MICROBIAL DIVERSITY, NUTRITIONAL VALUE AND ANTIOXIDANT ACTIVITY OF THE EDIBLE GIANT AFRICAN BULLFROG (*PYXICEPHALUS ADSPERSUS* TSCHUDI, 1838) MEAT FROM OSHANA REGION OF NAMIBIA

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Abstract

The seasonal, edible African Bullfrog *Pyxicephalus adspersus*, a notable indigenous food in Northern Namibia, is a good nourishment for human consumption and an alternative source of proteins. However, consumption of this species harvested after the first rain of the rainy season has been reported to be associated with severe dysuria. No study has been conducted to decipher its cause or consequent deleterious effects. Additionally, nutritional composition and the antioxidant properties of this meat species in Namibia have not been investigated. This study is a preliminary attempt to reveal the nutritional composition including antioxidant activities as well as the bacterial diversity associated with this species. Frog meat was analysed for crude protein, ash, crude fat and moisture content using standard analytical methods. Moreover, the antioxidant activity of methanol extract of the frog meat was evaluated using DPPH assay, nitric oxide as well as reducing power assay. The results showed that the highest crude protein recorded was 21±0.00%, ash was found to be 1.19±0.32%, crude fat was 1.65±2.71% while moisture content was 78.21±0.38%. Moreover, frog meat extracts exhibited antioxidant activity with the highest reducing power absorbance of 0.98±0.66 at 700nm, DPPH free radical scavenging of 51.13±18.26% inhibition, and a much lower nitric oxide inhibition of about 34.57±35.85%. Furthermore, metagenomics data reveal four phyla associated with Giant African Bullfrog meat, where *Firmicutes* is the most abundant accounting for nearly 90% of the total bacterial accumulation. In addition, metagenomics revealed a few pathogenic species such as *Lactococcus garvieae* which is related to urinary tract infection in humans. Moreover, *Lactococcus lactis* which is highly studied in food fermentation research is one of the dominant species in this study. These findings endorsed the consumption of this species as a source of protein and antioxidants. The results indicated that frogs might have a valuable chemical composition as compared to other types of meat. Therefore, frog meat has the potential of attracting the attention of food technologists and dieticians as a healthy and valuable source of food based on its chemical composition.

Keywords: Edible African bullfrog, nutritional composition, antioxidant, metagenomics, dysuria

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List of Abbreviations and/or Acronyms

16S Rrna -16S ribosomal ribonucleic acid

AMPs - Antimicrobial peptides

ANOVA - Analysis of variance

AOAC - Association of Official Analytical Chemists

ASV - Amplicon Sequence Variant

ATP - Adenosine triphosphate

BLAST- Basic Local Alignment Search Tool

CCS - Circular Consensus Sequences

CPS - UNAM Center for Postgraduate Studies

DADA2 - The Divisive Amplicon Denoising Algorithm version 2

DNA- Deoxyribonucleic acid

DPPH - 1,1-diphenyl-2- picrylhydrazyl

FAO - Food and Agriculture Organization

GS - Gas Chromatography

HPLC - High Performance Liquid Chromatography

IC_{50} - The half maximal inhibitory concentration

ITS - Internal transcribed spacer

MGS - Shotgun metagenomics sequencing

NCBI - National Center for Biotechnology Information

NGS -Next-generation sequencing

NIR - Near infrared reflectance

NMR - Nuclear Magnetic Resonance

NO - Nitric oxide

OTUS - Operational taxonomic units

PacBio - Pacific Biosciences

PCR - Polymerase Chain Reaction

pH - potential hydrogen

PICRUSt2 -Phylogenetic Investigation of Communities by Reconstruction of Unobserved States version 2

QIIME2 - Quantitative Insights into Microbial Ecology version 2

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

SMRT- Single-Molecule, Real-Time

SPSS - Statistics Package for Social Science

UNAM - University of Namibia

UREC - UNAM Research Ethics Committee

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Dedications

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Ondapandula Kalunga nemuyambeke.

Declaration

I, **Martha Ndapandula Hatutale** hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

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Martha Ndapandula Hatutale		**October 2022**
Name of Student	Signature	Date

1. CHAPTER ONE: INTRODUCTION

1.1. Background of the study

According to the United Nations Food and Agriculture Organization *et al*. (2019) in 2018 more than 12% of the global population were chronically undernourished and 98% of them live in developing countries. Africa is the region with the highest prevalence of undernourishment and this constitutes a huge setback towards achieving the Zero Hunger target by the year 2030 (FAO *et al*., 2019). According to the 2016/2017 Annual Vulnerability Assessment, poverty affects about 28% of the Namibian population whilst 27.8% of Namibia's population is food insecure (FAO, 2019).

Factors that have contributed to food insecurity have been the loss of indigenous foods and the related indigenous knowledge coupled with utilization of indigenous food sources that could improve food security significantly, hence reducing the poverty levels (FAO, 2019). One such source could be the African Bullfrog, *Pyxicephalus adspersus*, whose meat can be a contender as a source of protein and income for vulnerable communities. In Namibia, *P. adspersus* is considered to be a delicacy mostly by people of the Aawambo ethnic group (Okeyo, Kandjengo and Kashea, 2015). However, nutritional composition and the antioxidant properties of this species in Namibia is still unknown.

