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Differences in gut microbiota in rat models of cardiovascular disease

A dissertation submitted to the faculty of Health Science, University of the Witwatersrand, for the degree of Master of Science in Medicine

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Abstract

Approximately 25% of the world's adult population has hypertension. Hypertension is a major risk factor for cardiovascular disease, stroke, and heart and kidney failure; however, the cause remains unknown. Gut microbiota have been shown to have a causal role in the development of hypertension. In animal studies, it has been shown that eradication of certain gut microbiota leads to decreased blood pressure and that gut dysbiosis may cause an increase in blood pressure. Furthermore, there is a difference in microbial flora composition in hypertensive and normotensive rats. The aim of this study was to compare the diversity and abundance of gut microbiota in animal models of hypertension. Stomach, intestinal and faecal samples were harvested from spontaneously hypertensive rats (SHR), Dahl salt sensitive rats (SSR) and normotensive Dahl rats. The samples were cultured in microaerophilic conditions (5% O_2–10% CO_2–85% N_2) and identified by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). Genomic DNA isolation, 16S rRNA gene sequencing, and analysis of microbial composition were performed on the samples. There was decrease in microbial species diversity, richness, and abundance in the hypertensive rat models. In addition, there a was an increase in *Firmicutes* and *Bacteroidetes* ratio in the hypertensive rat models. The observed results demonstrate that a dysbiotic gut microbiota is associated with hypertension. Previous studies have shown that bacteria from *Bacteroidetes* and *Firmicutes* play a crucial role in development of hypertension and are needed for the maintenance of physiological homeostasis.

Declaration

I, Anza Thiba, declare that this dissertation is my own aided work, except where stated. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this university or any other university.

Anza Thiba, on this _____ day of _____ 2018

…….. ……..

Prof G Candy (Supervisor) Prof K Rumbold (Co-supervisor)

Date: ……. Date: …….

Dedication

I would like to dedicate my dissertation to my loving friends, family, colleagues and my supervisors. A special thanks to my parents, Mr and Mrs Thiba, who were fully supportive both emotionally and financially. My brothers, Divhani and Murendwa, who kept me laughing, made me feel loved and appreciated all the time.

I would like to thank my supervisor, Prof G Candy, who has been extremely patient, kind and quite understanding and for presenting me with such an opportunity. Working with you has been phenomenal. Umar and Siyanda, I know I annoy you guys so much and thank you for never running out of patience with me and for always being there when I needed you. Dr Nweke, my advisor, you have been both tough, pushing and quite encouraging. The impart you have made in my life is indescribable.

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Publications and Presentations

The work done on this project was presented at:

- The 45th Meeting of the Surgical Research Society of Southern Africa held at the University of the Witwatersrand, 6th – 7th July 2017, (International conference)
- 8th Cross-Faculty Symposium held at The Wits School of Public Health in October 2018, hosted by the Wits research department,
- Bert Myburgh Conference hosted by the department of Surgery at Wits University on the 29th of November 2018, and
- Molecular Bioscience Research Trust held at The Wits Alumni House on the 30th of November.
- South African Society of Biochemistry and Molecular Biology 2018 held at North West University on the 10th of July 2018.

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List of Abbreviations

ACN	Acetonitrile
ALD	Alcoholic liver disease
α_2-AR	α_2-Adrenergic Receptor
AVP	Arginine vasopressin
cGMP	cyclic guanosine phosphate
CHCA	Cyano-4- hydroxycinnamic acid
CO_2	Carbon dioxide
DOCA	Deoxycorticosterone acetate
FBS	Fetal Bovine Serum
GIT	Gastrointestinal tract
HCCA	α-Cyano-4-hydroxycinnamic acid
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease (IBD),
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectrometry
MIDs	Multiplex identifiers
N_2	Nitrogen
Na+	Sodium
NAFLD	Non-alcoholic fatty liver disease
NO	Nitric oxide
O_2	Oxygen
OTU	Operational Taxonomical Units
SHR	Spontaneously hypertensive rats
SSR	Salt sensitive rats
Sr RNA	small ribosomal ribonucleic acid
TFA	Trifluoroacetic acid
WHO	World Health Organisation
QIIME	Quantitative Insights into Microbial Ecology
QIIMD	Quantitative Insights into Microbial Diversity

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Chapter 1: Introduction

1.1 Introduction

1.1.1 Epidemiology of Hypertension

Hypertension accounts for a significant part of globally recognized leading causes of death in both mid-income and fully developed countries. Approximately one-third of the population worldwide have high blood pressure (Makridakis and DiNicolantonio, 2014). It is a major risk factor for cardiovascular diseases, heart and kidney failure, stroke and atherosclerosis (Rahimi et al., 2015). Hypertension is defined as a systolic blood pressure ≥ 140 mmHg and/or a diastolic pressure ≥ 90 mmHg (Bolívar, 2013).

The World Health Organization (WHO) has declared hypertension as the leading risk factor for disease, mortality and morbidity. Over the years, there has been an increasing number of people affected by hypertension. Currently, hypertension causes 51 % of deaths due to stroke and 45 % of deaths due to heart disease (WHO. 2015). In 2010, 40 % of people ≥ 25 years were diagnosed with hypertension with Africa leading the charts with + 46 % of adults living with hypertension (Lim *et al.,* 2012).

According to a study by Lawes *et al*, 2008 about 13,5 % of premature deaths and 6 % of disability adjusted life years (DALYs) globally have been attributed to high blood pressure. A higher percentage of those affected were in low and mid income region when compared to high income regions (Lawes *et al.,* 2008). Consequently, hypertension has been found to be the leading cause of death amongst non-communicable diseases globally.

Low income regions are showing an increasing prevalence of high blood pressure. In sub-Saharan Africa, approximately 6,3 million people are affected by hypertension, thus there is a rise in prevalence of cardiovascular disease (Addo et al., 2007). A quarter of the South African population is diagnosed with high blood pressure. When The Heart and Stroke Foundation surveyed individuals in the South African population they found that 21% of men, 15% of woman and a total of 24,4% were affected by hypertension (Steyn and Fourie, 2007) the impact of mortality in hypertensive patients with ischaemic heart disease was 41,7%, stroke 49,6%, malignant hypertension 71,5% and other cardiovascular diseases were 21,6% (Norman et al., 2007).

1.1.2 Pathophysiology of hypertension

High blood pressure can be described as a product of cardiac output and systemic vascular morbidity resistance (Steyn and Fourie, 2007). For patients with arterial hypertension, either the cardiac output or the systemic vascular resistance is higher and in certain cases both can be elevated. When the vascular tone is affected, it increases due to increased release of peptides like endothelin and angiotensin or by elevation α-adrenoreceptor stimulation. This causes vasoconstriction of smooth muscle due to an increase in cytosolic calcium (Foëx and Sear, 2004a). Studies show that young adults, especially those with borderline hypertension, have a higher cardiac output, raised heart rate and a normal vascular resistance but in older people there is increased vascular resistance (Foëx and Sear, 2004a).

Endothelins and angiotensin play a role in increasing vascular smooth muscle mass. Angiotensin II is proinflammatory, a vasoconstrictor and a procoagulant, thus is essential for remodelling blood vessels (Zuliani et al., 2005). Downstream, the increase in systemic vascular resistance and cardiac output leads to left ventricular diastolic dysfunction (Foëx and Sear, 2004a).

Other proposed mechanisms that contribute to pathophysiology and regulation of hypertension include; Endothelial mechanisms which primarily involves nitric oxide (NO). NO is synthesized from L-arginine and functions to prevent platelet and leukocyte aggregation, induces vasodilation and inhibits cell proliferation in vascular smooth muscle (Foëx and Sear, 2004a). Additionally, neurogenic control, kallikrein-kinin systems, atrial natriuretic peptide and reno-medullary vaso-depression have a nett effect of causing vasodilation and an efficient blood flow thus contributing to pathophisiology of hypertension (Bolívar, 2013; Foëx and Sear, 2004a; Steyn and Fourie, 2007).

With the majority of hypertensive cases reported, the aetiology of the hypertension is not fully understood. This is termed essential hypertension. Essential hypertension is described as a multi-factorial disease through combined effects of genetic, environmental and behavioural factors (Bolívar, 2013). Research has given insights on association of high dietary salt (sodium) and blood pressure. Other risk factors for essential hypertension include: obesity, diabetes, aging, sedentary life styles (smoking, drinking excessively and lack of physical exercise),

emotional stress and a low potassium intake (Bolívar, 2013; Foëx and Sear, 2004b; Steyn and Fourie, 2007).

A significant number of patients with hypertension, particularly those of African ancestry, have blood pressures affected by dietary salt intake (Weinberger, 1989). The effect of dietary sodium intake on the incidence and mortality from stroke, coronary heart disease and cardiovascular mortality is increased significantly in overweight individuals (Alderman, 2009). Although the mechanism is not fully understood, salt intake appears to modify blood pressure by decreasing the ability of the kidneys to excrete sodium through genetic and environmental factors (Fedorova et al., 2010).

1.1.3 Salt induced hypertension

High sodium intake has been associated with an elevation in blood pressure. This phenomenon has been studied for several years but the pathogenesis of salt induced hypertension remains unknown. Salt-induced hypertension is more prominent in the black/African population than in other ethnic groups (Fuchs, 2011). The long list of putative causes for this higher prevalence suggests that the real reasons are still unknown.

There are a few mechanisms that have been suggested for the development of salt induced hypertension. The slavery hypertension hypothesis for example, was used to explain the prevalence of high blood pressure in the African-American population (Curtin, 1992). It states that high blood pressure in African Americans is caused by one or more of these conditions: first, salt deficiency in the parts of Africa that supplied slaves for the Americas; second, the trauma of the slave trade itself; third, conditions of slavery in the United States (Curtin, 1997). This means that the prevalence of high blood pressure the African American population would have resulted from the ability to conserve salt. This mechanism of salt conservation protected them from salt-depleting illnesses such as vomiting and diarrhoea but consequently had the potential of causing hypertension and has been passed on from one generation to the next (Curtin, 1997).

There are genetic and environmental factors that have been associated with development of salt induced hypertension. Environmental factors that have been identified include socioeconomic status (discrimination and economic inequality included), stress, social network, and diet (Fuchs, 2011; Longo-Mbenza et al., 2015; Pereira et al., 2009). Genetic factors include sex and

race. Dahl and colleagues have studied how genetics plays a crucial role in pathogenesis of diseases (Dahl, 1961).

Dahl and colleagues investigated prevalence of hypertension varies across different populations, considering differences in salt intake (Dahl, 1961). They found that high blood pressure was more common in regions where the dietary salt intake was high. In populations that had a low salt intake, high blood pressure was rare. They also observed a group of individuals that were on a high salt intake but never developed high blood pressure and thus concluded that the development of hypertension was affected by both environmental factors and genetic factors (Dahl, 1961; Dahl et al., 1967a). Furthermore, animal studies showed that rat models fed a high salt diet develop hypertension. Some of the rat models fed a high salt diet were sensitive to salt intake but when fed a low salt diet maintained a stable blood pressure. Interestingly, another group of rats did not develop hypertension when fed a high salt diet. In fact, there were no observed changes in blood pressure when they were fed a high salt diet and these rats were labelled to be salt resistant (Dahl, 1961)

In 1967, Dahl and colleagues continued hypertension studies using rat models. They studied the influence of genetics on salt induced hypertension through parabiosis. Selective breeding with two different rat models was used to test the effect of the humoral factor on hypertension and its effect on the pathogenesis of hypertension. A key component of this experiment was the exchange of extracellular fluids between the two different rat models to determine humoral response. Parabiosis resulted in chronic hypertension even in salt-resistant rat models. This phenomenon only occurred when both strains were fed on a high salt diet (Dahl, 1961; Dahl et al., 1967a, 1972) proving that humoral influence can be transferred from one parabiosis strain to the other. Only through parabiosis do salt resistant rats develop chronic hypertension while salt sensitive parabionts had a significant drop in blood pressure. The results from this study were anomalous as previous studies showed salt sensitive strains to develop hypertension when fed a high salt diet whilst salt resistant strain fail to develop hypertension on a high salt diet (Dahl, 1961; Dahl et al., 1967a, 1972).

Although blood pressure, sodium intake and age have been found to be associated with cardiovascular disease and end- stage organ failure in hypertensive individuals, the influence of salt sensitivity in normotensive individuals was scarcely studied. In 2001, Weinberger and colleagues did a long-term study normotensive and hypertensive subjects were the effects of salt sensitivity on blood pressure was assessed. Several factors associated with development of

hypertension were measured in the duration of the study including; pulse pressure, age, gender, body mass index (but not body weight, systolic, diastolic, and mean arterial pressures, baseline renin levels and salt sensitivity) (Wienberger et al. 2001).These factors were found to be significantly associated with increased mortality in hypertensive and normotensive patients correlating to work done by other previous researchers. In this study, salt sensitive normotensive subjects had a higher mortality rate than salt resistant normotensive subjects. Additionally, salt sensitivity in hypertension was strongly associated with age in individuals < 25 years old. More research is needed to understand the mechanisms by which salt sensitivity may contribute to mortality (Wienberger et al., 2001, 2001).

1.1.4 Mechanism of development of salt-induced hypertension

Bayorah and colleagues, looked at plausible mechanisms that were involved in the development of salt induced hypertension using Dahl salt sensitive rat models over a 4-week treatment period. Cellular mechanisms, biochemical parameters and hemodynamic were investigated in Dahl salt sensitive rats. To achieve this, humoral regulators (arginine vasopressin and aldosterone), norepinephrine release, basal cGMP levels and sodium and potassium were measured in Dahl salt sensitive rats that are on both low and high salt diets. They hypothesized that blood pressure increase by salt sensitivity can be influenced by enhanced arginine vasopressin (AVP) release after an increase in sympathetic activity and leads to reduced cGMP production (Bayorh et al., 1998).

There was a significant blood pressure increase in salt sensitive rats fed on a high salt diet (8.0%) when compared to the group fed low salt diet (0.3 %). Heart rate was barely affected by this high blood pressure change. Reduction in blood flow, lower abdominal aortic and renal vascular resistance were strongly associated with a rise in blood pressure. In the group fed a high salt diet there was increased AVP. During the treatment no significant changes in sodium, potassium and aldosterone were observed. There was a decrease in cGMP and increase in release of norepinephrine as hypothesized suggesting AVP, cGMP and norepinephrine play a crucial role in salt induced elevation of blood pressure (Bayorh et al., 1998).

AVP is an antidiuretic hormone that has a key role in maintaining osmolality. AVP functions include; regulation of water retention by increasing water reabsorption, has a role in constricting blood vessels and in sodium reuptake in the kidney (Sharman and Low, 2008). AVP regulates extracellular fluid osmolality through modifying excess water excreted by the

kidney (Cheng et al., 2009; Sharman and Low, 2008). Its role in hypertension is not well understood, however, plasma AVP increases with high salt intake because Na^+ concentration determines plasma osmolality, which is a powerful regulator of AVP secretion. Kawano et al observed that there was no significant increase in plasma AVP in hypertensive patients compared normotensives (Kawano et al., 1997).

Previous studies have shown an association between high salt intake and plasma AVP in malignant hypertension and in salt induced hypertension. AVP has been reported to have a pressor effect on the animal models. In spontaneously hypertensive rats (SHR), AVP in plasma increased and was followed by an increase in urinary excretion. Furthermore, AVP increased more in Dahl salt sensitive rats than in Dahl resistant. The mechanism however has not been elucidated (Share and Crofton, 1982).

Norepinephrine is both a neurotransmitter and a hormone, may also play a crucial role in essential and salt-induced hypertension, however, the mechanism is not well understood. Norepinephrine is used to treat low blood pressure. Its physiological functions includes increasing the heart rate, blood flow and blood pressure (Goldstein et al., 1983; Hermsmeyer, 1976). In the kidney, it functions in Na^+ retention and release of renin. Most studies of role of norepinephrine are done on DOCA-salt hypertensive rat models. So far, no significant difference in norepinephrine release in both normotensive and hypertensive individuals (Goldstein et al., 1983; Hermsmeyer, 1976). The primary cause of hypertension remains unknown and the various risk factors increase the chance of developing hypertension. Figure 1.1 below lists several risk factors that have been identified.

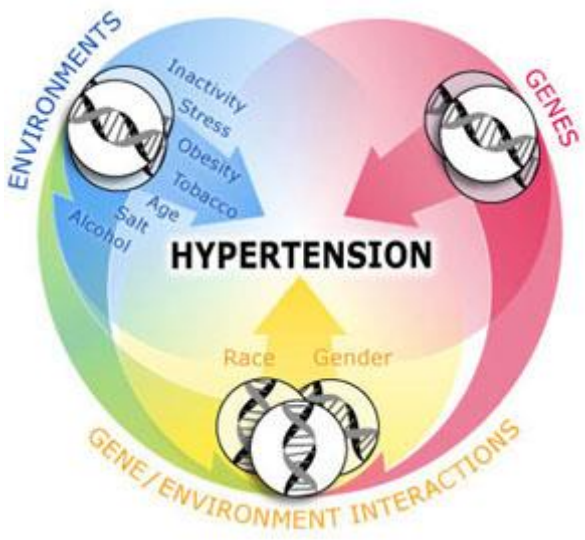


Figure 1.1: Various risk factors for hypertension(Oropesa, 2010). Both environmental and genetic factors are risk factors for hypertension. Inactivity, stress obesity, age, high alcohol and salt consumption are among the most prevalent risk factors that have been identified.

1.1.5 Gut microbiota

There is an increasing realization that gut microbiota play an important role in maintenance of physiological homeostasis such as acquiring energy and nutrients (Fujimura et al., 2010; Guinane and Cotter, 2013), are involved in various metabolic pathways (Rowland et al., 2017) and consequently are involved in the pathogenesis of various diseases (Gill et al., 2006; Qin et al., 2010; Yatsunenko et al., 2012). The gut microbiota can be described as all the microbes that reside along the gastrointestinal tract (Shreiner et al., 2015). How the gut microbiota cross-links with disease, other organs and the immune system has been a topic of interest in recent studies and reviews.

Gut microbiota have an extensive substantial and functional plasticity and great metabolic functionality (Clarke et al., 2014; Shreiner et al., 2015). Other functions of the microbiota include breaking down indigestible compounds, offering defence against pathogens by activating antibody production and initiating host defence mechanisms in the immune system (Zhou et al., 2017). Figure 1.2 below shows the different functions gut bacteria have.The gut microbiota is made up of over a trillion microorganisms; which is 10 times more cells than in the human body. It is composed of microbes from the following major Domains: Bacteria ,

Viruses, Archaea and Eukarya (Lozupone et al., 2012; Reyes et al., 2010). The gut has the most microbial diversity and richness (Gill et al., 2006; Lozupone et al., 2012).

