rank	1429867	0.000187189
265. *Magnetospirillum gryphiswaldense MSR-1 v2*	No Rank	1430440	0.000187189
266. *Ochroconis constricta UM 578*	No Rank	1442076	0.000187189
267. *Cladophialophora bantiana CBS 173.52*	No Rank	1442370	0.000187189
268. *Luteimonas huabeiensis HB2*	No Rank	1442599	0.000187189
269. *Carnobacterium alterfunditum DSM 5972*	No Rank	1449335	0.000187189
270. *Brettanomyces anomalus****	Species	37662	4.966698988
271. *Diaporthe longicolla**	Species	54899	0.1497516
272. *Stachybotrys chartarum*	Species	74722	0.06345724
273. *Ogataea polymorpha*	Species	460523	0.029201562
274. *Hypoxylon sp. E7406B*	Species	1489628	0.020778034
275. *Lacazia loboi*	Species	121752	0.006738822
276. *Epichloe aotearoae*	Species	170559	0.005615685
277. *Sporothrix schenckii*	Species	29908	0.005428495
278. *Trametes cinnabarina*	Species	5643	0.005054116
279. *Lasallia pustulata*	Species	136370	0.004866927

280.	*Tolypocladium sp. Salcha MEA-2*	Species	1355412	0.004305358
281.	*Corynebacterium phage P1201*	Species	384848	0.004118169
282.	*Dactylonectria macrodidyma*	Species	307937	0.003369411
283.	*Epichloe bromicola*	Species	79588	0.003182221
284.	*Galactomyces candidum*	Species	1173061	0.003182221
285.	*Acetobacter tropicalis*	Species	104102	0.002995032
286.	*Mrakia blollopis*	Species	696254	0.002807842
287.	*Wickerhamomyces ciferrii*	Species	1041607	0.002620653
288.	*Penicillium verrucosum*	Species	60171	0.002246274
289.	*Candida apicola*	Species	29830	0.002059084
290.	*Saccharomycetaceae sp. 'Ashbya aceri'*	Species	566037	0.001871895
291.	*Penicillium italicum*	Species	40296	0.001684705
292.	*Fusarium circinatum*	Species	48490	0.001497516
293.	*Thielaviopsis punctulata*	Species	72032	0.001497516
294.	*Ceratocystis platani*	Species	88771	0.001497516
295.	*Valsa mali*	Species	105487	0.001310326
296.	*Diaporthe ampelina*	Species	1214573	0.001310326
297.	*Torrubiella hemipterigena*	Species	1531966	0.001310326
298.	*Talaromyces islandicus*	Species	28573	0.001123137
299.	*Shiraia sp. slf14*	Species	665115	0.000935947
300.	*Capronia semiimmersa*	Species	5601	0.000748758
301.	*Ectromelia virus*	Species	12643	0.000748758
302.	*Penicillium expansum*	Species	27334	0.000748758
303.	*Mucor ambiguous*	Species	91626	0.000748758
304.	*Phaeomoniella chlamydospora*	Species	158046	0.000748758
305.	*Ralstonia pickettii*	Species	329	0.000561568
306.	*Hanseniaspora uvarum*	Species	29833	0.000561568
307.	*Penicillium solitum*	Species	60172	0.000561568
308.	*Taiwanofungus camphoratus*	Species	196114	0.000561568
309.	*Diplodia seriata*	Species	420778	0.000561568
310.	*Sporothrix pallid*	Species	431197	0.000561568
311.	*Fibroporia radiculosa*	Species	599839	0.000561568
312.	*Rhodotorula mucilaginosa*	Species	5537	0.000374379
313.	*Tilletia indica*	Species	43049	0.000374379
314.	*Atkinsonella texensis*	Species	51582	0.000374379
315.	*Penicillium capsulatum*	Species	69766	0.000374379
316.	*Trachipleistophora hominis*	Species	72359	0.000374379
317.	*Facklamia languid*	Species	82347	0.000374379
318.	*Aspergillus ochraceoroseus*	Species	138278	0.000374379
319.	*Exophiala oligosperma*	Species	215243	0.000374379
320.	*Stenotrophomonas rhizophila*	Species	216778	0.000374379
321.	*Verruconis gallopava*	Species	253628	0.000374379