In addition, studies on the microbiological safety of most edible frogs are rare, though Douglas and Amuzie (2017) reveal some common human pathogens associated with *Hoplobatrachus occipitalis*. Douglas and Amuzie (2017) explained that pathogens from frog meat can be transmitted to man both actively and passively causing diseases. Correspondingly, consumption of *P. adspersus* harvested proximately after the first rain in Namibia is reported to be associated with a severe dysuria (Okeyo *et al*., 2015). However, no studies have been conducted to decipher its cause. Thus, this study was

conducted to determine the nutritional value, antioxidant activity of this species as well as determining the association between the Northern Namibia's Giant African Bullfrog microbiome as the initial phase of investigating potential cause of this side effect upon consumption.

1.2. Statement of the problem

Though *P. adspersus* meat has been a delicacy and a potential source of energy, nutrients and bioactive compounds in northern Namibia, its nutritional composition and antioxidant quality have not yet been deciphered. Moreover, despite the painful urination experienced upon consumption of *P. adspersus* harvested from the first rainfall of the rainy season as reported in Okeyo *et al*. (2015), putative toxin producer associated microbiome in African bullfrog meat has never been explored.

1.3. Objectives of the study

Objectives of the study were to:

a) Determine the nutritional value of the Giant African bullfrog meat.

b) Assess the Giant African bullfrog meat for antioxidant activities.

c) Determine the microbial composition, diversity, function and assessing the putative toxin producer symbiotic microbiome associated with the Giant African bullfrog meat through metagenomics analysis.

1.4. Hypothesis of the study

It can be hypothesized that African bullfrogs from the first rainfall of the season are associated with certain microbe(s) which are linked to dysuria side effect upon

consumption. It can also be hypothesized that this species is associated with a great deal of nutritional and antioxidant activities.

1.5. Significance of the study

This research serves as a foundation towards a better understanding regarding the cause of the said painful urinary condition. In addition, determining the nutritional value and antioxidant activities may improve the consumption rate of this species benefiting more Namibians, and subsequently Africans and reducing poverty levels. Furthermore, the idea of propagation and growing of this particular species as a way of supplementing income streams for impoverished communities could be considered.

1.6. Limitations of the study

Metagenomics analysis was used to determine the presence of bacteria using 16S sequencing only as ITS gene for fungi failed to be amplified via Polymerase Chain Reaction (PCR). Therefore, the study was limited to toxin producing bacteria only. In addition, the microbial diversity might have been influenced by the location where the frogs have been harvested (De Assis, Barreto & Navas , 2017).

1.7. Delimitation of the study

The results are only applicable to Oshana region of Namibia where sampling occurred. Due to logistics, sampling was only possible to be done in one area.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. Types of frogs

2.1.1. Poisonous frogs

Omonona and Ekpenko (2011) described frogs as transitional animals that live partly in water and on land. Frogs belong to the Kingdom: Animalia, Phylum: Chordata, Subphylum: Vertebrata, Class: Amphibia, Order: Anura (Omonona & Ekpenko, 2011). Poisonous frogs , (family *Dendrobatidae*), are also called poison dart frogs, dart-poison frogs, or poison arrow frogs, or any of approximately 180 species of new world frogs characterized by the ability to produce extremely poisonous skin secretions (Zug, 2020). However, Saporito *et al*. (2012) disputed that the term ‘poison frog’ is not synonymous with the term ‘dartpoison frog,’ which is properly used only to describe poisonous frogs of three species in the genus *Phyllobates* (*Dendrobatidae*) that have been used in dart-poisoning. Saporito *et al*. (2012) highlighted that poison frogs are generally classified by an ability to sequester an alkaloid-based chemical defense from dietary arthropods and currently, poison frogs include members of certain genera in the families *Dendrobatidae, Bufonidae, Mantellidae, Myobatrachidae*, and most recently, *Eleutherodactylidae*. Saporito *et al*. (2012) note that *Dendrobatid* frogs represent the most species-rich group of poison frogs, including more than 90 species in eight genera (*Adelphobates, Ameerega, Dendrobates, Epipedobates, Minyobates, Oophaga, Ranitomeya*, and *Phyllobates*). In addition, *Phyllobates terribilis* Myers, Daly and Malkin, 1978 is labelled as one of the most toxic vertebrates (Stynoski, Schulte & Rojas, 2015).

Besides being poisonous, many poison frog species display bright colors and unique behaviors such as most are active in the daytime which is quite rare for frogs (Stynoski *et*

al., 2015). Coloration is an direct indicator of toxicity in some species, but not in others, and is associated with territorial aggressiveness and boldness in some cases (Stynoski *et al.*, 2015). The toxin found on the skin of *Dendrobatidae* is a type of alkaloid (Hare, 2019). The current theory is that toxicity of poison dart frogs is actually "exogenous" meaning the amphibians don't make the poison themselves (Hare, 2019). Poison frogs acquire defensive chemicals (lipophilic alkaloids) that are sequestered from dietary arthropods and stored in skin glands (Moskowitz *et al*., 2018). However, there is little evidence on the transmitting of such poison to human. Unlike frogs, ingestion of toad can lead to poisoning which may primarily manifest as gastrointestinal, mental, cardiac conduction, and arrhythmic disturbances (Gowda, Cohen & Khan, 2003).