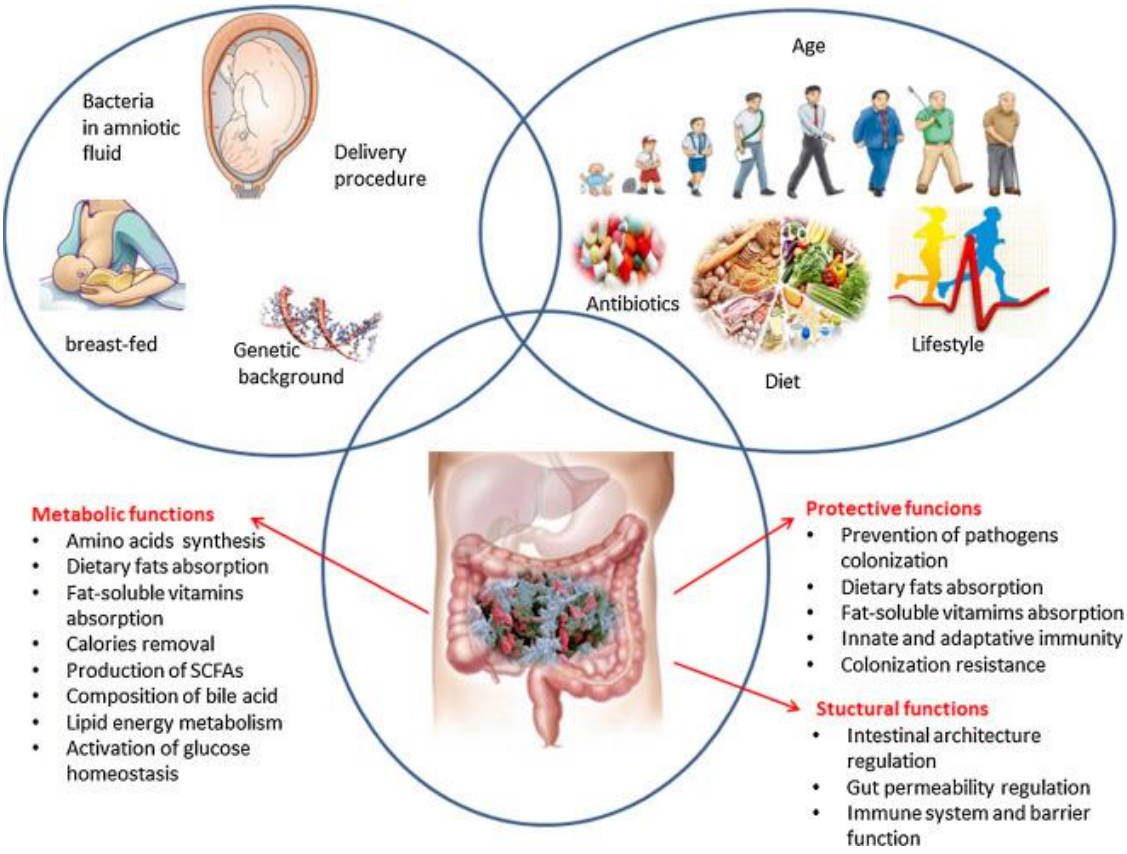


Figure 1.2: Functions of the gut microbiota and several factors affecting composition of the gut microbiota Source: (Blandino et al., 2016).

1.1.6 Factors affecting composition of the gut microbiota

Acquisition of a stable and healthy microbiota starts from infancy or early childhood (Dominguez-Bello et al., 2010) and requires microbe-microbe and microbe-host interactions. The early establishment of a healthy microbiota is crucial for health maintained throughout life (Dominguez-Bello et al., 2010; Yatsunenko et al., 2012). Factors that affect development of a healthy balanced microbiota are discussed below.

Birth- Several factors before, during, and after birth, can influence bacterial gut composition. Before birth, maternal conditions such as period of pregnancy, the environment in the uterus, stress and the use of certain drugs, and subsequently a caesarean or natural birth will affect the microbiota composition (Dominguez-Bello et al., 2010). After birth, contact with the mother, exposure to the environment, diet, life events such as illnesses and use of medication and

antibiotics play crucial roles in establishing the microbiota of an individual (Abrahamsson et al., 2014; Jakobsson et al., 2014; Munyaka et al., 2014).

During pregnancy the foetus is in a sterile uterine environment however, a few bacterial species have been identified in the amniotic fluid, (DiGiulio, 2012; DiGiulio et al., 2008), umbilical cord (Jiménez et al., 2008) and placenta (Satokari et al., 2009) The presence of these bacterial species is proof of mother to infant bacterial transmission (Jiménez et al., 2008). How an infant is born has an important role in acquisition of microbiota (Dominguez-Bello et al., 2010). Infants born vaginally will acquire the mother vaginal microbiota. They will also have a greater microbiome that is diverse and rich. Infants born through caesarean section will have bacteria composition that represents the mother's skin microbial flora. Infants born by caesarean section will have a less rich and diverse microbiome, this may attribute to immune related diseases such as asthma and diabetes in later stages of life (Abrahamsson et al., 2014; Dominguez-Bello et al., 2010; Jakobsson et al., 2014; Munyaka et al., 2014).

The gut flora changes constantly over time. Once the infant is born it acquires more bacterial species from the environment, food and interactions with the mother. How the child is fed highly contributes to development of the gut flora. Breast milk has nutrients that influence growth and colonization of gut flora. A study by Charbonneau and colleagues, found that oligosaccharides in breast milk are crucial for bacteria-dependent growth of the child and influence metabolism in the infant (Charbonneau et al., 2016).

Penders et al. (2006) investigated the influence of formula feeding on the development of gut microbiota and colonization. Infants fed with formula did not acquire bacterial species acquired by breast-fed infants, their guts were colonized with mostly bacteria such as *E. coli* and *C. difficile*, which are potential pathogens. It is therefore favourable to breast feed because of the development of a healthier bacterial ecology (Charbonneau et al., 2016; Penders et al., 2006).It takes about 2-3years for a healthy gut microbiota to be established after birth. The microbial flora (that utilizes lactate) develops to one highly representative of an adult. This development can be affected by biotic and abiotic factors thus illness, use of medication, antibiotic treatment and diet changes can alter the gut flora composition, growth and colonization (Lozupone et al., 2012; Yatsunenko et al., 2012).

Age- The adult gut flora changes with age. There is a significant difference in gut microbiota diversity and abundance in adults of different age groups (Ley et al., 2006; Maffei et al., 2017;

Odamaki et al., 2016). Life style, nutrition, geographic location, diet, illness and disease, antibiotic usage and other bacteria-altering medication influence the composition of the gut bacteria. For example, adults who have been exposed to antibiotics for long periods will have lower species diversity and ultimately a very poor microbial ecology (Ley et al., 2006; Maffei et al., 2017; Odamaki et al., 2016).

Geographical location and ethnicity- Populations from different regions have different gut composition. For example, children in rural areas interact more with the environment thus acquiring various bacterial species (De Filippoa et al. 2010; Lozupone et al. 2012 & Yatsunenko et al. 2012). Those in urban areas live in communities with little interactions with the environment therefore a reduced bacterial species abundance and diversity would be acquired compared to rural areas. A study by Yatsunenko *et al.*, (2012) compared gut microbiota composition between populations in Malawi and United States and found a significant differences in gut bacterial composition between the two populations (Yatsunenko et al., 2012).

Another study compared gut composition of gut bacteria in children of rural African village of Burkina Faso and children in urban parts Florence, Italy in Europe. The gut flora from African villagers had a higher abundance of *Bacteroidetes* and low abundance of *Firmicutes* while those from Europe exhibited a higher *Firmicutes* abundance and low on *Bacteroidetes* abundance (De Filippo et al., 2010).

Diet- Diet plays a crucial role in shaping the gut microbiome. The types of foods ingested will affect distribution of gut microbiota and influence species abundance and diversity(Singh et al., 2017). Studies show that population that have westernized diets; fried foods such as chips, burgers and fizzy drinks, have a lower abundance of bacterial species and have less species diversity and richness. These populations are more susceptible to diseases such as inflammatory bowel syndrome, asthma, obesity and metabolic dysfunction (Doggrell and Brown, 1998). Populations with diets high on carbohydrates, fibre, vegetables and non-animal proteins and oils have a more balanced microbiome with a rich species abundance and a high species diversity (De Filippo et al., 2010; Penders et al., 2006; Porter and Rettger, 1940; Yatsunenko et al., 2012). This populations are less prone to diseases that are influenced by a dysbiotic gut.

De Filippo and colleague studied the influence of diet on the gut composition these population. They compared influence of diet on the gut composition. The findings include a high abundance of *Firmicutes* and lower abundance of *Bacteroidetes* in European children. These children's diet consisted mostly of sugar, animal protein and fat and high calorie foods. Those in rural Africa had a high abundance of both *Bacteroidetes* and *Firmicutes* and displayed a healthier balanced gut microbiota. They concluded that westernized diets limit the growth and adaptive potential of microbiota (De Filippo et al., 2010).

1.1.7 Bacterial community in the gastrointestinal tract

Over a thousand-microbial species reside in the gastrointestinal tract (GIT) tract (Qin et al., 2010). Most of the bacteria falls under four major bacterial phyla; *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*. The gut is composed of mostly *Bacteroidetes* and *Firmicutes*. *Bacteroidetes* are a phylum that are predominantly gram-negative, rod-shaped, non-spore forming bacteria that either be anaerobic or aerobic. They reside in different environments; for example, they can be found in the soil, sea water, guts and epidermis of various animals. They are a crucial part of a healthy gut microbiome and some of them have been identified as opportunistic pathogens (Mor and Kwon, 2015).

The phylum *Firmicutes* is made of endospore forming, gram-positive bacteria. The bacterial cells from this phylum that can either be cocci or bacillus. There have been a few exceptions of bacteria that have a pseudo outer membrane which causes them to have a negative-gram stain. The endospores produced are desiccation resistant thus giving them the ability to survive very harsh environmental conditions. *Firmicutes* can be found in different environments and form the largest group of bacteria in the gut microbiome of humans and animals (Ley et al., 2006)

Actinobacteria are a phylum of gram-positive bacteria. They can be found in aquatic and terrestrial environments. They are not a broad group in the gut microbiota but are important for forestry and agriculture. They form a symbiotic relationship with plants and those that reside in the soil are needed for nitrogen fixation (Ghai et al., 2011).

The phylum *Proteobacteria* contains major human pathogens; *Salmonella, Helicobacter, Vibrio* and *Escherichia*. They are gram-negative bacteria some of which are non-pathogenic and are needed in the environment for nitrogen fixation. Most of the pathogenic bacteria from this phylum reside in the stomach. *Helicobacter* for example, has a urease enzyme and can

break down urea in the stomach to ammonia, carbon dioxide and water. This increases the pH in the stomach thus allowing growth of the bacteria. These pathogenic bacteria are of clinical significance. *Escherichia* causes diarrhoea, *Helicobacter* species have been implicated in gastric cancer, ulcers and inflammatory bowel syndrome and *Salmonella* infections cause diarrhoea, vomiting and fever (Mor and Kwon, 2015).

Gut microbiota can further be grouped into three types of enterotypes. An enterotype is a form of classification based on the bacterial ecosystem in which the bacteria are found. Enterotypes are clustered based on the long-term effects of a diet and are not affected by age, geographical location or gender. *Bacteroides*, type 1, are associated with a diet high in saturated fats and animal protein. *Prevotella*, type 2, are linked to diets high in simple sugars and *Ruminococcus*, type 3, currently under investigation (Arumugan et al., 2011; Zimmer, 2011).

1.1.8. Distribution of bacteria in the gastrointestinal tract

Bacteria are not evenly distributed along the gastrointestinal tract. The stomach has the least bacterial abundance and species diversity because of its harsh environment. The pH in the stomach is very acidic thus preventing various bacterial species to colonize it. Most bacteria that colonize the stomach are pathogens from *Proteobacteria* phylum. *Streptococcus*, *Staphylococcus*, *Lactobacillus*, and *Peptostreptococcus* are the dominant species found in the stomach.

The intestines have a high species diversity and high species richness. The small intestines are home to gram positive cocci/rod shaped bacteria. The duodenum, due to its proximity to the stomach, does not have a high species abundance. The ileum and jejunum have a higher pH thus allowing for gram negative bacteria to reside in it (Canny and McCormick, 2008; Porter and Rettger, 1940). The large intestines have the largest microbial diversity and a high species richness. Bacteria in the large intestines are obligate anaerobes and form part of *Bacteroides* enterotype. These bacteria assist in breaking down undigestible compounds in foods such as complex carbohydrates, starches, sugars and oligosaccharides. Whereas humans lack enzymes that can breakdown these compounds, bacteria ferment these carbohydrates to short chain fatty acids such as acetic acid, butyric acid and propionic acid. These short chain fatty acids have functions in regulating cellular processes including the differentiation, apoptosis, gene expression, chemotaxis and proliferation. Table 1.1 below shows common metabolites produced by gut microbiota (Stearns et al., 2011; Thursby and Juge, 2017; Xu et al., 2017).

Table 1.1 Gut microbiota and metabolites they produce

Phyla	Common Genera	Metabolites they produce	Metabolite function	References
Firmicutes	• *Staphyloccocus* • *Lactobacillus* • *Clostridium* • *Faecalibacterium*	Short Chain Fatty acids: Propionate, Acetate, Butyrate, isobutyrate, 2-methylpropionate, valerate, isovalerate, hexanoate and Tryptophan	decrease pH in the colon, inhibit lipolysis and lipogenesis in the liver, antimicrobial activity, helps with absorption of water and sodium, cholesterol synthesis, have role in development of obesity and colorectal cancer.	(Nicholson et al., 2012; Samuel et al., 2008; Scheppach, 1994; Wong et al., 2006)
Bacteroidetes	• *Bacteroides* • *Prevotella*	Short Chain Fatty acids: Propionate, Acetate and Butyrate Bile acids: cholate, hyocholate, deoxycholate, chenodeoxycholate, α-muricholate, b-muricholate, w-muricholate, taurocholate	-reduction of visceral fat, protection against pathogens and infections, involved in metabolism, immunomodulatory effect, protection against inflammation, mediating host-microbe interactions, energy and sugar homeostasis.	(Groh et al., 1993; Lin and Zhang, 2017; Nicholson et al., 2012; Ridlon et al., 2006, 2014; Swann et al., 2011)
Proteobacteria	• *Helicobacter* • *Escherichia* • *Salmonela* • *Vibrio*	Tryptophan, bile acids, lipids: cholesterol, polyamines: spermine and putrescine, cadaverine	enhancing the immune system, cholesterol production, induction of chronic systemic inflammation, anti-inflammatory and antitumoral effects. Impact intestinal permeability involved in glucose homeostasis and some have been identified to be potential tumour markers.	(Cani et al., 2007; Hanfrey et al., 2011; Matsumoto et al., 2012; Nicholson et al., 2012; Ridlon et al., 2014; Serino et al., 2011; Swann et al., 2011)
Actinobacteria	• *Streptomyces* • *Gardnerella* • *Bifidobacterium*	Tetracyclines, daptomycin aminoglycosides, tigecycline, tryptophan & vitamins	antimicrobial, antiviral and antifungal activity can function as immunomodifiers, they have antitumor activity and can provide alternate sources of vitamins.	(Janardahn et al., 2014; Mahajan and Balachandran, 2012; Ventura et al., 2007)

1.1.9 Gut dysbiosis in human diseases: role in hypertension

There is a link between gut microbiota and various diseases. The human gut microbiota has been associated with chronic diseases such as metabolic syndrome, obesity, alcoholic liver disease (ALD), inflammatory bowel disease (IBD), atherosclerosis, liver disease (NAFLD), diabetes mellitus and cirrhosis (Wang & Kasper, 2014). Most of these diseases develop from an imbalance in the gut microbiota, or dysbiosis, which may be caused by use of antibiotics, certain diets, the influence of certain diseases, the complex interactions amongst gut flora with diet, host genes and the host environment (Mell et al., 2015; Yang et al., 2015).

In metabolic disorders such as obesity, type 2 diabetes and cardiovascular disease, a dysbiotic gut influences production of various immune modulators which induces malfunction of several metabolic pathways (Blandino et al., 2016; Serino et al., 2011; Wu et al., 2015). However, there is very little evidence linking gut microbiota with hypertension (Li et al., 2017; Mell et al., 2015; Wang & Kasper, 2014; Yang et al., 2015). Studies have shown that a decrease in species diversity and microbial richness in hypertensive rat models affected blood pressure furthermore an increase in *Firmicutes/Bacteroidetes* ratio was reported in hypertensive rat models and hypertensive patients (Mell et al., 2015; Yang et al., 2015).

Yang et al., (2015) provided the first evidence of an association of hypertension and gut microbiota. They investigated gut dysbiosis, species diversity and microbial richness and compared gut flora in hypertensive and normotensive rat models and hypertensive patients. The findings were: a decrease in species diversity and microbial richness, an increase in *Firmicutes*: *Bacteroidetes* ratio and presence of dysbiotic gut in hypertensive patients (Yang et al., 2015). The results found in this study were correlating with the findings of this research. They further investigated and compared metabolites produced by gut bacteria in both hypertensive and normotensive models.

Mell et al., (2015) determined the species diversity and abundance on Dahl rat models, the Dahl resistant (R) and Dahl salt-sensitive rats (S). They used 16S rRNA sequencing for species identification. They compared distribution of gut bacteria (diversity and abundance) in both rat models. They observed a high abundance of *Firmicutes* and *Bacteroidetes* in R rats while S rats displayed less species richness and diversity and an increase in *Firmicutes: Bacteroidetes* ratio. When fed a high salt diet, blood pressure in R rats did not change while the systolic and mean arterial pressure increased significantly for S rats (Mell et al., 2015).

To investigate the relationship between hypertension and gut microbiota, cecal and faecal microbiota from S models were transplanted to R and vice versa, from S to S and R to R. S rats transplanted with R cecal content were hypertensive through the duration of the study. Mean blood pressure increased in R to S transplantation when compared to S to S and R to R transplantation. The expected outcome was for R to S transplantation to bring about normotension in S rats, and for S to R transplantation for an increase in blood pressure. These findings suggested that gut microbiota have little to no significant difference in development of salt induced hypertension in Dahl rat models (Mell et al., 2015). Very little remains known about the influence of gut microbiota in the development of salt sensitive hypertension. Many argue that salt sensitive hypertension is caused by genetic factors while there are reports that the gut microbiota may have a role in development of salt sensitive hypertension. This difference may attribute to the difference of pathogenesis in both animal models and is a field requiring further research.

Thus far, it is not known if gut dysbiosis influences development of hypertension or if hypertension causes gut dysbiosis. Few studies have described that there is a link between hypertension and gut microbiota. Such studies will contribute to strategies that may be used to understand development of hypertension.

1.1.10 Hypertension in humans compared to rat models

Animal models have been used to study diseases, the pathogenesis of disease and drug interventions to treat such diseases. For hypertension and cardiovascular diseases, spontaneously hypertensive rat models (SHR) and Dahl rat models have been widely used. In this study, rat models were used to study the differences in gut microbial diversity and species abundance in hypertension.

SHR develop hypertension 6-8 weeks after being bred and by 12 weeks they develop severe hypertension. The development of hypertension in SHR is similar to that in humans. First, there is a pre-hypertensive phase, with a systolic blood pressure ranging from 100-120mm Hg, then a hypertensive stage that will usually progress to end stage organ failure. In humans, hypertension follows a similar pattern thus making SHRs valuable for studies in hypertension. A disadvantage to using SHR is the lack in genetic variation in the rat models. In humans, genetic predisposition plays a crucial role in development of hypertension, therefore

development of hypertension may vary from one individual to the next (Doggrell and Brown, 1998; Pinto et al., 1998).

To study genetic variation in hypertension, Dahl bred salt sensitive and salt resistant rat models (Dahl, 1961; Dahl et al., 1967a, 1972). These were bred after an observation that salt ingestion affected blood pressure and these rats develop hypertension when fed a high salt diet. Dahl salt resistant rats are not affected by salt intake do not develop hypertension on a high salt diet. Some individuals, especially with African ancestry, are sensitive to salt and develop hypertension when they have a high salt intake. In contrast, other individuals and populations, do not develop hypertension on a high salt diet and thus the Dahl rats are useful to study salt sensitivity (Doggrell and Brown, 1998; Pinto et al., 1998).

Both rats and humans have the mouth, stomach, small and large intestines, cecum and anus. The stomach and intestines are lined with mucosa, Paneth cells, adaptive enterocytes, goblet cells and enteroendocrine cells. The major differences in the anatomy is the presence of a large cecum in rats, where significant metabolism occurs. In contrast, the appendix is the equivalent to the caecum, and is essentially a redundant part of the GIT in humans. The gut microbiota is fairly conserved in mammals, and as both humans and rats are omnivores, one would anticipate similarities in the composition of the microbiome of rats and humans. Studies have shown that both human and rat microbiota are dominated by *Bacteroidetes* and *Firmicutes* and have differences at a species level that reside in their gut (Ley et al., 2006; Nguyen et al., 2015; Rawls et al., 2006). Thus, the study of the microbiome in these rat models of hypertension may provide insight into in impact of the microbiome on hypertension in humans.

1.2 Problem statement

Hypertension is the leading cause of morbidity and mortality worldwide. The prevalence of hypertension is rapidly increasing in both developing and underdeveloped countries. Worldwide, the cost of treating hypertension and cardiovascular disease is $80-billion annually and yet the cause of essential hypertension remains unknown. Few studies have determined the associations between gut microbiota, blood pressure and development hypertension. The role of gut microbiota in health and disease has been a rapidly emerging field of research and has been implicated in the pathogenesis of various diseases. My study aimed to utilize rat models of hypertension and determine the association between composition and abundance of the gut microbiota/microbiome and hypertension and consequently to identify potentially important

bacteria associated with this disease. This study may provide further insight in the aetiology of hypertension and may suggest novel treatments for the reduction and potentially the prevention of hypertension.