322.	*Taphrina deformans*	Species	5011	0.000187189
323.	*Fusarium fujikuroi*	Species	5127	0.000187189
324.	*Amsacta moorei entomopoxvirus 'L'*	Species	28321	0.000187189
325.	*Camelpox virus*	Species	28873	0.000187189
326.	*Heliothis zea nudivirus*	Species	29250	0.000187189
327.	*Clonostachys rosea*	Species	29856	0.000187189
328.	*Erythrobacter litoralis*	Species	39960	0.000187189
329.	*Fusarium avenaceum*	Species	40199	0.000187189
330.	*Cryptococcus curvatus*	Species	57679	0.000187189
331.	*Trichoderma atroviride*	Species	63577	0.000187189
332.	*Buzura suppressaria nucleopolyhedrovirus*	Species	74320	0.000187189
333.	*Starmerella bombicola*	Species	75736	0.000187189
334.	*Exophiala spinifera*	Species	91928	0.000187189
335.	*Thermomucor indicae-seudaticae*	Species	101121	0.000187189
336.	*Kozakia baliensis*	Species	153496	0.000187189
337.	*Exophiala mesophila*	Species	212818	0.000187189
338.	*Phaeocystis globosa virus*	Species	251749	0.000187189
339.	*Cladophialophora immunda*	Species	569365	0.000187189
340.	*Terribacillus aidingensis*	Species	586416	0.000187189
341.	*Exophiala sideris*	Species	1016849	0.000187189
342.	*Paenirhodobacter enshiensis*	Species	1105367	0.000187189
343.	*Basidioascus undulatus*	Species	1230402	0.000187189
344.	*Herpotrichiellaceae sp. UM238*	Species	1240657	0.000187189
345.	*Lachancea lanzarotensis*	Species	1245769	0.000187189
346.	*Choristoneura rosaceana entomopoxvirus 'L'*	Species	1293539	0.000187189
347.	*Pyrenochaeta sp. UM 256*	Species	1295359	0.000187189
348.	*Talaromyces cellulolyticus*	Species	1472165	0.000187189
349.	*Biatriospora mackinnonii*	Species	1489893	0.000187189



Appendix 6. 16S ion torrent metagenomics sequencing of Kombucha.

Appendix 7: A picture gallery of some key elements of the experimental set-up for the in-vivo experiment

Appendix 8: Fasting Blood Glucose levels

Duration	Fasting blood glucose levels (mmol/L)						
	Normal control	Diabetic Control	Diabetic + 5 mg/kg Gli	Diabetic + 10 mg/kg Met	Diabetic + 5 mg Kom	Diabetic + 25 mg/kg Kom	Diabetic + 100 mg/kg Kom
Day 1	4.18 ± 0.31*	28.60 ± 3.40	23.00 ± 5.07	26.05 ± 5.12	20.33 ± 4.89	20.82 ± 4.88	20.70 ± 5.99
Day 7	6.63 ± 0.38*	22.93 ± 5.03	15.78 ± 3.56	25.03 ± 6.08	15.18 ± 5.49	5.47 ± 0. 30*	4.80 ± 1.10*
Day 14	6.28 ± 0.43*	30.00 ± 1.76	18.83 ± 6.73	19.47 ± 6.56	21.80 ± 4.51	5.43 ± 0.15*	6.43 ± 0.49*
Day 28	5.43 ± 0.24*	24.47 ± 2.54	13.67 ± 5.41	16.80 ± 5.14	13.55 ± 6.20	4.93 ± 0.17*	6.17 ± 0.77*

Appendix 9: Relative organ/body weight ratio

Organ	Organ weight/Body weight ratio (%)						
	Normal Control	Diabetic Control	Diabetic + 5 mg/kg Gli	Diabetic + 10 mg/kg Met	Diabetic + 5mg/kg Kom	Diabetic + 25 mg/kg Kom	Diabetic + 100 mg/kg Kom
Kidney	0.63 ± 0.01*	1.11 ± 0.07	0.79 ± 0.04*	0.80 ± 0.08*	0.71 ± 0.05*	0.65 ± 0.01*	0.62 ± 0.03*
Diaphragm	0.27 ± 0.02	0.17 ± 0.04	0.23 ± 0.02	0.19 ± 0.00	0.28 ± 0.02*	0.19 ± 0.03	0.21 ± 0.03
Liver	2.74 ± 0.33	4.17 ± 0.21	3.09 ± 0.13	2.93 ± 0.28	3.31 ± 0.24	2.88 ± 0.21*	2.74 ± 0.19*
Pancreas	0.22 ± 0.03	0.18 ± 0.02	0.28 ± 0.04*	0.28 ± 0.11	0.18 ± 0.02	0.25 ± 0.08	0.34 ± 0.04