Furthermore, like most animals, frogs are associated with skin microbiota. Mutnale, Reddy and Vasudevan (2021) highlighted the putative role of frog skin microbiota in affording resistance to *Batrachochytrium dendrobatidis* (*Bd*) infections. However, this is not unique to poisonous frogs, besides bacterial communities on the amphibian skin are lineage specific, and may be influenced by environmental factors (De Assis, Barreto & Navas, 2017). Moreover, frog microbiota is responsible for producing antimicrobial peptides (AMPs) (Grogan *et al*., 2018) but there is no evidence linking skin microbiota to toxicity in poisonous frogs.

2.1.2. Edible frogs and their nutritional composition

In literature, edible frogs refers to those frogs whose meat are considered for human consumption (Neveu, 2004). Scientifically, all edible frogs belong to the class of *Amphibia* (4000 known species), and are placed in the order *Anura* (3500 species) and for

the most part within the family *Ranidae* (700 species), among the genus *Rana* (250 species) (Neveu, 2004).

According to Neveu (2004) more than 50 species of frogs are harvested from nature for human consumption worldwide. In Africa particularly, the most consumed species are *Pyxicephalus adspersus* Tschudi, 1838; *Pyxicephalus edulis* Peters, 1854; *Hoplobatrachus occipitalis* Günther 1858; *Trichobatrachus robustus* Boulenger, 1900; *Conraua spp. or Ptychadena spp.* which are typically considered as delicacies (Mohneke, Onadeko & Rödel, 2009). Akinyemi, Akinyemi and Ogaga (2015) argue that most of the frogs utilized for consumption in developed countries are sourced from developing countries. Onadeko, Egonmwan and Saliu (2011) affirmed what Ashton *et al*. (1988) has reported frog legs being a popular delicacy in Europe and were even eaten in countries where it is legally prohibited to hunt frogs.

Nutrients are generally classified as macronutrients and micronutrients (Rosmawati *et al*., 2018). According to Chen, Michalak and Agellon (2018) micronutrients, which include vitamins and minerals, are needed only in small amounts, and are required for the proper function of important proteins and enzymes. Whereas, macronutrient which include carbohydrates, proteins, and fats, are usually needed in large amounts (Chen, Michalak & Agellon, 2018). Edible frogs have been found to be very nourishing and represent an alternative source of animal protein particularly in areas where fish and other protein sources are either in short supply or relatively more expensive (Daniel *et al*., 2016). In many African countries, frogs have been collected on a local scale as an essential source of protein (Mohneke *et al*., 2009).

According to Onadeko *et al*. (2011), the amino acid composition of frog meat can be compared to those of fish such as *Clarias* and Tilapia species with Glutamic acid being

the major amino acid followed by aspartic acid. In addition, Blé, Yobouet and Dadié (2016) revealed that *Hoplobatrachus occipitalis,* an edible frog from Midwest areas of Côte d'Ivoire, is a good source of protein with dietary minerals such as calcium and potassium. Furthermore, Daniel *et al*. (2016) concluded that the lipid contents in frog meat are considerably low and healthy for human consumption. Therefore, several studies have in tandem recommended frog meat for functional and health benefits such as treatment of gastrointestinal diseases, allergies, and in diets with sodium, fat, and calorie restrictions (de Oliveira *et al*., 2017). Despite that, more studies still need to be conducted on proximate composition and mineral content of most edible frogs (Blé *et al*., 2016).

In addition, besides being used for consumption, some of the benefits of frog meat are very persuasive on health. Some of these benefits are summarized by Ainun, Fadhillah and Silalahi (2019) :

1. Animal Protein Sources. Frog meat is good for health because it is a source of animal protein which is high in nutritional content.

2.Treating Impotence in Men. Frog meat served in the form of juice; it actually has properties to treat impotence in men.

3. Overcoming Heart Damage. It turns out that frog meat also has the potential to treat wounds caused by heart disease.

4. Prevent Asthma and bronchitis. In addition to treating impotence, frog juice derived from frog meat is also able to prevent other diseases such as asthma and bronchitis.

5. As an antibiotic, due to frog skin antimicrobial and alkaloids properties.

6. Overcoming Stroke Damage and Cancer. Frog meat has the potential to treat conditions that require rapid repair of blood vessels, such as healing from damage caused by stroke (Ainun *et al*., 2019).

Edible frogs thus only represent a very small proportion of all *Amphibia* and *Ranidae*, since many species are too small or, more importantly, considered too toxic for human consumption or vitally considered in traditional medicine (Neveu, 2004).