1.3 Research questions

- Is there a difference in the gut microbiome composition between hypertensive and normotensive rat models?
- Is this difference associated with hypertension?

1.4 Hypothesis

There is a difference in gut microbial composition (abundance and diversity) between hypertensive and normotensive rat models and this difference is associated with hypertension.

1.5 Aim

The aims of this study were to;

1) determine the differences in the diversity and abundance of the composition of the gut microbiome in the different rat models of hypertension;
2) determine the association between the diversity and abundance of the gut microbiome and hypertension in these rat models.

1.6 Objectives

- To harvest the gastrointestinal system of different rat models of hypertension and control rats and isolate the gut microbiome;
- To culture bacteria and identify culturable gut microbiota from the gastrointestinal sections using biochemical tests and Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALD-TOF MS);
- To isolate the bacterial genome and use 16S rRNA gene sequencing to analyse the microbiota composition, abundance and diversity of both cultural and non-culturable bacteria.

Chapter 2: Material and Methods

2.1 Materials

Table A1-A5 shows materials used for the studies. Materials include reagents, consumables, software and equipment.

2.2 METHODS

2.2.1 Overview

Ten rats representing models of hypertension (3 SHR, 3 SSR and 4 Control) were used in this study. The gastrointestinal tract (stomach, small intestine and large intestine) was harvested from each rat. There was a total of 34 samples. The work was done in triplicates for reproducibility. Culturable bacteria were isolated and identified using biochemical tests and MALDI-TOF MS. Furthermore, DNA was extracted from the harvested GIT samples and 16s SRNA sequencing was performed using Illumina Miseq. Data analysis was performed using CLC Microbial Genomics tools. Furthermore, analysed data from 16S rRNA sequencing was represented in the form of pie charts generated from Microsoft excel. Figure 2.1 below is a diagram representing the work flow.

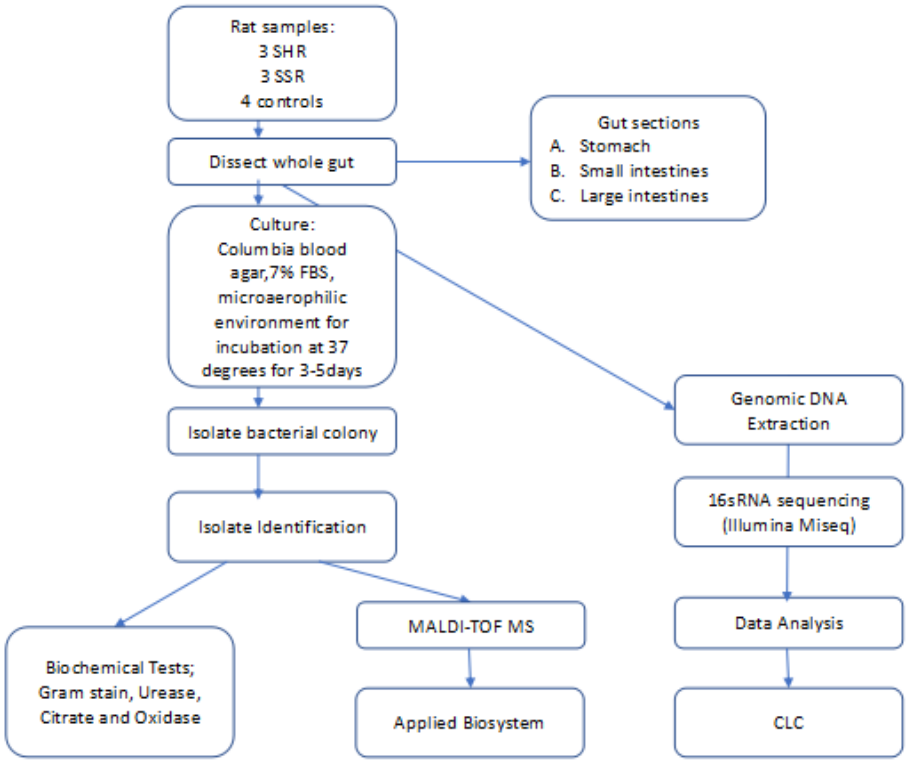


Figure 2.1: A flow diagram showing the work flow of this project.

2.2.2 Animal models

The study protocol was approved by the University of Witwatersrand Animal Research Ethics Committee (Ethics Waiver 08-05-2017-O). Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Animals, 2011). Salt sensitive rats (SSR), spontaneously hypertensive rats (SHR) and control Dahl rats were received from a study by Profs GR Norton and AJ Woodiwiss from The Wits Cardiovascular Pathophysiology Research Unit. All the rats were males and were 3months old when terminated. They were fed a standard diet as noted in the introduction, SSR rats develop hypertension when fed a high salt diet (1.8 g/ml more salt than the controls) and SHRs are genetically modified to develop hypertension from infancy.

2.2.3 Sample harvesting

The rats were dissected at the University of the Witwatersrand Central Animal Services Unit. Once the rat models were declared to be hypertensive, they were euthanized. Whole gut biopsies from the stomach, small and large intestines were collected from 3 SSR, 3 SHR and 4 control rats. Half of the biopsy sample was used for culture and the other half for DNA extraction. Biopsies for DNA extractions were stored in 10ml saline while biopsies for culture were stored in 10ml Brain Heart Infusion broth transport media. The samples were stored in 50ml tube at 4˚C for 2hours and taken to the lab for culture.

2.2.4 Culture and growth of Isolates

Culturing biopsies was performed to isolate bacteria that will be used for further experiments and to also study the biochemical profiles of the isolates. Biopsies were disrupted and homogenised using the vortex-bead beating method described by OPS Diagnostics (https://opsdiagnostics.com/products/beadbeating.htm). The biopsies were vortexed until they became a homogenous suspension. Two types of plates were prepared for culture;500 ml selective Columbia blood agar base (skirrows:10 mg/l vancomycin, polymyxin B 2500 units/l and trimethoprim 5 mg/l) and 500 ml non-selective Columbia blood agar base. Selective media was used to select for *Helicobacter* and *Campylobacter* species. Both media were supplemented with 5% FBS. Spread plates were prepared from 200 µL of the homogeneous suspension. They were incubated in micro-aerophilic conditions (5% O_2;10% CO_2;85% N_2) for 3-7 days in anaerobic jars at 37˚C incubation until colonies appeared. Individual colonies were

isolated from the spread plates. The isolates were sub-cultured continuously until pure colonies were achieved for each isolate.

2.2.5 Identification of microbial isolates

Biochemical and morphological tests

The gram stain, urease test, oxidase test and catalase test were used to identify the microbial isolates. Table 2.1 shows how results of the various biochemical tests were interpreted.

Table 2.1: How results from biochemical tests will be interpreted

Biochemical Test	Positive results (+)	Negative results (-)
Gram stain	Purple/violet colour	Pink colour
Urease Test	Media turns pink	No colour change
Oxidase Test	Blue colour	No colour change
Catalase Test	Bubble formation	No bubbles

2.2.6 Matrix-assisted laser desorption/ionization-Time of flight Mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS allows rapid identification of microorganisms based on the mass spectral pattern of the bacterial proteins. This mass spectra are specific for a given microorganism and can be used to identify bacteria, fungi and yeast by comparing the spectra to a reference database. MALDI-TOF MS can differentiate microorganisms at a genus, species and subspecies level (Zhou et al., 2017).

Sample preparation

To obtain cellular extracts, pure bacterial colonies (isolated by Loop last® inoculation loops) were washed with 70% ethanol. Forty microliters of 70% formic acid and pure ACN were added in a 1:1 (v/v) ratio to the bacterial pellet, and the mixture was vortexed for 30 seconds then centrifuged on a micro-centrifuge for 2 mins at 1300 rpm. The supernatant was transferred to new Eppendorf tube and were used to spot on the MALDI plate. The 96-well stainless steel MALDI target plate was spotted with a drop of the supernatant and a smeared with the respective bacteria colony the protein was extracted from. The spots and smears were left at room temperature to dry. The spotting and colony smearing were done in duplicated to check result accuracy.

To reconstitute the matrix (HCCA), α-cyano-4- hydroxycinnamic acid (CHCA) (5 mg/ml) in a 50:48:2 acetonitrile (ACN): water: trifluoroacetic acid (TFA) matrix solution was prepared. A microlitre of the matrix was overlayed on the spots and colony smears and left at room temperature to dry. Once dry, the target plate was placed in the MALDI-TOF MS and identification was performed.

MALDI-TOF MS sample analysis

Sample analysis was done at the National Health Laboratory Services, Johannesburg, South Africa. The experiment was carried out as described by the Bruker user manual (www.bruker.com).

Mass spectra were obtained using an Auto flex II MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Measurements were performed in linear positive ion mode, using a nitrogen laser (337 nm) at 50 Hz frequency. The acceleration voltage was 20 kV, with delay time acquisition and mass range of 3000–20,000 *m/z*. External calibration was performed prior to each analysis using a mix of insulin (5734.51 *m/z*), ubiquitin I (8565.76 *m/z*) and cytochrome c (12,360.97 *m/z*) (Protein Calibration Standard I – Bruker Daltonics). Automated spectra acquisition was performed using the Auto Execute tool of Flex Control 3.0 (Bruker-Daltonics) with fuzzy control of laser intensity. For each sample a total of 1000 laser shots were accumulated in 100 laser shots steps in 10 different regions of the same sample. The raw data were converted into a peak list using Flex Analysis 3.0 software (Bruker Daltonics, Bremen, Germany), peak picking was performed using the method centroid of the peak, height 80%, peak with 0.1 *m/z* and signal/noise greater than 3. This was followed by one round of base line subtraction and smoothing.

MALDI-TOF MS data analysis of isolated bacteria was done using SPECLUST. The program calculates the peak match score, giving the probability that the two peaks originated from the same peptide. The clustering procedures calculate the similarity score for each pair of peak lists, followed by calculating distances based on the similarity scores. Finally, a linkage procedure was applied to merge the clusters and construct the dendrogram. The SPECLUST parameters were set as follows: “liberal distance” for spectra grouping; “average” for distance calculation between groups; and a mass tolerance error of ±8 Da (Stets et al., 2013).

2.2.7 Genomic DNA Isolation and 16S rRNA Gene Sequencing

16S rRNA gene sequencing is a technique used to identify microorganisms in different taxonomic levels. Identification and characterization of microorganisms is based on the presence of the hyper variable regions of the 16S rRNA gene. In this region there is a specific

signature sequence unique in each microorganism thus allowing for species identification. 16S rRNA sequencing is also widely used to measure species abundance and species diversity in prokaryotes. 16S rRNA gene sequencing was performed to identify bot culturable and non-culturable bacteria (Logares et al., 2014).

Genomic DNA from gut biopsies were isolated using the Qiagen QIAamp DNA Mini Kit supplied by ThermoFischer Scientic®. This was performed as described by the user manual. To prepare a library, the V3–V4 regions of 16S rRNA genes were amplified using universal primers as described in the 16S Metagenonic Sequencing Library Preparation protocol;

Forward Primer = 5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'

Reverse Primer =

5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC
C-3'

Adapter sequences that were locus specific were used:

Forward overhang: 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'

Reverse overhang:5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

Libraries were pooled using Nextera XT indices. Sequencing was performed on Illumina Miseq, this yields over20 million reads then analysed on Miseq CLC Reporter.The number of reads generated can be seen in Appendix A table A16. The Metagenomics Workflow performs a taxonomic classification using the Greengenes database showing genus or species level classification in a graphical format as described in the 16S Metagenomic Sequence Preparation protocol.

Data Analysis

Reads in FASTQ format were imported as pairs to CLC Genomics Workbench v. 5.1 (CLC Bio) and trimmed using a minimum phred score of 20, a minimum length of 50 bp, allowing no ambiguous nucleotides and trimming off Illumina sequencing adaptors if found. Amplicon based analysis was performed. Reads within 97% similarities were clustered together and represented as one sequence, the cluster of reads is referred to as Operation Taxonomic Units (OTU). These were used to generate abundance tables that show abundance of all samples

and OTU. To determine which organisms are in a metagenomic sample and how abundant they are, taxonomy analysis was performed. Taxonomic profiler tools generated a list of the taxons. This data is represented in an abundance table. The data is represented in a stacked bar. Furthermore, abundance tables from the different samples were merged and a stacked graph was generated.

Statistical analysis

Two analysis of this merged data were Alpha diversity and Beta diversity. Alpha diversity was used to estimate the number of species in the different samples and Beta diversity was used to examine changes in species diversity between the samples. The Beta diversity tool calculations generate principal coordinate analysis (PcoA) on distance matrices. PERMANOVA (PERmutational Multivariate Analysis of Variance, also known as non-parameteric MANOVA) was used to measure effect size and significance on beta diversity for a grouping variable and was used as a measure for statistical significance. A heat map tool was used to generate a heat map showing the abundance of each feature in each sample and to show the sample clustering and/or feature clustering as a binary tree over the samples and features, respectively. More details of the protocol can be found on the link below: http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/702/User_Manual.pdf

Chapter 3: Results

3.1 Results overview

Alteration of the gut microbiota is associated with hypertension. This is marked by an imbalance in composition, abundance and diversity of bacterial species in the gut. To investigate this, the gut microbiome in rat models of hypertension was studied. The stomach, small and large intestines were harvested. These were used for both culture and DNA extractions. In culture, all isolates were identified using biochemical tests and MALDI-TOF MS. For an invasive and more accurate representation of the gut microbiota, 16S rRNA gene sequencing was performed using an Illumina Miseq sequencer. CLC genomics workbench was used for data analysis and results were represented in tables, bar graphs and pie charts.

In culturable isolates, there was an abundance of *Escherichia coli* and *Lactobacillus murinus* in all the rat models. Control rat models had a higher species abundance and higher species diversity when compared to SSRs and SHRs. Interestingly, there was the absence of bacterial species from *Bacteroidetes* in hypertensive rat models and a high abundance of species from *Firmicutes*. A balance in the *Firmicutes* to *Bacteroidetes* ratio is crucial for maintaining a healthy gut microbiome. An increase in *Firmicutes* and decrease in *Bacteroidetes* is indicative of a dysbiotic gut. Because dysbiosis was only present in hypertensive rat models this hints to an association between gut microbiota and hypertension.

16S rRNA gene sequencing gives more information on both culturable and non-culturable bacteria. The results from sequencing were classified according to taxonomy. The results revealed that normotensive rat models had a higher species abundance and more species diversity when compared to the hypertensive rat models. The results obtained correlated with results achieved from culture; there was a decrease in *Bacteroidetes* and increase in *Firmicutes* abundance. Results from this study have showed the presence of a dysbiotic gut in hypertensive rat models, thus associating gut microbiota with hypertension. In the following sections, these results are described in detail.

3.2 Identification of culturable isolates

Routine preliminary identification of gut bacterial isolates prior to downstream analyses done included; gram stain, urease, oxidase and catalase tests. This was then followed by MALDI TOF MS analysis. Table 3.1 shows results from biochemical tests and MALDI-TOF MS identification.

Table 3.1: Biochemical test and MALDI-TOF results from top three isolated bacteria

Rat model	MALDI-TOF MS	Biochemical tests			
	organism	Gram stain	Urease	Catalase	Oxidase
SHR	*Escherichia coli*	-	-	-	-
	Staphylococcus sciuri	+	+	+	+
	Klebsiella pneumoniae	-	+	+	-
SSR	*Pseudomonas aeruginosa*	-	-	+	+
	Bacillus cereus	+	-	-	+
	Escherichia coli	-	-	-	-
Control	*Escherichia coli*	-	-	-	-
	Bacillus cereus	+	-	-	+
	Lactobacillus murinus	+	+	-	-

3.3 Phyla present in rat models of hypertension.

The data generated from MALDI-TOF MS was represented in tables using Microsoft excel. The species represented in table 3.1 were grouped according to the phyla they belong to. Table 3.2 below shows the different phyla that were identified from the different models of hypertension. All the rat models had bacterial species from *Firmicutes* and *Proteobacteria*. SHR and SSR had a slightly higher abundance of *Firmicutes* and *Proteobacteria*, while the control rat models had bacterial species from *Firmicutes, Proteobacteria* and *Bacteroidetes* (Table 3.2).

Table 3.2: Different phyla identified from culture in different rat models of hypertension

Rat model	**Organism**	**Phylum**
SHR	*Escherichia coli*	*Proteobacteria*
	Staphylococcus sciuri	*Firmicutes*
	Bacillus cereus	*Firmicutes*
SSR	*Bacillus cereus*	*Firmicutes*
	Escherichia coli	*Proteobacteria*
	Staphylococcus sciuri	*Firmicutes*
Control	*Myroides odoratimimus*	*Bacteroidetes*
	Bordetella petrii	*Proteobacteria*
	Enterococcus faecalis	*Firmicutes*

3.4 Comparison of bacterial species abundance and diversity in SHR, SSR and control rat models.

To further investigate the difference in gut composition between hypertensive and normotensive rat models, data retrieved from MALDI-TOF MS were grouped according to species. *Escherichia coli, Lactobacillus murinus, Enterococcus faecalis, Staphylococcus sciuri* and *Bacillus cereus* were the species that were common in all the rat models (Figure 3.3). The control group had the highest species abundance and diversity with an abundance of *Escherichia coli, Lactobacillus murinus, Enterococcus faecalis* and *Bacillus cereus*. SHRs had an abundance of *Klebsiella pneumonia, Escherichia coli, Staphylococcus sciuri and Enterococcus faecalis*. *Pseudomonas aeruginosa, Escherichia coli, Lactobacillus murinus* and *Bacillus cereus* were the most abundant in SSRs. SHR and SSR had less species abundance and diversity when compared to control rat models indicating that hypertensive and normotensive rat models have a different microbial gut composition.

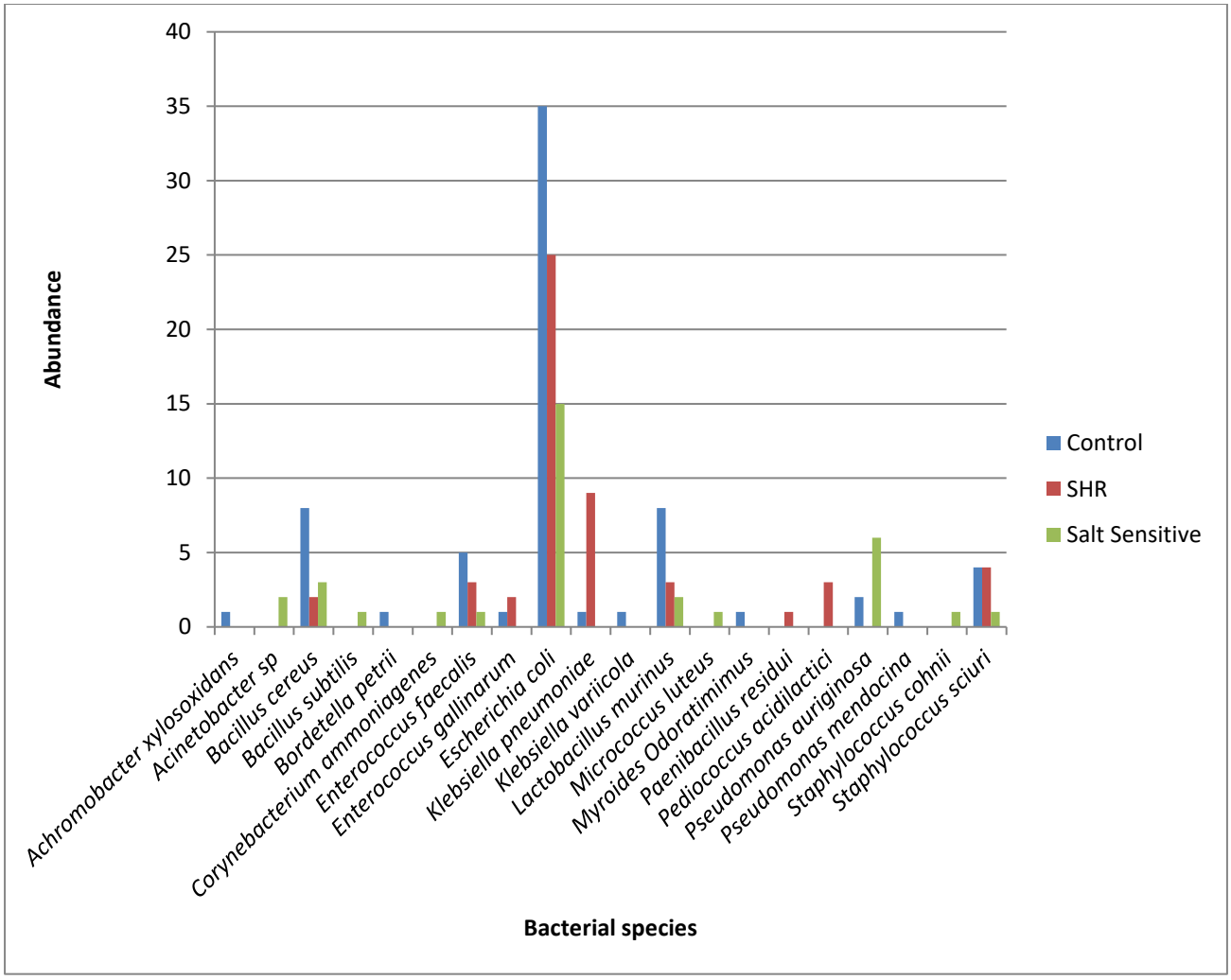


Figure 3.3: **Bar graph showing bacteria species identified in control, SHR, and salt sensitive rats.**

Furthermore, the bacterial isolates identified were grouped according to the different phyla (Figure 3.4). All the rat models had species from *Firmicutes*, *Proteobacteria*. The control rat models (normotensive group), had bacterial species from all four major phyla; *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* showing that normotensive rat models do not have dysbiosis.

SSR and SHR models had a lower abundance of species and had a low species diversity, this was marked by an increase in prevalence of *Firmicutes* and lack of *Bacteroidetes.* SHRs had an abundance of species from *Proteobacteria* and *Firmicutes* whereas no species were identified from *Actinobacteria* and *Bacteroidetes*. Several species of *Proteobacteria* have an association with development of hypertension. In SSR, only species from *Bacteroidetes* were not present. Unlike SHR, a few species of *Actinobacteria* were identified in SSR. These differences in bacteria abundance and diversity in the different rat models is indicative of a dysbiotic gut.

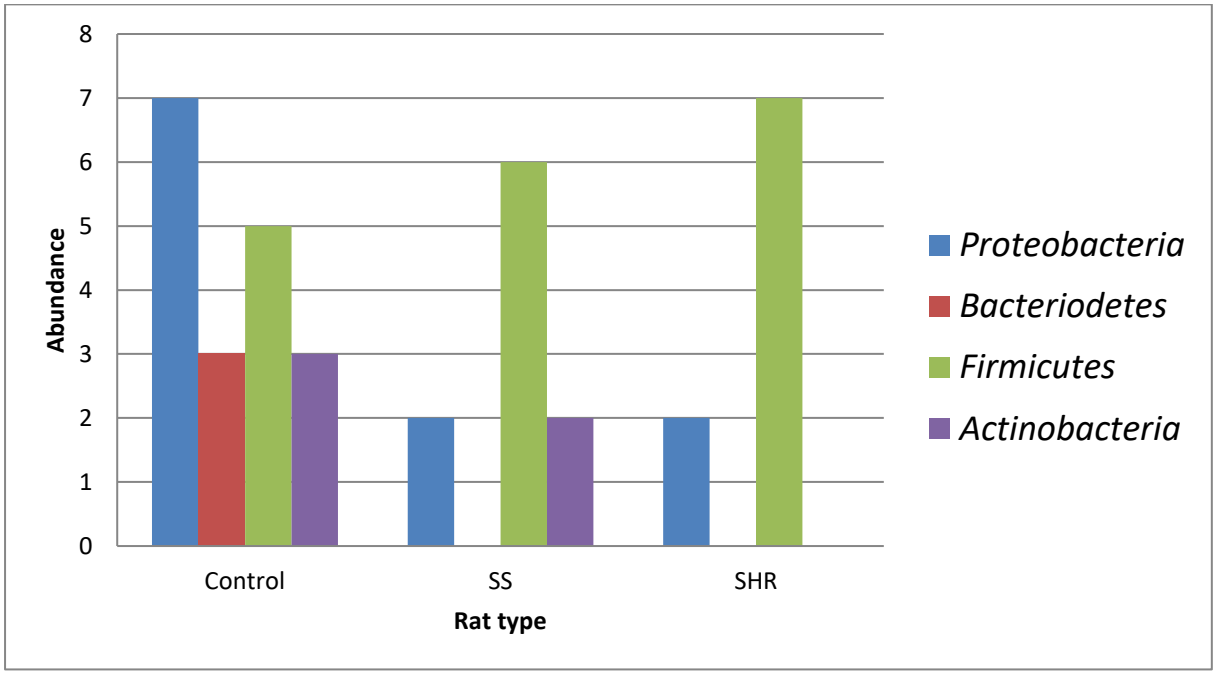


Figure 3.4: **Bar graph showing the different Phyla of bacteria that were isolated from Control, SSR, SHR and control Dahl rats**.

3.5 Species abundance and diversity in different regions of the gut of rat models of hypertension

There was a difference in the composition of species in the different regions of the gut that were sampled. To investigate this, biopsies from the stomach, small and large intestines were harvested for each rat model. The cultured isolates were identified using MALDI-TOF MS (Figure 3.5, 3.6). The stomach of all the rat models showed a lower species abundance with lack of species of diversity. *Escherichia coli*, *Lactobacillus* and *Enterococcus* species were isolated from the stomach of all the rat models. Several of these species were urease positive demonstrating how they can survive in a harsh environment (Table 3.1).

Consequently, the small and large intestines had more species abundance when compared to the stomach. In the small intestines, only *Escherichia coli* was common in all rat models. The large intestines had the most species abundance and diversity. The large intestines have a higher pH and have a high nutrient content thus supporting bacterial growth. Although both hypertensive models, SHR and SSR, had bacteria species diversity.

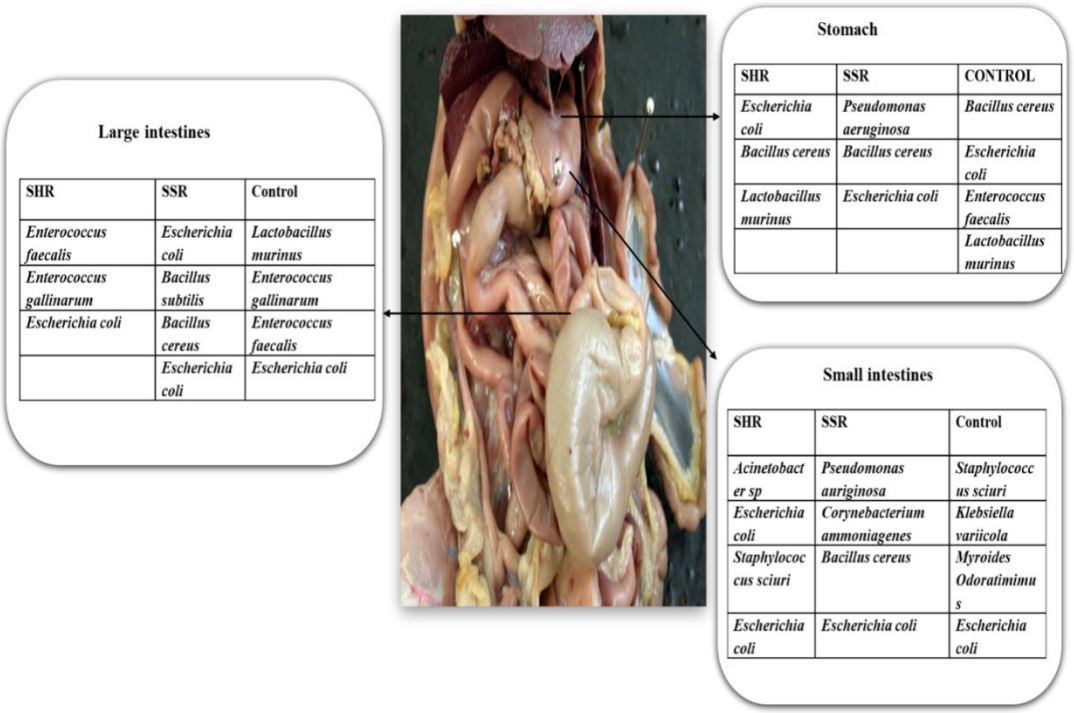


Figure 3.5: Bacterial isolates identified in the different regions of the gut in rat models of hypertension. The picture was taken by the author at the Central Animal Services unit at the University of the Witwatersrand.

When looking at the isolates at phylum level, all the rat models had bacterial species from *Proteobacteria* and *Firmicutes* across the different regions of the gut. The stomach had a high abundance of *Proteobacteria* and *Firmicutes*. Species from *Proteobacteria* are well-known to inhabit the stomach. The large intestines of control rats had species across all the phyla whilst SHR and SSR had the lack of bacterial species from *Bacteroidetes*. The lack thereof might suggest dysbiosis in hypertensive rat models.

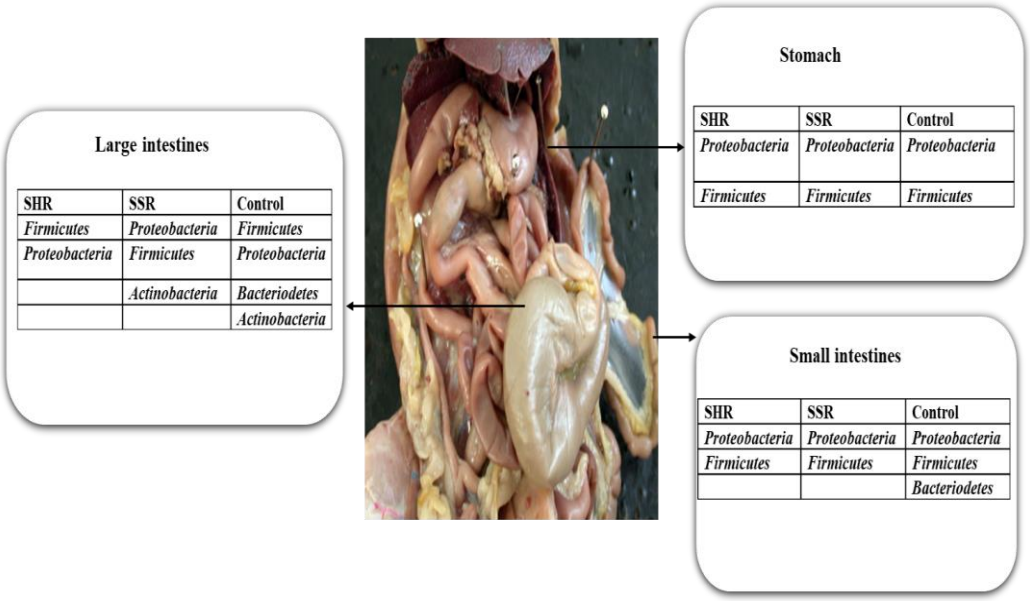


Figure 3.6: Different phyla identified along the GIT of rat models of hypertension. The picture was taken by the author at the Central Animal Services unit at the University of the Witwatersrand.

3.6 Unculturable species of the gut in rat models of hypertension.

To investigate if the lack of species diversity and low species abundance is associated with hypertension, 16S rRNA gene sequencing was performed. DNA was extracted from the biopsies harvested from the different rat models. 16S rRNA gene sequencing was performed and results were analysed using CLC Genomics and Microsoft excel was used to represent the results in pie charts and tables. Table 3.3 below shows the quality of reads from Illumina Miseq sequencer.16S rRNA gene sequencing was performed to give information on non-culturable bacteria.

Table 3.3: Total number of reads achieved from SHR, SSR and control rat model biopsies from the large intestine, small intestine and stomach.

Rat model	Region on the gut	Total Reads	Reads Passing quality filtering	% Reads Passing quality filtering
SHR	large intestine	44109	39443	89,40%
	Small intestines	30191	27140	89,90%
	Stomach	40541	36473	90%
SSR	Large intestine	74943	67482	91.1%
	Small intestines	47906	43722	91.3%
	Stomach	73452	66599	90.7%
Control	Large intestines	112174	102871	91.7%
	Small intestines	7404	6685	90.3%
	Stomach	77943	71156	91.3%

3.6.1 Identification at taxonomic level

At Kingdom level, Bacteria was the most abundant accounting for over 80% of the species identified (Figure 3.7). SHR model had a low number of species that were identified taxonomically. This might be due to several reasons including the amount and quality of DNA and technique used for analysis. There were no species of Viruses and Archea identified because of the specific gene sequence that was targeted for sequencing.

3.6.2 Abundance and diversity of bacterial species at phyla level in rat models of hypertension

Bacteria were identified at phylum level in rat models of hypertension and results are represented in Figure 3.8 below. Both hypertensive and normotensive rat models had an abundance of species from all four phyla*; Bacteroidetes, Proteobacteria*, *Firmicutes* and *Actinobacteria*. The difference between the hypertensive and normotensive rat models is the kind of species that are abundant in both rat models. Hypertensive rat models, SHR and SSR have a high prevalence of *Firmicutes* and *Proteobacteria* while normotensive rat models have a high abundance of *Bacteroidetes* and *Firmicutes* species.

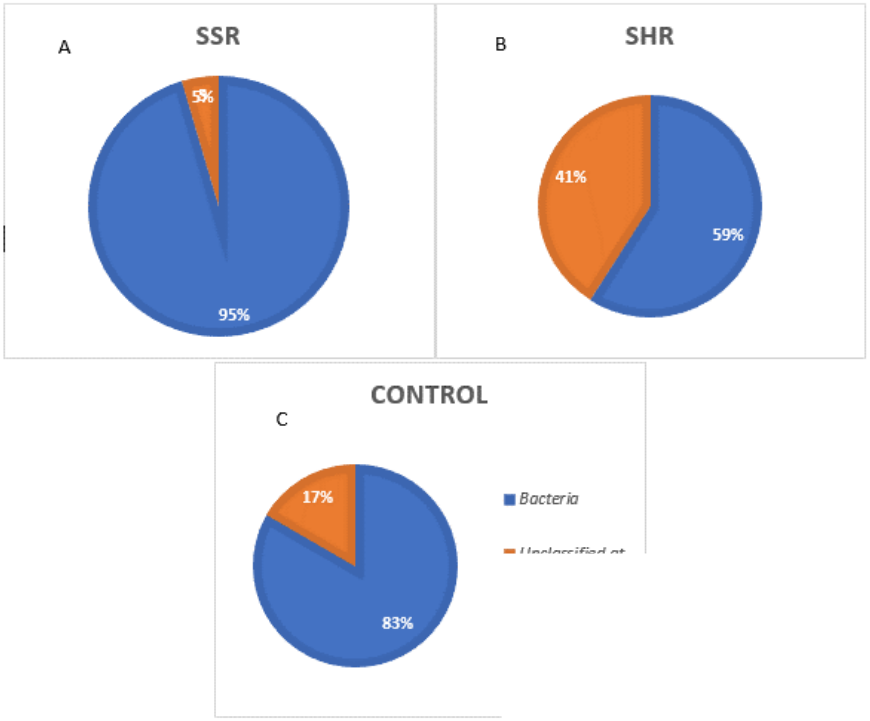


Figure 3.7: **Pie chart showing identification at Kingdom level in SSR (A), SHR (B) and control (C) rat models**. Blue is Bacteria and orange is for unclassified species at Kingdom level.

In hypertensive rat models there is an increase in the *Firmicutes*: *Bacteroidetes* ratio. In SHR models, *Proteobacteria* species had the highest abundance followed by *Firmicutes*. There was a low abundance of *Bacteroidetes*. While in SSR, *Firmicutes* were the most abundant followed by *Bacteroidetes*. Compared to SHR *Proteobacteria* abundance was very little. A decrease in abundance of *Bacteroidetes* with an increase of abundance of *Firmicutes* is a key marker for a dysbiotic gut. Since this was only observed in the hypertensive rat models, it suggests that a decrease in species abundance and diversity is associated with hypertension. *Actinobacteria* species were not identified in any of the rat models. They are not common in animal models and are had to identify since they are not common.

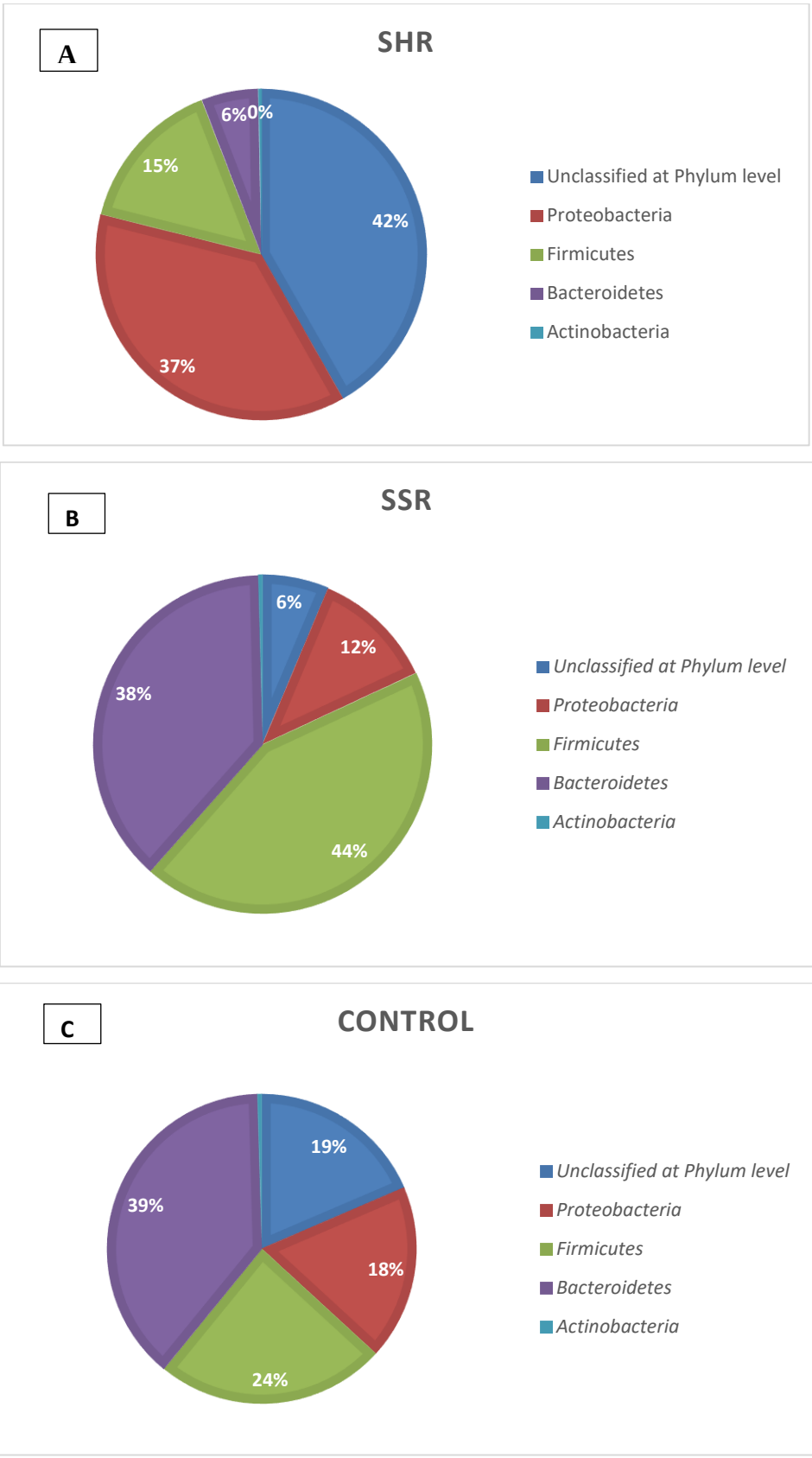


Figure 3.8: **Pie chart showing identification at Phyla level in SHR (A), SSR(B) and control (C)**.