2.2. *Pyxicephalus adspersus*

2.2.1. Biology, distribution and ecology

The genus *Pyxicephalus* currently comprises four species distributed throughout sub-Saharan Africa: The Giant African Bullfrog (*Pyxicephalus adspersus* Tschudi, 1838), Narrow-headed Bullfrog (*P. angusticeps* Parry, 1982), Edible Bullfrog (*P. edulis* Peters, 1854) and Calabresi's Bullfrog (*P. obbianus* Calabresi, 1927) (Scott *et al*., 2013). According to Okeyo, Kashea and Kandjengo (2014) the Giant African Bullfrog (*Anura: Phyxicephalidae*: *Pyxicephalus adspersus* Tschudi, 1838) is also referred to as "African Pyxie Frog" or "Pyxie Frog" or "Giant Pyxie" or "The Giant Bullfrog" or "African Bullfrog" or "African Burrowing Frog". *Pyxiecephalus adspersus* is recognized as a corpulent olive-green frog with darker skin ridges and a large head and mouth (Terry, 2002). Adult *Pyxiecephalus adspersus* are also known for their aggressive disposition and tendency to bite using the canine-like projections on their lower jaw (Yetman, 2012). In addition, this species is characterized by a non-webbed fingers, and a number of longitudinal elevated skin folds which are more prominent in the dorsum of large specimens (Okeyo *et al*., 2014).

The Giant African Bullfrog is distributed widely throughout Southern and Eastern Africa: Namibia, Angola, Botswana, South Africa, Zimbabwe, Zambia, Mozambique, Malawi, Tanzania and Kenya as stated by Channing 1991 in (Okeyo *et al*., 2014). In Southern Africa, the Giant Bullfrog is considered the largest reaching 200 mm in length (Okeyo *et al*., 2014). Furthermore, they confirmed that in Namibia, the species is reported to occur in the central and northern areas where it is commonly seen in the flooded plains. According to Terry (2002) African Giant Bullfrogs live mostly in a variety of arid and semiarid habitats, such as savanna, steppes, bushlands, and semi deserts.

Terry (2002) highlighted that the species spend most of their life underground during the dry season. They go into a long "sleep" to avoid the harsh summer conditions common in sub-Saharan Africa and when heavy rains come, the frogs congregate in shallow pools of water to spawn (Terry, 2002). Okeyo *et al*. (2014) explained that *P. adspersus* life begins during the early times of any rainy season. First the eggs are laid in shallow water of either ponds, pools, swamps or streams (rivers) with slow moving water. Eggs hatch into tadpoles and after a month or so, they turn into frog lings (Okeyo *et al*., 2014). At the time, the frog appears in various sizes: the sub-juveniles and the juveniles. If they survive predators, the Giant African Bullfrog can live, inhabiting close immediacies with water, or in hibernation to a ripe age of about forty years (Yetman, 2012). It is assumed that they aestivate for much of the year and come to the surface after the first rains to feed and breed (Okeyo *et al*., 2014).

A study done by Okeyo *et al*. (2014) revealed that food items eaten by the Giant African Bullfrogs occurring in the flooded plains in northern Namibia are of all sizes ranging from as tiny as ants to beetles and tadpoles. Consequently, due to a variety of food sizes that the African Bullfrogs have consumed, the study suggest that the Bullfrog seems to eat

anything that fits in its mouth or anything it can overpower (Okeyo *et al*., 2014). Equally important, it was concluded that the African Bullfrog's diet tends to be mainly carnivorous, though large frogs may sometimes become cannibalistic and feed on small frogs. (Okeyo *et al*., 2014). Furthermore, Conradie *et al*. (2010) study on the diet of metamorphosed Giant African Bullfrogs from a semi-aquatic habitat in the Karoo, South Africa revealed that metamorphosing Bullfrogs appear to consume anything in their immediate environment in order to gain mass before they enter winter dormancy. The study has reported that insects accounts for the greatest prey diversity, with Coleopterans (11 families) dominating the 29 insect families (Conradie *et al*., 2010).

2.2.2. Preparation and consumption of *P. adspersus* in Northern Namibia

The consumption and preparation of *P. adspersus* is unfortunately poorly studied (Daniel *et al*., 2016). In Africa particularly, *P. adspersus* has been reported to be widely consumed and features in the diet of many local communities in Nigeria and Namibia (Daniel *et al*., 2016 ; Okeyo *et al*., 2015). In Namibia, the African Bullfrog (*P. adspersus*) is considered to be a delicacy by local people, mostly people from the Aawambo ethnic group (Okeyo *et al*., 2015). According to local people as reported in Okeyo *et al*. (2015), African Bullfrogs are traditionally harvested after a heavy rainfall or upon at least a second rainfall of the season. Okeyo *et al*. (2015) reported that the intestines and all viscera are removed except the fat prior to cooking. Traditionally when cooking frogs, pieces of the bark from the stalks of Maize (*Zea mays* L.) or Pearl millet (*Pennisetum glaucum* [L.] R. Br.) locally known as omapungu and omahangu respectively or twigs from edible medicinal plants such as *Spirostachys africana* Sond. are laid on the base of a traditional cooking pot (normally made up of clay) (Okeyo *et al*., 2015). The barks or twigs are said to prevent

the frog meat from sticking to the bottom of the pot. In addition, traditional edible plants are reported to aid in preventing the side effects associated with consumption of the frogs harvested proximately after the first rainfall (Okeyo *et al*., 2015).