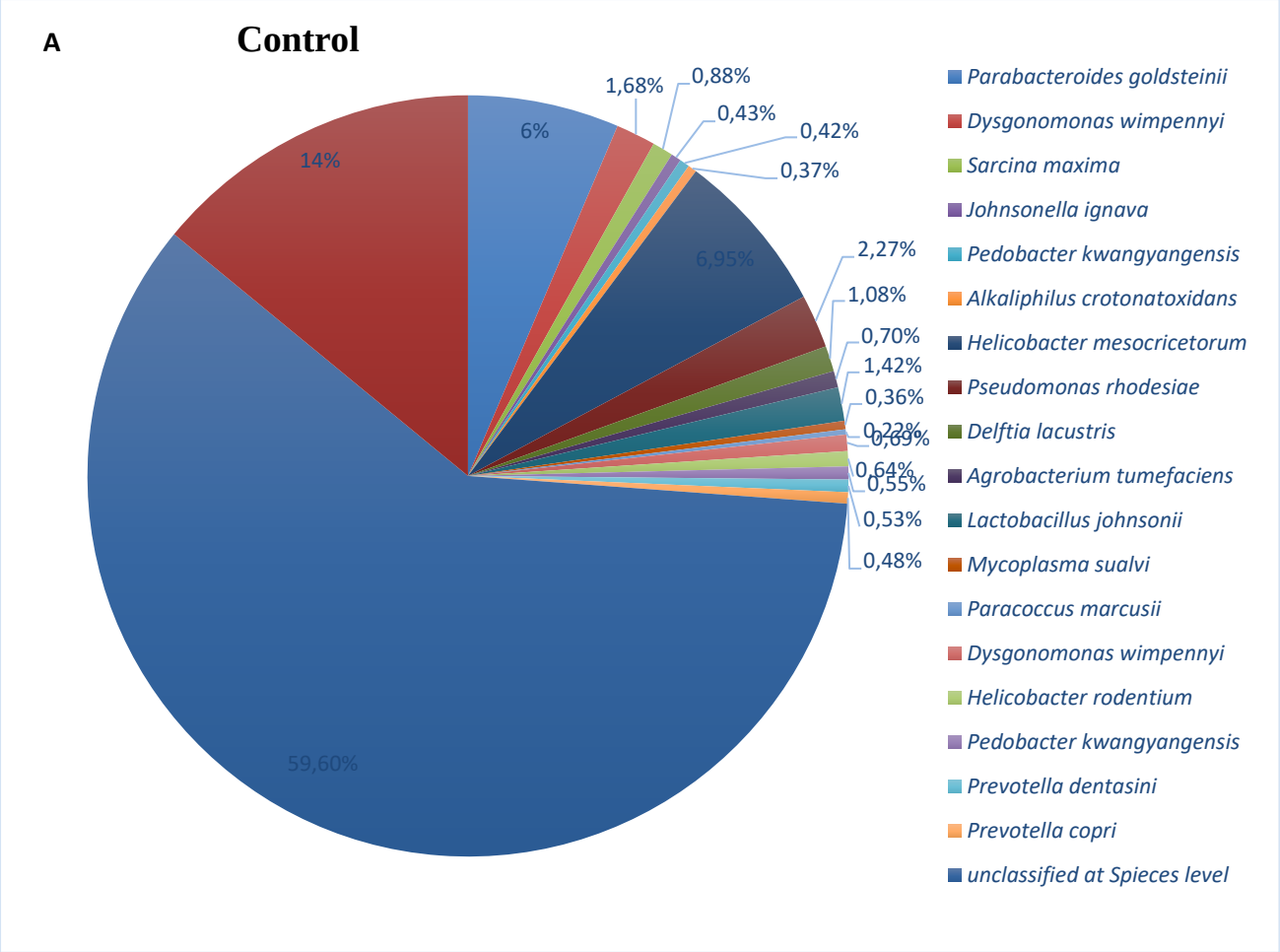
3.6.3 Species level identification in rat models of hypertension

Identifying microbiota at species level using 16S sequencing is limited because of the V3-V4 regions that are targeted in the bacterial genome. These regions are present in all bacterial species and are difficult to differentiate at species level. Only 40% of the bacteria in Control and SSRs were identified. In SHRs only 30% of the species were identified and this can be seen in figure 3.9 below.

Bacterial species common in all the three rat models were; *Parabacteriodes goldsteini, Helicobacter mesocricetorum* and *Helicobacter rodentium.* These are species from *Bacteroidetes* and *Proteobacteria* phyla. The relative abundance of *Helicobacter* species in the rat models was different. SHR models had a high prelavance of *Helicobacter mesocricetorum* and *Helicobacter ganamni*, 15% and 5% respectively. Control models had an abundance of *Helicobacter mesocricetorum with an abundance of 7%*. While SSR, had a low abundance of Helicobacter species, having both *Helicobacter rodentium* and *helicobacter mesocricetorum* at a 1% abundance. The differences in abundance of *Helicobacter* species maybe linked the differential patterns for the development of hypertension.

As expected, the control rat models, normotensives, had a high species abundance and high species diversity (Figure 3.8A). As Compared to control rats, SSRs and SHRs had a lower species diversity and species abundance (Figure 3.8 B, C). SSR had an abundance of *Provetella* species which form part of *Bacteroidetes*, the rest of the species identified, although in low abundance were from *Proteobacteria* and *Firmicutes*. Species from *Bacteroidetes* were not identified in culture, hence the lack of representation in the results from MALDI-TOF MS.

SHRs had a high abundance of *Helicobacter species*, these are part of *Proteobacteria* phylum. In all the rat models, SHRs had the least species abundance and species diversity accompanied by a high prevalence of *Proteobacteria*. The results obtained correlate with the data reported from culturable species in this study. SSR and SHR had various species similarities. For example; both had an abundance of *Paraprevotella spp, Dysgonomas wimpennyi, Hellicobacter rodentum* and *Helicobacter mesocriceterum.* And both had a lower species abundance and diversity when compared to the control rat models. This is indicative that there was gut dysbiosis in hypertensive rat models thus providing evidence that gut microbiota are associated with hypertension.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Control
6%
1,68%
0,88%
0,43%
0,42%
0,37%
6,95%
2,27%
1,08%
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0,69%
0,64%
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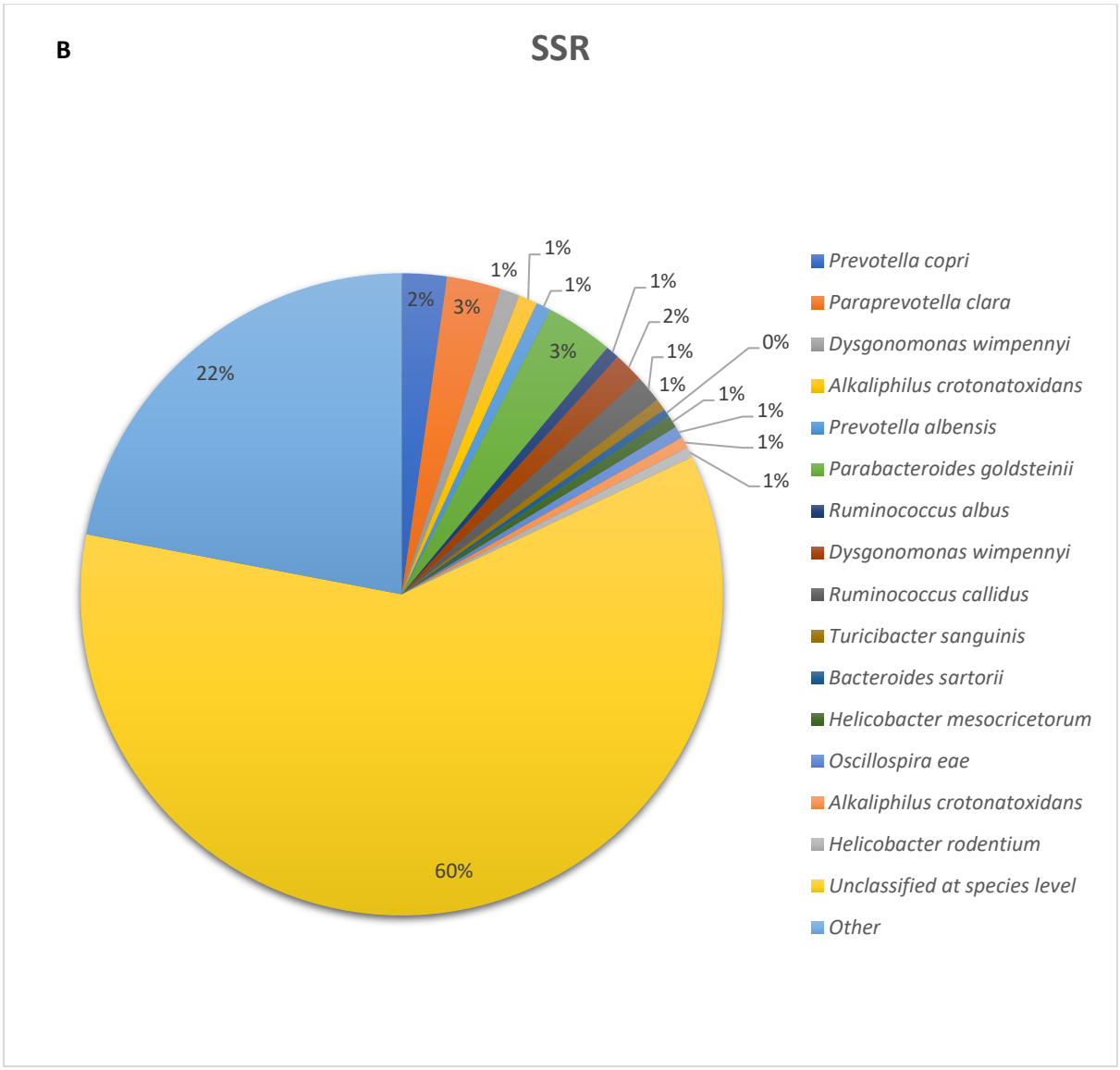
0,53%
0,48%
59,60%
14%
Parabacteroides goldsteinii
Dysgonomonas wimpennyi
Sarcina maxima
Johnsonella ignava
Pedobacter kwangyangensis
Alkaliphilus crotonatoxidans
Helicobacter mesocricetorum
Pseudomonas rhodesiae
Delftia lacustris
Agrobacterium tumefaciens
Lactobacillus johnsonii
Mycoplasma sualvi
Paracoccus marcusii
Dysgonomonas wimpennyi
Helicobacter rodentium
Pedobacter kwangyangensis
Prevotella dentasini
Prevotella copri
unclassified at Spieces level


B
SSR
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3%
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2%
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1%
1%
22%
60%
Prevotella copri
Paraprevotella clara
Dysgonomonas wimpennyi
Alkaliphilus crotonatoxidans
Prevotella albensis
Parabacteroides goldsteinii
Ruminococcus albus
Dysgonomonas wimpennyi
Ruminococcus callidus
Turicibacter sanguinis
Bacteroides sartorii
Helicobacter mesocricetorum
Oscillospira eae
Alkaliphilus crotonatoxidans
Helicobacter rodentium
Unclassified at species level
Other

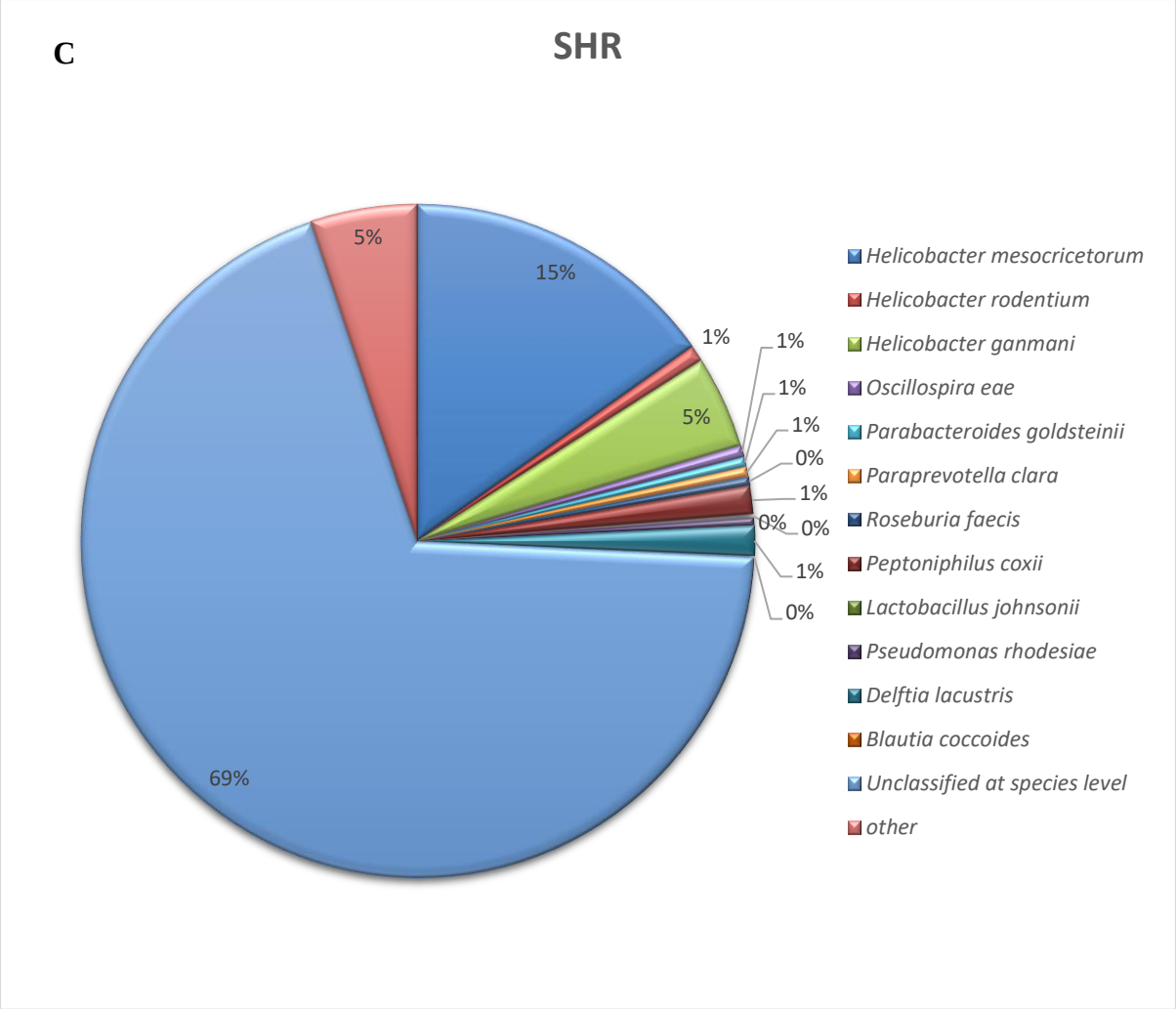

Figure 3.9**: Pie chart showing the number of species identified in Control (A), SSR (B) and SHR (C)**.

3.7 Taxonomic clustering of bacteria species

Based on the data obtained from both MALDI-TOF MS and I6s rRNA sequencing, there are observed differences in the gut microbiome of hypertensive versus control rat models. To further illustrate this, Operational Taxonomical Units (OTU) were clustered from the raw data retrieved from 16s rRNA sequencing. The six taxonomic levels namely; Domain, Kingdom, Phylum, Class, Order, Family and genus were used to cluster the microbial community and then shown in a stacked bar chart (Figure 3.10). There was a relative abundance of *Firmicutes* and *Bacteroidetes* in all the rat models. *Bacteroidetes* was more abundant in the Dahl control models, while *Firmicutes* were more abundant in the SHR rat models. Hypertensive rat models tend to have an abundance of *Firmicutes*, while normotensives have a higher abundance of *Bacteroidetes*.

Epsilonbacteraeota, a class of *Proteobacteria* was mostly abundant in SHRs with an abundance of *Helicobacter* and *Campylobacter* genus. *Proteobacteria* have been linked with hypertensive rat models. *Bacteroidia* and *Clostridia* were the most abundant class identified in both hypertensive and normotensive rat models. *Bacteroidia* are under *Bacteroidetes* phyla and were abundant in the control rat models. They are the major constituent of gut microbiota. *Clostridia* are under *Firmicutes*, which as previously described, are more abundant in hypertensive rat models.

The genus *Provotella*: of the *Bacteroidetes* phylum were abundant in control rat models. *Provotella* has been reported to contribute to about 50% of gut microbiota in a healthy balanced gut microbiome. In the hypertensive rat models the Genus *Clostridium* was highly abundant. *Clostridium* has a lot of pathogenic bacteria and is important for development of disease

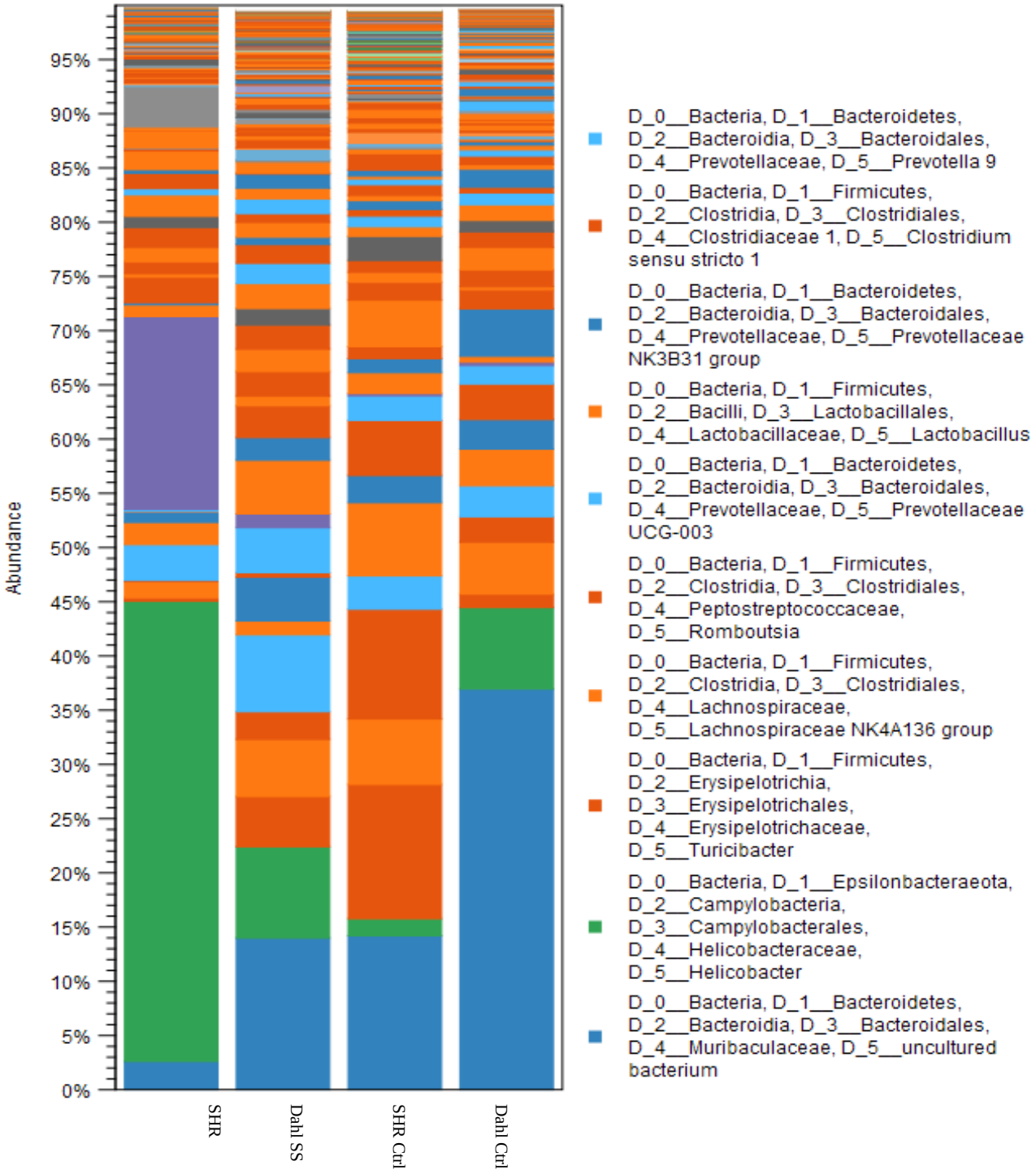

Figure 3.10: **A stacked bar of microbial community abundance in 4 different rat models of hypertension**. The results are representative of the top 10 adundant isolates classified according to taxonomic levels. D_0 represents Kingdom, D_1 Phylum, D_2 Class, D_3 Order, D_4 Family and D_5 Genus. Ctrl stands for control. The colours are stacked according to the colour scheme set out on the right-hand side of the plot

Furthermore, the taxonomic clusters were identified in the different regions of the GIT tract (Figure 3.11). Samples were collected from the stomach, large intestines and small intestines in SHR, SSR and control models. DNA was extracted and 16s rRNA sequening was done. A stacked bar graph was presented using Amplicon-based OTU clustering.

In the control rat models, the stomach and large intestines had a high abundance of *Bacteroidetes*, followed by *Firmicutes* then *Epsilonbaceteraeota.* The stomach had a lower abundance of species compared to the large intestines because of it has a hostile environment (such as low pH) that does not support growth of certain bacterial species.

Firmicutes and *Bacteroidetes* had a relatively similar abundance in SSR models. When compared to the controls, the hypertensives had less abundance of *Bacteroidetes* and an increased abundance of *Firmicutes* in the different regions of the gut. Compared to large intestine, the small intestines and the stomach had lower species abundance and were dominated by bacteria from *Bacteroidetes*, *Firmicutes* and *Epsilonbaceteraeota.* The large intestine contains the largest bacterial ecosystem in the human body. It has a high abundance of bacteria from *Bacteroidetes.*

In SHRs, there was a high abundance of *Firmicutes* and low abundance of *Bacteroidetes*. Eighty percent of the microbial species in the stomach of SHRs fell under *Firmicutes*, the small and large intestines have about 40% *Firmicutes*. SHRs have a lower species abundanced compared to controls and Dahl SS rat models, this may be attributed to the abundance species from the genus *Clostriduim*. These are pathogenic and can outgrow good bacteria in the gut.

In the SHR controls there was a really high abundance of *Epsilonbacteraeota*. SHRs are expected to have more bacteria from *Proteobacteria* compared to its control because the species from this phylum are highly associated with the development of hypertension. *Helicobacter* species were expected to be highly abundant in the stomach than the intestines. Most *Helicobacter* species have been isolated from the stomach because they are well adapted to survive the harsh environmental conditions of the stomach.

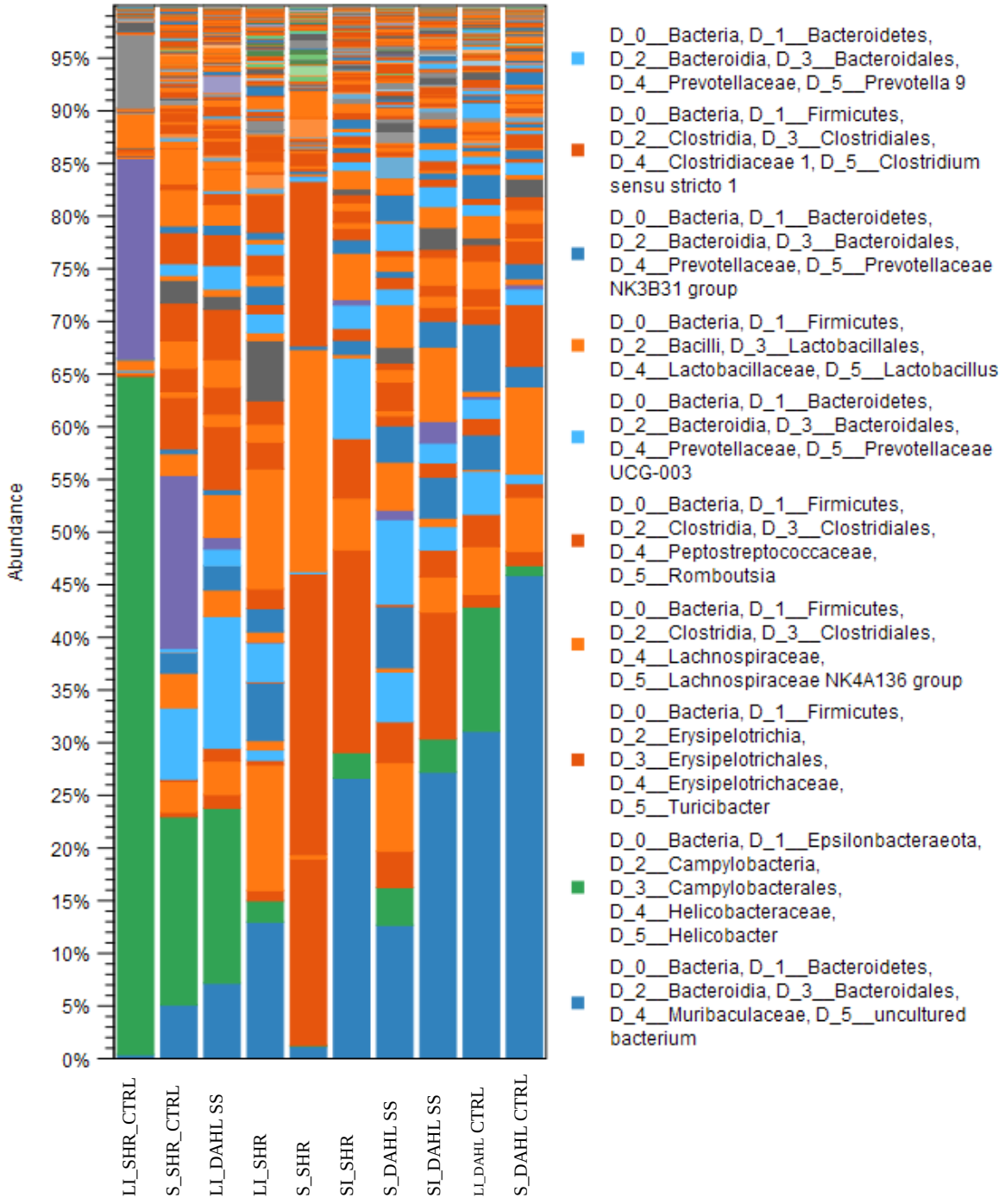

Figure 3.11: A stacked bar of microbial species abundance in different regions along the GIT of 4 different rat models of hypertension. The results are representative of the top 10 abundant isolates classified according to taxonomic levels. D_0 represents Kingdom, D_1 Phylum, D_2 Class, D_3 Order, D_4 Family and D_5 Genus. LI large intestines, S stomach SI small intestines and CTRL control. The colours are stacked according to the colour scheme set out on the right-hand side of the plot.

3.8 Differential abundance analysis

To analyse the abundance of species between the hypertension and normotensive rat models, Alpha diversity, Beta diversity and a heat map tool from CLC genomics were used. Alpha diversity analyses species richness/diversity within an ecosystem (the ecosystem being the different locations of the gut). We assessed species richness between the hypertensive and normotensive rat models. Figure 3. 12 below gives distribution of species richness between the hypertensive and normotensive rat models according to the different regions of the gut they were isolated from. As observed previously (figure 3.10 &3.11), the normotensive rat models (Rat 8 & 1) had a greater species richness compared to the hypertensive models. The hypertensive models, (Rat SSR & Rat 3 SHR), had a low species richness.

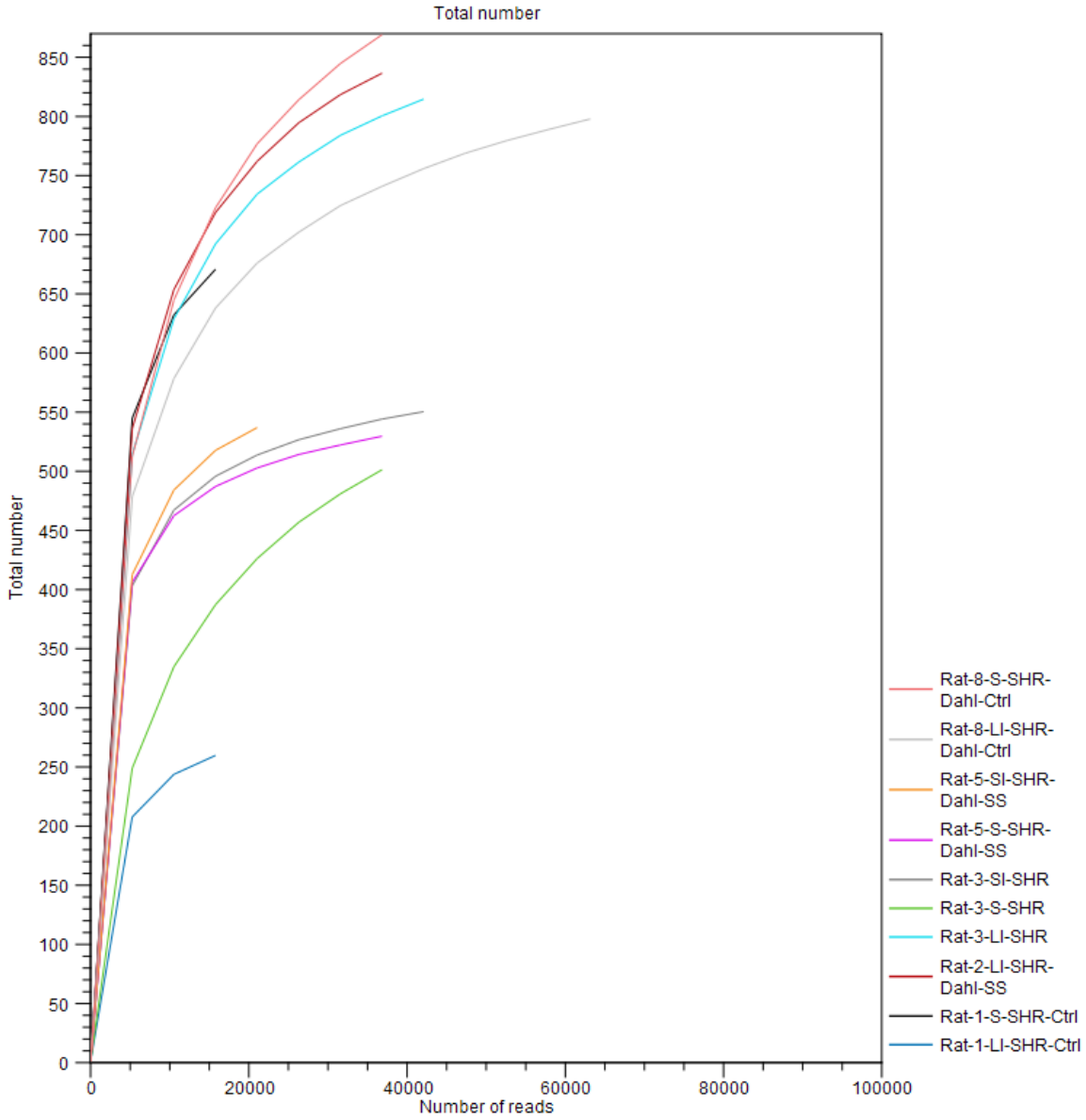

Figure 3.12: Alpha Diversity graph of bacterial richness in rat models of hypertension.

Beta diversity unlike Alpha diversity, measures the degree of similarity (e.g., phylogenetic relatedness) between pairs of communities. We therefore compared the similarities between species in the hypertensive and normotensive rat models. The results support prior observations regarding bacterial distributions between hypertensive and normotensive models (Figure 3.10 & figure 3.11). Samples differed in membership primarily based on whether they are derived from the stomach, small and large intestines and differed based on whether they were hypertensive or normotensive (figure 3.13). Among the hypertensive samples (Rat 3 SHR & Rat 5 SSR), there was a separation between samples derived from the stomach, small and large intestines, this was also observed in the normotensive model, rat 1 and 8 (Dahl controls). These results suggest that differences in microbial ecosystem is important in evaluating microbial diversity.

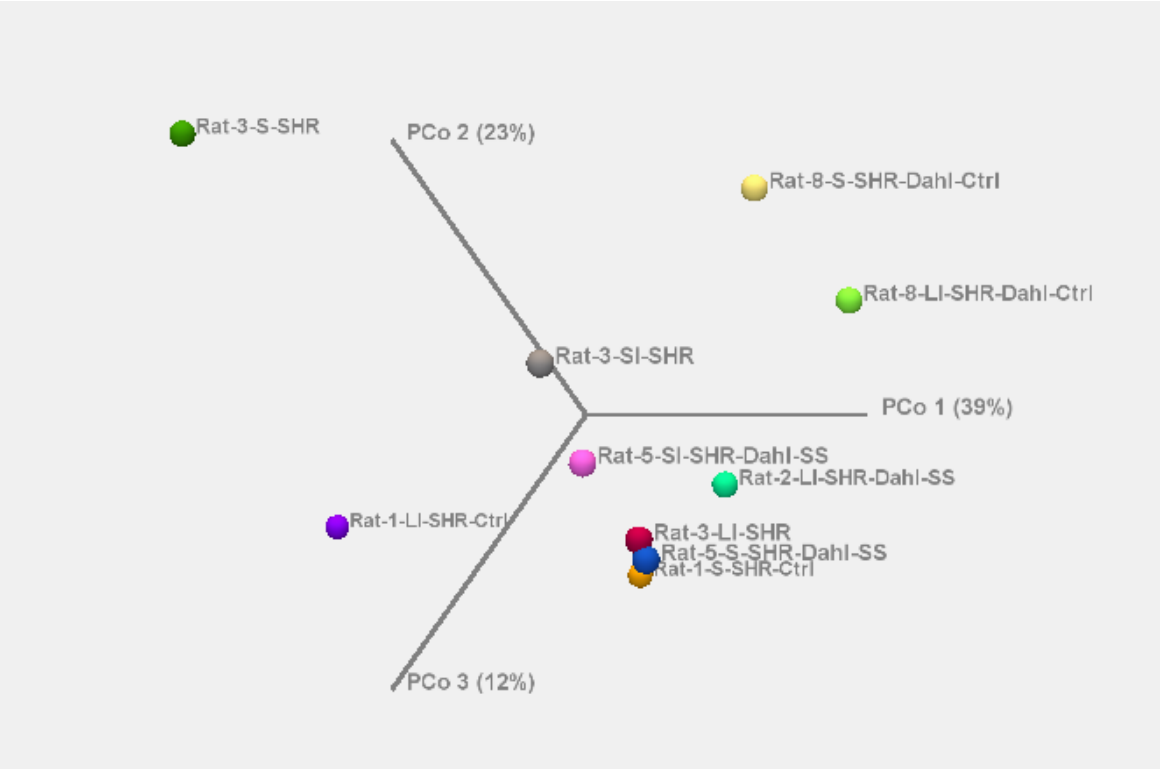

Figure 3.13: Beta diversity results seen as a 3D PCoA for the similarities in species distribution between hypertensive and normotensive rat models.

Furthermore, a heat map was generated from the abundance OUT inputs. In a heat map, hierarchical clustering is grouped according to similarities of genomes over the set of samples, and by sample genome similarities over features. The phylogenetic tree is generated as follows: the tool considers each feature or sample to be a cluster, calculates pairwise distances between all clusters, and joins the two closest clusters into one new cluster, the process is repeated until

there is only one cluster left, which contains all the features or samples and then the tree is drawn so that the distances between clusters are reflected by the lengths of the branches in the tree.

The heat map below, figure 3.14, represents species relatedness and abundance in both hypertensive and normotensive rat models. The results were grouped according to the different regions of the gut they were isolated from. Rat 5 and 3, SSR and SHR, were clustered together showing that they are more closely related. In these, there was an abundance of *Bacteriodes acidifaciens, Listeria monocytogenes* and *Turicibacter sp*. The stomach of SHRs (rat 3), was more distantly related to the rest of the samples. As shown previously (Figure 3.10 & figure 3.11), the stomach of SHRs is abundant with species of *Firmitcutes* and *Proteobacteria*. In the map, *Helicobacter muridarum, micrococcus luteus* and *Rodentibacter ratti.* In the control models, there was an abundance of *Lactobacillus reuteri,Clostridium phoneceensiss* and *Helicobacter apodemus*. Rat 1 and 8 were expected to clustered close to each other since both are hypertensive models but the map shows that they are not closely related. These results show that although samples are both hypertensive they can be unrelated.

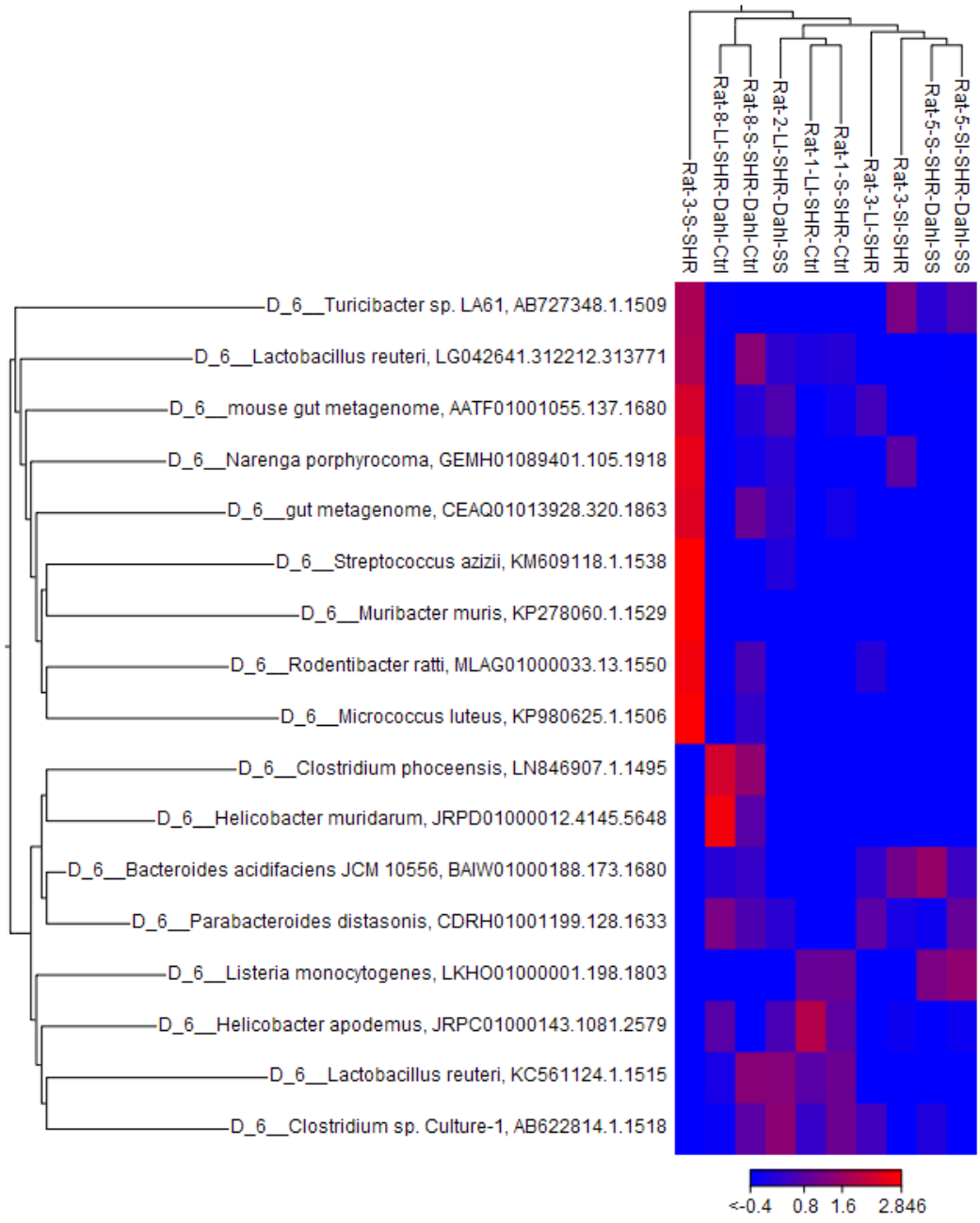

Figure 3. 14: Heat map depicting bacterial diversity and relative abundance in rat models of hypertension.

Chapter 4: Discussion

Discussion

Overview

Gut dysbiosis has been associated with pathogenesis of various diseases including obesity, diabetes and cardiovascular disease (Carding et al., 2015). This dysbiosis has been marked by an increase in *Firmicutes* and decrease of *Bacteroidetes*. In this study we investigated the association of gut microbiota and hypertension by evaluating the abundance and diversity of species in animal models of hypertension. Furthermore, the distribution of bacterial species in different regions of the gut (stomach, small and large intestines) was investigated.