In general, frogs are mostly consumed smoked or fresh cooked in sauce with skin after evisceration. For instance, In Burkina Faso, the frogs are properly cleaned and eviscerated then fried before consumption; while in Nigeria, they are frequently sundried or smoked and seldom fried prior to consumption (Kia *et al*., 2017). However, it is not clear whether the same practice is being done in Namibia as *P. adspersus* is observed to be consumed only during the rainy season. Though spicing frying or roasting of frogs is common today, most local people of the Aawambo ethnic group have been reported to prefer frog meat that retains its texture and shape during cooking , hence only salt is added for taste (Okeyo *et al*., 2015). In contrast, Europeans and in other parts of the world only skinless Frog legs are eaten either fried or roasted (Kusrini & Alford, 2006). Besides having high level of protein (Kusrini *et al*., 2006), it is not clear as why most Europeans prefer only the frog legs than other parts of the frog for consumption purposes. De Oliveira *et al*. (2017) emphasize that frog meat is a highly digestible food which justifies its use in special diets, however the importance of its use and forms of preparation must be divulged.

2.2.3. Side effects associated with consumption of *P. adspersus* meat harvested after the first rain and their putative causes

According to Okeyo *et al*. (2015) consumption of the Giant African Bullfrogs that are harvested proximately after the first rain in Namibia are associated with a condition locally known as “oshitekateka” translated into English as dysuria. It affects both male and female of all age groups and the symptoms include acute inflammation and pain when passing

urine (Okeyo *et al*., 2015). Okeyo *et al*. (2015) further reported that local people reveal that "oshitekateka" condition can be avoided by not harvesting frogs when they are too young or before the matured one start calling to mate. In addition, The "oshitekateka" condition is said to be avoided traditionally by cooking the frogs with pieces of bark from specific plants such as *Spirostachys africana* (Okeyo *et al*., 2015). Furthermore, treatment can be achieved traditionally though people also seek professional medical treatment from local clinics and other health centers (Okeyo *et al*., 2015).

Although local people have indigenous knowledge on how to avoid and treat the said condition, there is lack of scientific information and understanding on the actual cause of that condition. Besides, it is unclear whether similar conditions caused by *P. adspersus* harvested and consumed after the first rain occurs elsewhere in the world.

2.3. Determination of nutritional contents: Proximate analysis

Nutritional proximate analysis is a quantitative analysis of macromolecules in food which includes lipid/fat content, moisture content, crude protein, ash content and carbohydrates as analyzed in several studies (Efenakpo, Ijeomah & Eniang, 2015; Mathew *et al*., 2015).

2.3.1. Fat content

Fat is a diverse class of compounds that contribute to the organoleptic, physiochemical, nutritional aspects of foods and is one of the major source of energy in the diet (Srigley & Mossoba, 2017). In addition, fat has a great influence on the maintenance of muscular tissue reducing protein breakdown and contributes to palatability, tenderness, juiciness, and flavor of meat (Beriain *et al*., 2021). According to Nielsen (2010) the total lipid content of foods are commonly determined by organic solvent extraction methods, Gas Chromatography (GC) analysis, non-solvent wet extraction methods, such as the Babcock

or Gerber, or using instrumental methods, such as NMR, infrared, and Foss-Let. Soxhlet extraction is a common crude fat determination method in many food commodities (Nielsen, 2010). The method has been successfully used as part of proximate analysis to determine crude fat content in frog meat (Efenakpo, Ijeomah & Eniang, 2015; Ibietela & Amadi, 2019). Soxhlet method is usually preferred because it is simple to use and it is officially recognized by the Association of Official Analytical Chemists (AOAC) as the standard method for crude fat analysis (Nielsen, 2010).

2.3.2. Moisture content

According to Fairulnizal *et al*. (2020) moisture content is one of the most crucial components in food analysis as it defines the quality, shelf life and sensory features in food. The moisture analyzer and the drying oven are the most common techniques used in moisture analysis (Efenakpo *et al*., 2015). Moreover, Microwave Radiation Method and Near Infrared Reflectance (NIR) method has been effectively used as described in (Fairulnizal *et al*., 2020). Though, the oven drying methods has been used for moisture determination in frog meat (Efenakpo *et al*., 2015; Mathew *et al*., 2015), the method is slowly being replaced by a moisture analyzer. Arezou, Maria and Mehrdad (2020) emphasized that the drying oven method is time consuming as the drying time is significantly more, and additional instruments, such as a precise measuring scale and sample containers, are required. Meanwhile, the moisture analyzer is a portable automated unit that minimizes user inaccuracies (Arezou *et al*., 2020). Moreover, a moisture analyzer is energy efficient as it stops the drying process once no significant changes in the specimen weight are detected (Arezou *et al*., 2020).