Differential abundance and diversity of bacteria between hypertensive and normotensive rats was observed. Interestingly, *Helicobacter* species were highly abundant in SHR rat models compared to controls and SSR. Also, decrease in microbial species diversity and abundance in hypertensive rat models. This might be attributed to the bioactive metabolites produced by *Bacteroidetes* that have a hypotensive effect on blood pressure. Additionally, a dysbiotic gut in hypertensive rat models marked by an increase in *Firmicutes: Bacteroidetes* ratio, suggesting that gut dysbiosis is linked with hypertension (Li et al., 2017; Mell et al., 2015; Yang et al., 2015). An increase in *Firmicutes: Bacteroidetes* ratio has been reported in obesity. These bacteria play a crucial role in fermenting undigestible compounds in the gut. The end product of fermentation produces various metabolites, these metabolites have a differential effect on blood pressure, mostly depending on which bacteria they are produced by. Finally, differences in distribution of bacteria along the gut in the different rat models of hypertension.

4.1 Microbial species diversity and abundance is decreased in hypertensive rat models

To compare microbial species diversity and abundance in hypertensive and normotensive rat models in culture, biopsy samples from SSR, SHR and control rat models were harvested then cultured using the spread plates. Biochemical tests and MALDI-TOF MS were used to identify the isolates. There was a low species abundance and low species diversity in hypertensive rats. SHR and SSR showed a lack of species from *Bacteroidetes* and *Actinobacteria* and had a high abundance of *Firmicutes* and *Proteobacteria* (Figure 3.4). Looking at both culturable and non-culturable bacteria, DNA extractions were performed on the biopsy samples and 16S rRNA sequencing was performed. As observed by MALDI-TOF MS, there was a decrease in species abundance and diversity in hypertensive rat model (Figure 3.10). Furthermore, SHR had the least species abundance in all the rat models. A low species abundance and diversity indicates a dysbiotic gut and may be caused by diet and/ genetic variation between the rat models

(Bayorh et al., 1998; Dahl, 2005). For SSR, high salt intake contributed to the decrease in microbial species and in both SHR and SSR the genetic variation might have played a role in the decrease of species abundance. Studies have shown an association between genetics and gut composition. In a study by Mell, salt sensitive and salt resistant rats were found to have a genetic variation between then and this genetic variability contributed to microbial composition (Mell et al., 2015).The genetic variability between SHR and SSR and control rat models was not investigated in this study.

Both hypertensive and normotensive rat models lack species from *Actinobacteria*. This result was unexpected. *Actinobacteria* species form part of the healthy microbiome and was expected to be identified in the normotensive rat models. The lack of species from *Actinobacteria* from the 16S rRNA sequencing results might be due to errors in methods utilized. However, due to the variability in rRNA operon copy number in bacteria, the proportion of 16S rRNA gene copies cannot be directly transformed into the number of bacteria (Logares et al., 2014).

Both hypertensive and normotensive models had species from *Helicobacter*. Interestingly these species were highly abundant in the SHR models, more than SSR and control models. *Helicobacter* species have been associated with high blood pressure thus the presence of *Helicobacter* species was expected to be relatively low in controls and highly abundant in both SHR and SSR, not just the SHR (Migneco et al., 2003). The presence of *H. mesocriceorum* and *H. rodentium* in both hypertensive and normotensive rat models may resemble that these species form part of the normal gut microbiota. Studies on the influence of *Helicobacter* in hypertension have yielded contradictory results. Some studies have shown that *H. pylori* may increase systolic pressure (Shankar et al., 2012), while epidemiological studies have shown that there is no association between being *H. pylori* positive and having hypertension (Kopacova et al., 2014). Thus, the influence of *Helicobacter* on hypertension requires further study.

4.2 Species abundance and diversity was different in the hypertensive rats: SHR vs SSR

To compare the diversity and abundance of bacterial species in SHR and SSR models, the bacterial composition of the rat models was identified. Results from MALDI-TOF MS and 16S rRNA sequencing showed there were differences in microbial composition in hypertensive rats. The SHRs and SSRs had a low species abundance and diversity compared to the control rat (Figure 3.3 and 3.8). Furthermore, SHR models had the least species diversity and abundance. These rat models were bred in the same environment, thus environmental factors did not

contribute to the observed difference in gut microbiota of these rat models of hypertension. The diet however, was different between the SSR, SHR and control models. The SSR models were fed a high salt diet. The influence of salt in bacterial composition may account for the difference of gut microbiota. Salt is absorbed in the intestines; this process causes an increase in osmotic pressure resulting in a change in the intestinal environment, thus, making it hard for enteric bacteria to survive/grow in these conditions (Hu et al., 2017). Consequently, this leads to a change in the microbial ecosystem. A study by Hu and colleagues showed a difference in cecal microbiota of pathogen free mice fed a high chronic salt diet. The chronic intake of salt caused a decrease in enteric bacteria thus causing a difference in gut microbiota composition in mice fed a high salt diet and control rats (Hu et al., 2017). This correlates with the results obtained from this study. Another factor that might contribute to differences in species diversity and abundance between hypertensive and normotensive rat models might be the genetic make-up of the rat models.

Genetic make-up between SHR and SSR are vastly different. SSR models are genetically modified and bred to respond to salt intake to develop hypertension and SHR models were bred to develop hypertension from a very young age. The α_2-AR receptor has been identified as one of the important receptors in the central nervous system involved in interactions between adrenergic neurons and vasopressinergic neurons in the development of salt induced hypertension. Furthermore, the α_2-adrenergic receptor gene polymorphisms have been identified in African-American that are hypertensive and in the elderly black population. Although the effect of the α_2-AR gene in salt induced hypertension is not fully understood, it has been shown that mice that lack this gene do not respond to a chronic salt intake thus not developing hypertension. In a study by Makaratsis and colleagues, they suggested that this gene is crucial for the development of salt-induced hypertension. This conclusion was made after observing that knock-out mice lacking α_{2B}-AR genes and those have subtypes of the of α_{2B}-AR genes did not respond to salt induced blood pressure elevation (Makaritsis et al., 1999). Mell *et al.,* concluded that since there were no variants in the *Olfr78* and *Gpr41,* genes that have been reported to be associated with hypertension, other genes in the hosts genome maybe the ones associated with hypertension(Mell et al., 2015). The influence of genes in the development of hypertension still needs to be investigated.

The mechanism behind the development of salt induced hypertension has been controversial. While it has been proven that gut microbiota is altered in hypertensive SSRs, it is not known if

gut dysbiosis is the main reason behind development of hypertension (Bayorh et al., 1998; Dahl, 1961, 2005; Dahl et al., 1967b). Though the mechanism underlying salt sensitive hypertension is not well understood, genetics has mapped pathways for blood pressure responses to salt intake. Bayorh and colleagues demonstrated an elevated blood pressure accompanied by development of hypertension after a prolonged a high salt diet. This was associated with a fourfold increase of AVP in subjects fed a high salt diet compared to those on a low salt diet (Bayorh et al., 1998). This supports the role played by AVP in regulation of blood pressure (Cheng et al., 2009; Kawano et al., 1997; Share and Crofton, 1982; Sharman and Low, 2008). Other factors that have been associated with salt induced hypertension include; decreased endothelial NO production, NO has a vasodilatory effect on cells and is important in regulation of blood pressure (Arnal et al., 1992), the renin/angiotensin/aldosterone pathways (Dzau, 2001), decreased or inability to dilate in renal vasculature after pro-longed high salt intake (Hall, 2016) and kidney dysfunction (Hall, 2016). Although the primary cause of hypertension is not well understood, our research findings provided evidence of presence of a dysbiotic gut in hypertensive rat models thus showing an association of gut dysbiosis and hypertension.

In hypertensive rat models, the development of hypertension has been associated with the Y chromosome. Ely and Turner compared offspring bred from SHR female x male rat and WKY female x SHR male (Ely and Turner, 1990). They found that the male offspring had an elevated blood pressure when the father was SHR than when the mother was SHR (Ely and Turner, 1990). This might be attributed to the presence of a mutation on the Y gene that allows for a blood pressure increase. Other genes in SHR models that have been investigated include the prostaglandin E receptor 4 (*Ptger4*), albumin (*Alb*), angiotensin II receptor-associated gene (*Agtrap*), angiotensin II receptor type-1B (*Agtr1b*) and chymase 1 (*Cma1*). These genes have all been found to contribute to the development of hypertension in both SHR and stroke prone –SHR (Yoshida et al., 2014). Further research in the influence of genetics on blood pressure will be required.

4.3 A dysbiotic gut in hypertensive rat models is marked by an increase in Firmicutes: Bacteroidetes ratio

To investigate if a decrease in species abundance and species diversity was linked to hypertension, the microbial composition between hypertensive and normotensive rat models were compared from results obtained from MALDI-TOF MS and 16S rRNA sequencing. A decrease in species abundance and diversity was only observed in the hypertensive rat models. Both SHR and SSR models showed an increase *Firmicutes* and a decrease in *Bacteroidetes*. This signifies the presence of a dysbiotic gut.

Bacteria from *Firmicutes* and *Bacteroidetes* are key in synthesis of bioactive metabolites that influence progression of hypertension. These bioactive metabolites have differential effects on blood pressure and might be the key to the association of gut dysbiosis with hypertension (Kasubuchi et al., 2015; Lin and Zhang, 2017). For example, *Firmicutes* produced metabolites which include short chain fatty acids, 2-methylpropionate, valerate, isovalerate, hexanoate, lactate and tryptophan (den Besten et al., 2013; Scheppach, 1994). These have functions including antimicrobial activity, a role in absorption of water and sodium, cholesterol synthesis, having a role in development of obesity and colorectal cancer (Table 1.1). A high plasma concentration of lactate has been associated with an increase in blood pressure, however the mechanism is not well understood (Juraschek et al., 2015). Thus, in these models of hypertension *Firmicutes* were abundant, increasing the *Firmicutes: Bacteroidetes* ratio to indicate dysbiosis.

Bacteroidetes in the large intestines break down indigestible compounds, carbohydrates and oligosaccharides, to short chain fatty acids (SCFA) (Wong et al., 2006). SCFAs are secondary metabolites that play a role in physiological homeostasis. Common SCFAs produced in the large intestine include propionate, acetate and butyrate (den Besten et al., 2013; Wong et al., 2006). They have roles in the reduction of visceral fat, protection against pathogens and infections, involved in metabolism, immunomodulatory effect, protection against inflammation, mediating host-microbe interactions, energy and glucose homeostasis (Groh et al., 1993; Lin and Zhang, 2017; Nicholson et al., 2012; Ridlon et al., 2006, 2014; Swann et al., 2011). Mell *et al.*, (2015) provided the first evidence of the role of SCFAs in hypertension observing that acetate and heptanoate were more abundant in salt resistant rat models (Mell et al., 2015). Yan *et al.*, (2017) further showed a decrease in production in SCFA in the hypertensive study cohort, which suggests that a low SCFAs production is associated with a

dysbiotic gut. It is safe to conclude that gut dysbiosis linked hypertension is associated with a decrease of bacteria that produce SCFA especially acetate and butyrate. Further studies investigating the bacteria and metabolites they produce, may be useful to elucidating mechanism of hypertension.

4.4 Differences in distribution of bacteria along the gut in the different rat models of hypertension.

In addition to studying species abundance and species diversity of the gut microbiota between the rat models of hypertension, the composition of species in the different regios of the gut was evaluated. The composition of bacteria from the stomach, small and large intestines were analysed using CLC genomics workbench from data obtained by 16S rRNA gene sequencing. The stomach of both normotensive and hypertensive rat models had very little species abundance and low species diversity (Figures 3.5, 3.6 and 3.11). The stomach has a low pH and is too harsh for bacteria to reside in it. Interestingly, the bacteria species observed to be present in the stomach region tested positive for urease (Table 3.1). These bacteria species include *Lactobacillus murinus, Klebsiella pneumoniae* and *Staphylococcus sciuri.* The ability of the bacteria to breakdown urea in the stomach to ammonia, water and carbon dioxide increases the pH in the stomach (Vince et al., 1973). The increase in pH makes the stomach less acidic thus supporting growth and residence of certain bacterial species (Graham et al., 1992). Various studies have reported that only *Helicobacter/Campylobacter* species are urease-positive (Graham et al., 1992), but this has been refuted by Osaki and colleagues. They showed the presence of several non-*Helicobacter* species that are urease positive in the gut (Osaki et al., 2008). This study corroborates with that of Osaki *et al*., providing evidence that some non-*Helicobacter* species found in the gut are urease positive. The prevalence of various urease positive bacteria in the gut is a subject to be investigated distinctly in future studies.

The small and large intestines are known to harbour majority of the microbial community, with the large intestines having the largest microbial community(Gill et al., 2006). We found a great species abundance and high species diversity in the intestine of normotensive rat models, with bacteria from various phylum with a higher prevalence of *Bacteroidetes*. In SHRs and SSR, the species diversity and abundance were low in both small and large intestines compared to the normotensives. The SHRs had the least species abundance in the intestines, with bacteria mostly from *Firmicutes* and *Proteobacteria*. These results were expected since a decrease in

species abundance and diversity was observed in the hypertensive rat models. Bacteria in the large intestines are important for several physiological function including fermentation of carbohydrates for example to produce SCFAs. As explained previously, SCFAs have a role in maintaining physiological homeostasis including vasodilatory effects on cells (den Besten et al., 2013; Scheppach, 1994; Wong et al., 2006). Therefore, if there is a reduction in bacteria that produce SCFAs there will be an impact in the physiological functions. Enteric bacteria have antimicrobial activity, this prevents invasion from pathogenic bacteria (Jandhyala et al., 2015; Lin and Zhang, 2017; Wu et al., 2015). These bacteria also compete for nutrients in the intestines this helps with avoiding having excess nutrients in the gut that may disturb the ecology of the intestines (Gérard, 2013; Ridlon et al., 2014). Furthermore, bacteria in the large intestine synthesize bile acids (Gérard, 2013; Ridlon et al., 2014). Bile acids have been associated with a decrease in hypertension (Arab et al., 2017).

Other functions of gut bacteria in the large intestines that have been described in literature include synthesizing of certain vitamin (Vitamin B, biotin, folate, vitamin B12 and vitamin k), interacts with hosts immune system, breaking down indigestible compounds such as oligosaccharides to make gases, metabolites and nutrients for both the bacteria and the host (Bäckhed et al., 2005, 2005; Canny and McCormick, 2008; Murdoch and Detsky, 2012). This study further provides evidence that there are different bacteria in the different regions of the gut. To the author's knowledge, this study is the first of its kind to describe bacterial distribution along the gut in hypertensive rat models.

Conclusion

In conclusion, this study has described the microbial flora composition of hypertensive and normotensive rat models, and gave a comparison of gut microbiota between hypertensive and normotensive rat models. We observed a dysbiotic gut microbiome in SHR and SSR models of hypertension compared to control rats. These data suggest an association between gut microbiota, dysbiosis and hypertension. Our study implicates that gut microbiota may have a causal role in the pathogenesis of hypertension. Our findings will contribute towards finding a plausible mechanism underlying development of hypertension, thus contributing to developing new ways to eradicate and prevent not only hypertension but other cardiovascular diseases.

Limitations of the study

There was a small number of rat samples, in the future having a greater sample size will help increase the validity of the conclusions. Time and financial constraints prohibited us from adding more objectives to the study. Lastly, the lack of species identification because of the techniques used. Different media need to be considered to allow the growth of a wider range of microorganisms. In sequencing, further data analysis techniques can be used to reduce the number of unclassified species.

Future studies

Going forth, we hypothesize that the bacteria present in the hypertensive rat models are synthesizing cardiotonic steroids from secondary metabolites in the gut. These steroids block the ATPase pump causing an increase in blood pressure downstream. Therefore, future studies will include us undertaking metabolomic and meta-transcriptomic studies. Metabolomics is the study of metabolites produced during metabolism in an organism. This will help understand the impact of metabolites on the difference in composition of microbiota in the rat models of hypertension. Meta-transcriptomics describes the transcriptome of a group of interacting species. Studying transcripts, gene expression and how this influence microbial activity will contribute to understanding the underlying molecular mechanisms in the interaction of metabolites, bacteria and blood pressure.

Chapter 5: References

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Appendix

Table A1: Microbiological inoculation and culture supplies

Product	Manufacturer	Catalogue number
Disposable inoculating loops	ThermoFisher™	QL10
Petri-dishes	ThermoFisher™	172931
Microaero Gas packs	ThermoFisher™	R681005
Anaerobic jars	Oxoid™	AG0025A
Fetal bovine serum	Thermo Scientific™	16140089
Growth media/Agar	Thermo Scientific™	CM0331R

Table A2: Biochemical tests consumables

Product	Manufacturer	Catalogue number
Gram stain Kit	Sigma-Aldrich	77730
Catalase test solution	Sigma-Aldrich	88597
Urease	Sigma-Aldrich	U4883

Oxidase test strips	Sigma-Aldrich	40560

Table A3: MALDI-TOF MS consumables + Equipment

Name	**Manufacturer**	**Catalogue number**
Reusable polished steel MALDI target plate	Bruker Daltonics	8280800
Bruker Matrix HCCA, portioned	Bruker Daltonics	8255344

Table A4: DNA extraction and metagenomics

Product	**Manufacturer**	**Catalogue number**
QIAamp DNA Mini Kit	QIAGEN	51306
Illumina Sequencer	Services: NICD (Core sequencing unit NHLS)	

Table A5: Rat models used

	Rat label	sex	Genetic make up
1	C13	Male	SHR control
2	C14	Male	SHR control
3	R13	Male	SHR
4	R14	Male	SHR
5	R15	Male	SHR
6	DSA1	Male	SS
7	DCA1	Male	SS control
8	DCA2	Male	SS control
9	DSA2	Male	SS
10	DSA3	Male	SS

* SHR- Spontaneously hypertensive rats * C- Control, R- running exercise *ss- Salt sensitive

Table A6: Results from the urease, catalase, oxidase test and Gram stain

Sample	Urease test	Oxidase test	Catalase test	Gram stain
1.1 SI9 CBA	+	+	+	-, pink, coccus
2.1 SI9 BHI+S	+	+	+	+ coccus
3.2 SI9 BHI	+	+	+	- rods
4.3 SI9 BHI	+	+	+	+ rods
5.1S8 TSA+S	++	+	+	
6.1 SI6 BHI	+	+	+	
7.2SI6 BHI	+	+	+	- rods
8.3SI6 BHI	+	+	+	- rods
9.6 SI9 CBA	+	+	+	- rods
10.5 SI9 CBA	+	+	+	+ rods
11.4 SI9 CBA	+	+	+	+ spiral
12.3 SI9 CBA	+	+	+	- coccus
13.2 SI9 CBA	+	+	+	- coccus
14.1 SI8 CBA	+	+	+	- rods
15.2 SI8 CBA	+	+	+	- rods
16.1 SI9 CBA+S	+	+	+	-. Rods
17.1 SI7 BHI	+	+	+	-, rods
18.2 LI7 BHI	+	+	+	
19.2 SI8 CBA+S	+	+	+	
20.LI7 TSA + S	+	+	+	+ coccus
21.2 SI2 CBA	+	+	+	

22. SI7 TSA+S	+	+	+	
23.SI8 CBA	+	+	+	- coccus
24.1 SI2 CBA	+	+	+	
25.2SI2 CBA+S	+	+	-	
26.1 SI6 CBA+S	+	+	+	
27.1 SI2 CBA+S	+	+	+	
28.3 S8 CBA	+	+	+	
29.4 SI2 CBA	+	+	+	
30.1 SI8 BHI+S	+	+	+	
31.2 S8 BHI	+	+	+	
32.3 S8 BHI	+	+	+	- rods
33.1 S8 BHI	+	+	+	- rods
34. 2 SI8 BHI+S	+	+	+	
35.1 SI9 TSA	+	+	+	
36.2 LI7 CBA	+	+	+	-, rods
37.3 LI7 CBA	+	+	+	
38.S8 BHI+S (1a)	+	+	+	+ rods
39.S8 BHI+S (1b)	+	+	+	
40. 1 TSA+S	-	-	-	- spiral
41.3 S8 CBA	+	+	+	- coccus
42.2 S8 CBA	+	+	+	+ rods
43. 1 S8 CBA (a)	+	+	+	-coccus