2.3.3. Proteins

Proteins are considered essential for general health and wellbeing, biological functions and cell structure (Hayes, 2020). It is therefore crucial to find a protein analysis method that is reliable to determine protein content in the human diet. Several methods exist to quantify protein content, including the Kjeldahl, Lowry, Bradford and total amino acid content methods (Hayes, 2020). However, protein analysis in food is susceptible to several imprecisions due to several reasons. According to Mæhre *et al*. (2018) food composition, food structure, or matrix, and interactions between the different nutrients may reduce the accessibility of the proteins leading to underestimation of the protein content.

Furthermore, some methods determine protein either directly or indirectly by performing protein extraction prior to protein determinations. Mæhre *et al*. (2018) explained that direct protein determination is when protein content is calculated based on the analysis of amino acid residues whereas indirect protein determination can for instance be inferred succeeding the determination of the nitrogen content, or after chemical reactions with functional groups within the protein. The Kjeldahl method has been used in several studies (Efenakpo *et al*., 2015; Ibietela & Amadi, 2019) to determine protein content in frog meat. According to Mihaljev *et al*. (2015) Kjeldahl method is internationally used as the standard method against all other methods due to it is high precision and very low variation interval. However, a study by Hayes (2020) suggested that the Kjeldahl method and other methods that determine protein content in food based on nitrogen conversion factors overestimates the protein content even when the species-specific conversion factor for nitrogen was used. Mæhre *et al*. (2018) affirmed with Hayes findings and disputed that among all the methods for protein determination in food, amino acid analysis is the only method where interfering substances do not affect the results.

2.3.4. Ash content

Ash content is described as an inorganic residue remaining after water and organic matter have been removed by presence of oxidizing agents (Fairulnizal *et al*., 2020). Ashing is the primary step in preparing a food sample for specific elemental analysis (Nielsen, 2010). Therefore, ash may provide an estimation of the total amount of minerals within the food item (Fairulnizal *et al*., 2020). There are two major types of ashing as described by Nielsen (2010) namely, dry ashing and wet ashing. Dry ashing is primarily for proximate composition while wet ashing (oxidation) is a preparation for the analysis of certain minerals such as Iron, Copper, Phosphate and Zinc (Nielsen, 2010). In addition, microwave systems are available for both dry and wet ashing to speed the processes (Nielsen, 2010). Dry ashing has been used efficaciously as part of proximate analysis in frog meat analysis (Burubai, 2016; Ibietela & Amadi, 2019). However, Soylak *et al*.(2004) claimed that the dry and wet ashing methods are more time consuming and complicated than the microwave method.

2.3.5. Carbohydrates

Carbohydrates are a major source of energy, impart crucial textural properties in food, and are dietary fiber which influences physiological processes in the body (Nielsen, 2010). As explained in Nielsen (2010) carbohydrates can be determined by various techniques including High Performance Liquid Chromatography (HPLC), GC as well as Enzymatic methods (Nielsen, 2010). However, before analyzing for any class of carbohydrate, it is vital that the sample must be prepared so as to remove substances such as fats, proteins, pigments, vitamins and minerals that can interfere with analysis (Cui & Brummer, 2005).

According to Cui and Brummer (2005) the GC analysis of carbohydrates is advantageous over other methods as it requires small sample sizes and it is very sensitive.

2.4. Antioxidants

Oxygen is one of the crucial elements in life, however, "when cells use oxygen to generate energy, free radicals are formed as a consequence of adenosine triphosphate (ATP) production by the mitochondria" (Pham-Huy, He & Pham-Huy, 2008). Qazi and Molvi (2018) defined free radicals as "atoms or molecules or molecular fragments containing one or more unpaired electrons in their atomic or molecular orbitals." The human body generates two types of free radicals as a result of cellular redox processes: Reactive Oxygen Species (ROS) as well as Reactive Nitrogen Species (RNS) (Pham-Huy *et al*., 2008). As summarized by Qazi and Molvi (2018) ROS includes; Superoxide, Hydrogen peroxide, Hydroxyl radical, Peroxyl radical, Alkoxyl radical, Hydroperoxyl radical, Singlet oxygen and Ozone while RNS are Nitric oxide, Nitrogen dioxide, Nitrous acid, Dinitrogen tetroxide, Dinitrogen trioxide, Peroxynitrite, Peroxynitrous acid, Alkyl peroxynitrites, Nitronium cation and Nitryl chloride.

Free radicals are generally produced naturally in humans through biological processes, such as breathing, digesting food, metabolizing alcohol and drugs, and turning fats into energy (Sharifi-Rad *et al*., 2020). Based on that context, the immune system, metabolic processes, stress, dietary factors, environment factors, toxins and some drugs are all factors responsible for generation of free radicals (Sarma *et al*. as cited in Qazi and Molvi (2018). According to Qazi and Molvi (2018), free radicals may be both toxic and beneficial compounds. At low or moderate levels, free radicals exert beneficial effects on cellular responses and immune function such as phagocytosis, apoptosis, detoxification

reactions as mediator and executioner of precancerous and infectious cells (Qazi & Molvi, 2018). However, at high concentrations, they cause oxidative stress, and subsequent damage to proteins, lipids, and DNA subsequently resulting in the development of chronic and degenerative conditions such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases (Pham-Huy *et al*., 2008). Figure 1 below gives an illustration of the causes and effects of free radicals in the body.

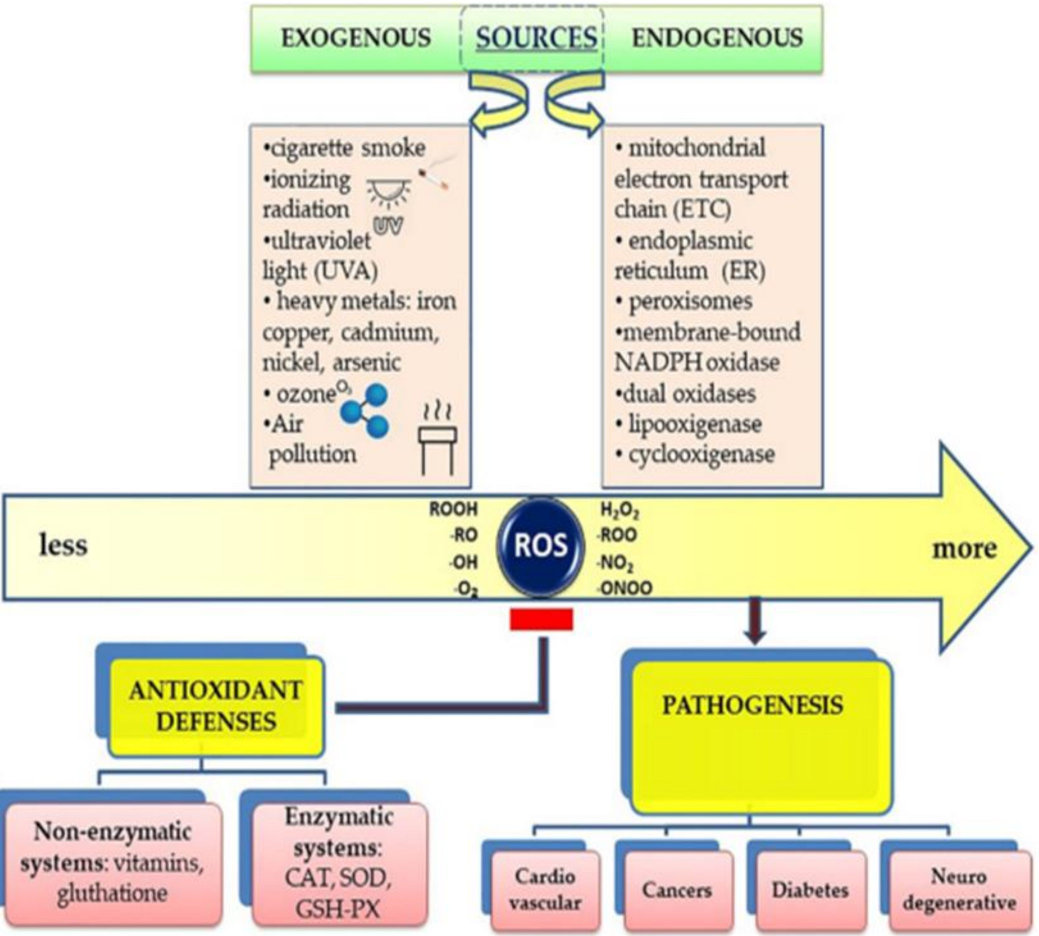


Figure 1: Main sources of free radicals and their effects on the human body (Sharifi-Rad *et al*., 2020).

Logically, since free radicals are necessary for life, the body has several mechanisms to minimize radically induced damage and to protect against excessive production of free

radicals by means of antioxidants (Sharifi-Rad *et al.,* 2020). Therefore, oxidative stress only occurs when there is a serious imbalance between the production of ROS and RNS on one hand, and the levels of antioxidant defenses on the other (Elsayed Azab *et al*., 2019). Elsayed Azab *et al*.(2019) defined an antioxidant as “a molecule which has the ability to prevent or slow the oxidation of macromolecules.” It is well established that the roles of antioxidants in a human body is to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention (Elsayed Azab *et al*., 2019). According to Halliwell (1990) as cited in Carocho and Ferreira (2013) another property that a compound should have to be considered an antioxidant is the ability after scavenging the radical, to form a new radical that is stable through intramolecular hydrogen bonding on further oxidation.

There are two main types of antioxidant; those that occur natural in food (natural antioxidant) and those that are added to food so it can withstand various treatments and conditions as well as to prolong shelf life (synthetic antioxidant) (Carocho & Ferreira, 2013). Natural antioxidants are mainly polyphenolic compounds which inhibit free radical reaction by stabilizing free radicals and these are classified into mineral, vitamins and phytochemical (Qazi & Molvi, 2018). Meanwhile synthetic antioxidants are just synthetic phenolic compounds that inhibit free radical chain reaction by interacting with free radicals (Qazi & Molvi, 2018). Antioxidants are further classified as enzymatic (primary and secondary) and non-enzymatic as illustrated in Figure 2 below. The primary enzymatic antioxidants such as glutathione peroxidase prevent the formation of free radical by neutralizing them while secondary enzymatic antioxidants such as glutathione reductase prevent the formation of free radical by generating a reducing compound which neutralizes them instead (Qazi & Molvi, 2018). On the other hand, non-enzymatic

antioxidants include chemical molecules of low-molecular-weight that directly act as antioxidants (Sharifi-Rad *et al.,* 2020). Though their action is not catalytic, they require antioxidant regeneration or supplementing from the diet (Sharifi-Rad *et al.,* 2020).