44. 1 S8 CBA (b)	+	+	+	
45.S8 CBA+ S	+	+	+	+ rods
46.2 LI7 BHI+S	+	+	+	- rods
47.1 LI7 BHI+S	+	+	+	
48.1 SI8 BHI	+	+	+	
49.2 SI8 BHI	+	+	+	-coccus
50. 4 SI6 BHI	+	+	+	+rods
51.SI9 BHI+S (B)	+	+	+	
52.4 SI6 BHI	+	+	+	
53.2 SI8 CBA+S	+	+	+	- coccus
54.3 SI9 BHI+S	+	+	+	+ rods
55.SI8 CBA+S (a)	+	+	+	
56. SI8 CBA+S (b)	+	+	+	- coccus
57.1 LI7 CBA	+	+	+	-coccus
58.2 SI9 TSA	+	+	+	-spiral
59.3 SI9 TSA	+	+	+	
60.4 SI9 TSA	+	+	+	-rods
61.5SI9 TSA	+	+	+	-rods
62.6 SI9 TSA	+	+	+	
63. 2 SI7 CBA+S	-	-	-	+ coccus
64.3 SI9 TSA+S	+	+	+	-coccus
65.2 SI9 TSA+S	+	-	+	

66.1 SI9 TSA+S	+	+	+	+spiral
67.1 TSA	+	+	+	-rods
68. 2 S8 TSA	+	+	+	-rods
69.3S8 TSA	+	+	+	-spiral
70.4 S8 TSA	+	+	+	-rods
71. 1SI7 BHI	+	+	-	-rods
72.2 SI7 BHI	+	-	+	-rods
73.1 SI7 CBA+S	+	+	+	+ rods
74.1 SI9 BHI	+	+	+	-rods

BHI Brain heart infusion broth

BHI+S Brain heart infusion broth+ skirrows

Group 2 results

Table A7: Biochemical test results for group 2 samples

Sample	Urease	Oxidase	Citrate	Gram stain
1. 1 LI9 CBA	-	+	+	- rods
2. 2LI9 TSA+S	-	-	+	- rods
3. 3 LI9 TSA+S	+	+	-	- rods
4. 1 LI9 TSA+ S	-	-	+	- rods
5. 3 LI6 CBA+S	+	-	+	- rods

6. 2 SI8 TSA+S	-	-	+	- rods
7. 3 SI7 TSA	-	-	+	+ rods
8. 1 SI7 TSA	+	-	+	- coccus
9. 1 LI8 TSA	-	-	+	- rods
10. 2 SI2 TSA	+	+	+	- coccus
11. LI8 BHI+S	-	-	-	+ rods
12. LI8 TSA	-	-	+	- rods

MALDI-TOF

Table A8: Identification of results by MALDI-TOF MS

SAMPLE	Isolate
1. LI9 CBA	*Bacillus subtilis*
2. LI8 TSA+S	*Escherichia coli*
3. 3 LI9 TSA+S	*Escherichia coli*
4. 1 LI9 TSA+S	*Escherichia coli*
5. 2 LI9 TSA+S	*Escherichia coli*
6. 3 SI7 TSA	*Escherichia coli*
7. 2 SI8 TSA+S	*Escherichia coli*
8. 3 LI6 CBA+S	*Bacillus cerues*
9. 1 SI7 TSA	*NO ID*
10. LI8 BHI+S	*Lactobacillus murinus*
11. 2 SI2 TSA	*Myroides Odoratimimus*

12. 1 LI8 TSA	*Escherichia coli*
13. 2 SI2 CBA+S	*Bordetella petrii*
14. 1 SI2 CBA+S	*Klebsiella variicola*
15. 3 SI2 CBA	*Staphylococcus sciuri*
16. 1 SI8 BHI+S	*Escherichia coli*
17. 1 SI8 CBA+S	*Psuedomonas aeruginosa*
18. 1 SI9 TSA	*Acinetobacter sp*
19. 1 SI2 CBA	*Escherichia coli*
20. 2 S8 BHI	*Escherichia coli*
21. 4 SI2 CBA	*Escherichia coli*
22. 6 SI9 TSA	*Escherichia coli*
23. 1 SI6 BHI	Pseudomonas aeruginosa
24. 2 SI9 TSA+S	Acinetobacter sp
25. 2 SI8 BHI+S	Bacillus cereus
26. S8 BHI+S	Escherichia coli
27. 3 LI7 CBA	Escherichia coli
28. S8 BHI+S	Bacillus cereus
29. 2 SI6 BHI	Escherichia coli
30. 3 SI9 TSA	Escherichia coli
31. 1 SI9 TSA+S	NO ID
32. 3 SI9 TSA+S	Escherichia coli
33. 2 S8 TSA	Bacillus cereus

34. 1 LI7 CBA	Bcillus cereus
35. 1 SI9 BHI	NO ID
36. 4 SI9 CBA	NO ID
37. 3 SI9 BHI	Escherichia coli
38. 6 SI9 CBA	Escherichia coli
39.2 SI7 CBA+S	Escherichia coli
40. 1 SI7 BHI	NO ID
41. 1 TSA	NO ID
42. 2 LI7	NO ID
43.2 LI8 CBA	No peaks
44. S8 TSA+S	No peaks
45. 1 SI9 TSA+S	No peaks
46. 2 SI9 CBA+S	No peaks
47. S8 BHI+S	No peaks
48. 2 SI8 CBA+S	Escherichia coli

Group 3 Results

Table A9: Biochemical test results from isolates

Sample	Urease	Citrate	Oxidase	Gram stain
1. 3 LI2 CBA	+	+	-	-, coccus
2. 3 LI2 CBA	+	+	-	-, coccus
3. 2 LI7 CBA	+	+	+	-, coccus
4. 1 LI2 TSA+S	-	+	+	+, rods

5. 2 LI2 CBA+S	+	+	+	-, coccus
6. 1 LI2 CBA	-	+	+	+, coccus
7. 2 LI2 TSA+S	+	-	-	+, coccus
8.1 SI3 BHI	-	+	+	-, coccus
9. 2 SI3	+	+	-	-, rods
10. S6 TSA	+	+	-	-, rods
11. S6 BHI	+	+	-	-, rods
12. 1 LI2 TSA	-	+	-	+, coccus
13.2 LI2 TSA	-	+	-	-, rods
14. 1 LI2 BHI+S	+	+	+	-, rods
15. 1 LI1 TSA	-	+	+	-, rods
16. LI2 BHI	-	+	+	+, coccus
17. 2 LI2 BHI	-	+	-	-, rods
18. 2 LI2 BHI+S	-	-	-	-, spiral
19. 2 LI2 CBA	-	-	+	+, coccus
20. 1 LI2 CBA	-	-	-	-, coccus
21. 1 LI2 CBA+S	+	+	-	-, rods
22. 1 LI2 CBA+S	-	-	-	+, spiral
23. 2 LI2 CBA+S	-	-	-	-, spiral
24. 2 LI2 BHI	-	-	-	-, coccus

25. 1 LI2 BHI	-	-	-	+coccus
26. 4 S1 CBA	-	-	-	-, rods
27. 3 S1 CBA	-	-	-	-, coccus
28.2 S1 CBA	-	+	-	+, coccus
29. 2 li2 TSA	-	-	-	+, coccus
30. 1 LI2 TSA	-	-	-	-. Rods
31. 2 S1 CBA+S	-	-	-	+, rods
32.3 LI2 TSA	-	-	-	-, coccus
33. S6 BHI+S	+	+	+	
34. 1 S1 CBA	+	+	+	
35. S6 CBA	+	+	+	+, rods
36. S6 TSA+S	+	+	+	+, rods
37. S6 TSA+S	+	+	+	+, rods
38. S6 CBA+S	+	+	+	+, rods

Group 3 MALDI RESULTS

Table A10: MALDI TOF MS results

Sample	Position	ID
1 LI2 CBA+S	1/2A	NO ID
2. 1 LI2 CBA	3/4 A	Enterococcus faecalis

3. 2 LI2 CBA	5/6A	Enterococcus faecalis
4. S6 CBA+S	7/8 A	Pseudomonas aeruginosa
5. S6 BHI+S	9/10 A	NO ID
6. S6 CBA	11/12 A	Pseudomonas aeruginosa
7. 2 LI2 TSA	1/2 B	Enterococcus faecalis
8. 1 LI2 TSA	3/4 B	Escherichia coli
9. 2 S1 CBA+S	5/6 B	Lactobacillus murinus
10. 3 LI2 TSA	7/8 B	Escherichia coli
11. S6 BHI+S	9/10 B	NO ID
12. 1 S1 CBA	11/12 B	Bacillus cereus
13. 1 LI2 CBA+S	1/2 C	Lactobacillus murinus
14. 2 LI2 CBA+S	3/4 C	Lactobacillus murinus
15. 2 LI2 BHI	5/6 C	Escherichia coli
16. 1 LI2 BHI	7/8 C	Enterococcus faecalis
17. 4 S1 CBA	9/10 C	Escherichia coli
18. 3 S1 CBA	11/12 C	Enterococcus faecalis
19. 2 S1 CBA	1/2 D	NO ID
20. 1 LI2 CBA	3/4 D	NO ID
21. 1 LI2 TSA+S	5/6 D	Lactobacillus murinus
22. 2 LI7 CBA	7/8 D	NO ID
23. 3 LI2 CBA	9/10 D	Escherichia coli
24. S6 TSA+S	11/12 D	Pseudomonas aeruginosa

25. 3 LI2 CBA	1/2 E	Staphylococcus sciuri
26. 2 LI2 TSA+S	3/4 E	NO ID
27. S6 TSA	5/6 E	Pseudomonas aeruginosa
28. 2 SI3	7/8 E	Escherichia coli
29.1 SI3 BHI	9/10 E	NO ID
30. S6 BHI	11/12 E	Pseudomonas aeruginosa
31. 2 LI2 CBA+S	1/2 F	NO ID
32. 1 LI2 TSA	3/4 F	Staphylococcus sciuri
33. 2 LI2 TSA	5/6 F	Escherichia coli
34. 1 LI2 BHI+S	7/8 F	Klebsiella pneumoniae
35. 1 LI7 TSA	9/10 F	NO ID
36. LI2 BHI	11/12 F	NO ID
37. 2 LI2 BHI+s	1/2 G	Lactobacillus murinus
38. 2 LI2 BHI	3/4 G	Escherichia coli

Group 4 results

Table A11: Biochemical test results

sample	Urease	Citrate	Oxidase	Gram stain
1. S7 BHI 1	-	-	-	-
2. S7 BHI 2				-
3. S7 TSA+S	-	-	-	-

4. S1 TSA 1	-	+	-	+
5. S1 TSA 1	-	-	-	+
6. CBA S1 1	-	-	-	+
7. CBA S1 2	+	+	+	+
8. SI1 BHI+S	-	-	-	+
9. LI6 BHI+S	+	+	+	-
10. 2 SI3 CBA				-
11. 1 SI3 CBA	-	-	-	-
12. SI3 TSA 1	-	-	-	-
13. SI3 TSA 2	-	-	-	-
14. SI1 TSA	-	-	-	-
15. TSA LI6	+	+	+	-
16. 1 S7 TSA				-
17. 2 S7 TSA	-	+	+	+
18. 1 LI1 CBA+S	-	+	+	+
19. 2 LI2 CBA+S	-	+	-	-
20. 2 LI2 BHI	-	+	-	+
21. 1 LI7 BHI	-	-	+	+
22. 2 SI3 CBA+S	-	-	+	+
23. 1 LI7 BHI	-	-	+	-

24. 2 LI7 BHI	-	-	+	-
25. BHI S1 (1)	-	-	+	-
26. BHI S1 (2)	-	-	+	-
27. CBA (1)	-	-	-	-
28. CBA (2)	-	-	+	-
29. 1 S9 TSA+S	+	+	+	-
30. S7 BHI	+	-	+	-
31. 1 BHI LI6	+	+	+	-
32. LI1 BHI	_	-	-	-
33. LI1 BHI+S	_	-	-	+
34. 1 SI3 CBA+S	+	+	+	+
35. 2 BHI LI6	-	-	-	_
36. LI7 CBA	-	-	-	-
37. BHI LI6	-	-	-	+
38. LI7 CBA	-	-	-	+
39. S9 BHI+S	-	-	-	-
40. 3 CBA+S S7	-	-	+	-
41. 2 S7 CBA+S	-	-	+	+

42. 1 BHI SI7	-	-	+	+
43. 1 SI1 CBA	-	-	+	+
44. SI2 BHI+S	-	-	+	-
45.2 SI1 CBA+S	-	-	+	-

GROUP 4 MALDI RESULTS

Table A12: MALDI-TOF results

Sample	Position	Identification
1. LI1 CBA	1/2 A	NO ID
2. LI6 BHI	3/4 A	NO ID
3.LI1 CBA	5/6 A	*Escherichia coli*
4. 2 BHI LI6	7/8 A	NO ID
5. 1 SI3 CBA+S	9/10 A	NO ID
6. 1 LI7 BHI+S	11/12 A	*Lactobacillus murinus*
7. LI1 BHI+S	1/2 B	NO ID
8. LI1 BHI	3/4 B	NO ID

9. 1 BHI LI6	5/6 B	NO ID
10. 3 CBA+S S7	7/8 B	NO ID
11. S9 BHI+S	9/10 B	NO ID
12. CBA+S S7	11/12 B	NO ID
13. 1 BHI SI7	1/2 C	NO ID
14. CBA SI1	3/4 C	NO ID
15. BHI+S SI2	5/6 C	*Escherichia coli*
16. 1 SI1 CBA+S	7/8 C	*Escherichia coli*
17. 1 S7 TSA	9/10 C	*Escherichia coli*
18. 2 S7 TSA	11/12 C	NO ID
19. 2 LI1 CBA+S	1/2 D	NO ID
20. 2 SI3 CBA+S	3/4 D	NO ID
21. LI1 BHI	5/6 D	NO ID
22. 2 LI2 BHI	7/8 D	*Pseudomonas mendocina*
23. 2 BHI LI1	9/10 D	NO ID
24. BHI S1	11/12 D	NO ID
25. BHI S1	1/2 E	*Escherichia coli*
26. S7 BHI	3/4 E	NO ID
27. 1 S9 TSA+S	5/6 E	NO ID
28. CBA 1	7/8 E	NO ID
29. CBA 2	9/10 E	NO ID
30. CBA S1	11/12 E	*Pseudomonas aeruginosa*

31. CBA S1	1/2 F	NO ID
32. TSA SI1	3/4 F	NO ID
33. TSA LI6	5/6 F	NO ID
34. BHI S7	7/8 F	NO ID
35. 1 SI3 CBA	9/10 F	NO ID
36. SI3 TSA	11/12 F	NO ID
37. S7 BHI	1/2 G	NO ID

MALDI-TOF results

Sample	Isolate
1. 1 SI8 CBA	No ID
2. 1 S8 CBA	*Escherichia coli*
3.4 SI6 BHI (1a)	*Corynebacterium ammoniagenes*
4.1 S8 TSA+S	No ID
5.1 S8 TSA+S (b)	No ID
6.SI8 CBA+S (1a)	NO ID
7. 2 SI8 BHI	*Escherichia coli*
8. 1 LI7 BHI+S	*Lactobacillus murinus*
9.1 SI9 CBA	*Escherichia coli*
10.1 SI9 CBA	*Escherichia coli*
11.1 SI7 CBA+S	*Bacillus cereus*
12. 1 SI7 BHI	*Escherichia coli*

13.1 S8 BHI	*Escherichia coli*
14.4 S8 TSA	*Escherichia coli*
15.3 SI6 BHI	*Pseudomonas auriginosa*
16.2 SI8 CBA	*Bacillus cereus*
17.4 SI6 BHI	*Bacillus cereus*
18.SI8 CBA+S	*Achromobacter xylosoxidans*
19.1 SI9 CBA+S	*Escherichia coli*
20.2 S8 TSA+S	*Escherichia coli*
21.1 SI9 BHI+S	NO ID
22.S8 TSA+S (1b)	NO ID
23. SI9 BHI+S (1b)	Bacillus cereus
24. 2 SI7 BHI+S	NO ID
25.S8 CBA+S	*Escherichia coli*
26.2 LI7 BHI+S	*Escherichia coli*
27.1 S8 CBA	*Bacillus cereus*
28.2 LI7 CBA	*Escherichia coli*
29.SI8 CBA	*Escherichia coli*
30.2 SI8 CBA	*Staphylococcus sciuri*
31.3 S8 CBA	NO ID
32.1 TSA+S SI9	NO ID
33.SI7 TSA	*Bacillus cereus*
34.2 SI9 CBA	*Escherichia coli*

35.1 S8 BHI	*Escherichia coli*
36.3 S8 TSA+S	*Escherichia coli*
37.2 SI9 CBA+S	NO ID
38.4 LI7 CBA	NO ID
39.LI7 TSA+S	*Enterococcus gallinarum*
40.4 SI9 TSA	*Staphylococcus sciuri*
41.3 SI9 BHI+S	*Escherichia coli*
42.5 SI9 TSA	*Escherichia coli*

MALDI-TOF MS identification of isolated grouped according to the phylum they belong to.

SHR (Spontaneously hypertensive)

Table A13: Bacteria Identification in SHR

Organsism	No of times isolated	*Phylum*
Escherichia coli	25	*Proteobacteria*
Staphylococcus sciuri	4	*Firmicutes*
Klebsiella pneumoniae	9	*Proteobacteria*
Bacillus cereus	2	*Firmicutes*
Lactobacillus murinus	3	*Firmicutes*
Paenibacillus residui	1	*Firmicutes*
Enterococcus faecalis	3	*Firmicutes*
Pediococcus acidilactici	3	*Firmicutes*
Enterococcus gallinarum	2	*Firmicutes*

Table A14: Bacteria identification in SSR

Organsim	No. of times isolated	*Phylum*
Corynebacterium ammoniagenes	1	*Actinobacteria*
Pseudomonas auriginosa	6	*Proteobacteria*

Bacillus cereus	3	*Firmicutes*
Escherichia coli	15	*Proteobacteria*
Staphylococcus sciuri	1	*Firmicutes*
Acinetobacter sp	2	*Actinobacteria*
Bacillus subtilis	1	*Firmicutes*
Micrococcus luteus	1	*Actinobacteria*
Lactobacillus murinus	2	*Firmicutes*
Staphylococcus cohnii	1	*Firmicutes*
Enterococcus faecalis	1	Firmicutes

Table A15: Bacteria identification from Control rat models

Organism	No. of times isolated	*Phylum*
Myroides Odoratimimus	1	*Bacteroidetes*
Bordetella petrii	1	*Proteobacteria*
Klebsiella variicola	1	*Proteobacteria*
Staphylococcus sciuri	4	*Firmicutes*
Escherichia coli	35	*Proteobacteria*
Enterococcus faecalis	5	*Firmicutes*
Lactobacillus murinus	8	*Firmicutes*
Pseudomonas mendocina	1	*Proteobacteria*
Klebsiella pneumoniae	1	*Proteobacteria*
Pseudomonas aeruginosa	2	*Proteobacteria*
Enterococcus gallinarum	1	*Firmicutes*
Bacillus cereus	8	*Firmicutes*
Achromobacter xylosoxidans	1	*Proteobacteria*

Table A16: sRNA sequencing results

0		Classification	Number of reads	% Reads

SSR	Large intestine	Bacteria	65537	97,12%
		Unclassified at Kingdom level	1944	2,88%
		Archea	1	0,00%
	Small intestines	Bacteria	40239	92,03%
		Unclassified at Kingdom level	3477	7,95%
		Archea	1	0,01%
	Stomach	Bacteria	64736	97,20%
		Unclassified at Kingdom level	1858	2,79%
		Archea	5	0,01%
SHR	Large intestines	Bacteria	23,121	58,63%
		Unclassified at Kingdom level	16,321	41,38%
		Archea	1	0,00%
	Stomach	Bacteria	24806	68,01%
		Unclassified at Kingdom level	11664	31,98%
		Archea	2	0,01%
		Viruses	1	0,00%
	Small intestines	Bacteria	13720	50,55%
		Unclassified at Kingdom level	13420	49,45%
Control	Large intestines	Bacteria	102279	99,42%
		Unclassified at Kingdom level	591	0,57%
		Archea	1	0,00%
	Small intestines	Bacteria	3464	51,82%
		Unclassified at Kingdom level	3221	48,18%
		Archea		
	Stomach	Bacteria	70429	98,98%
		Unclassified at Kingdom level	725	1,02%