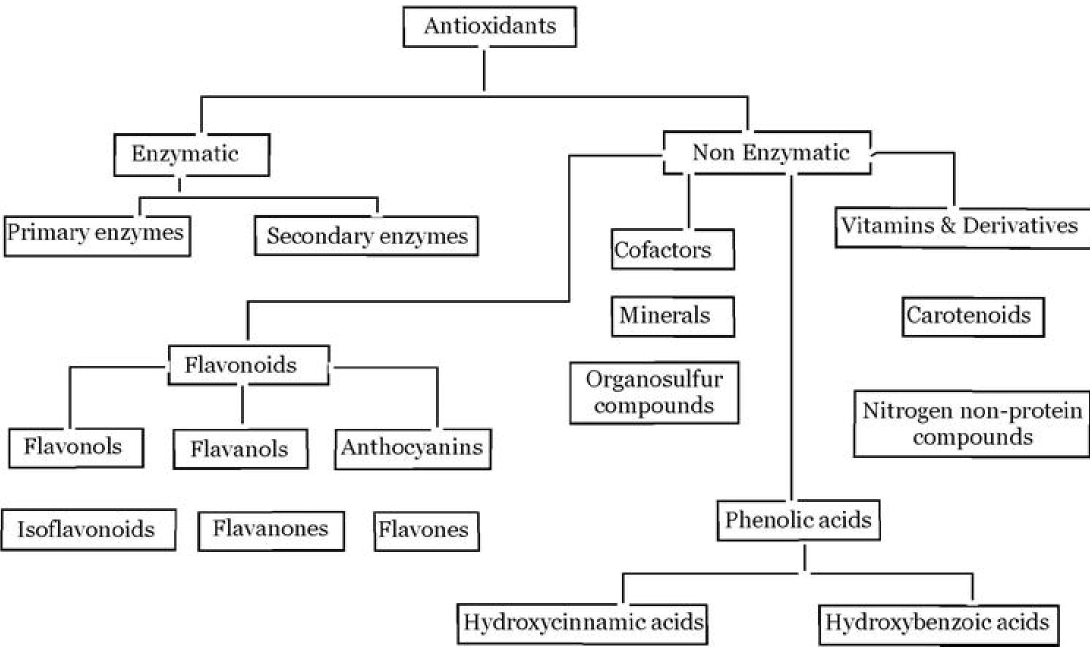


Figure 2: Broad classification of antioxidants adapted from Carocho and Ferreira (2013)

It is vital to maintain the balance of the antioxidants in human diets to reduce the oxidative stress. Antioxidants are mostly abundant in colored fruits, vegetables, as well as other foods including nuts, grains, poultry and fish (Hamid *et al*., 2010). In general, plants are a rich source of antioxidants as they protect them from ultraviolet damage and against lipid peroxidation (Reynertson, Basile & Kennelly, 2005). Additionally, Polyphenolic compounds such as phenolic acids, flavonoids, anthocyanin's and tannins are naturally produced as secondary metabolites by plants and are said to possess remarkable antioxidants and anticancer activities (Prasad *et al*., 2009). Meanwhile, there are few studies on meat as a source of antioxidants. Most studies either use synthetic or natural antioxidants (plant based) to enhance quality or to prevent lipid oxidation in meat and

meat products (Alvarez-Parrilla *et al*., 2014; Arshad *et al*., 2017). Nonetheless, Mirzaei, Afshoon and Barmak (2017) confirmed presence of antioxidant in meat from chicken and goat.

2.4.1. Methods for determination of antioxidant activity

According to Alam, Bristi and Rafiquzzaman (2013) tests for antioxidant activity are performed both in vivo and in vitro. Assays performed in vivo included lipid peroxidation, reduced glutathione, superoxide dismutase and catalase assay (Alam *et al*., 2013). Most studies however test for the antioxidant activity through in vitro procedures which includes 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging assay, superoxide anion radical scavenging activity, reducing power, ferric thiocyanate assay and total antioxidant activity (Prasad *et al.,* 2009; Aliyu *et al*., 2019).

2.4.1.1. 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging assay

Alam *et al*. (2013) describe DPPH as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. Furthermore, Alam *et al*. (2013) explain that when a solution of DPPH is mixed with that of a substrate that can donate a hydrogen atom it gives rise to the reduced form with the loss of this violet color. The color change is usually measured spectrophotometrically at 517 nm (Chanda & Dave, 2009). The percentage of inhibition can therefore be calculated using the following formula as described in (Chanda & Dave, 2009):

$$Inhibition\ (\%) = \frac{A0 - A1}{A0} \times 100$$

Where; A0 is the absorbance of control and A1 is the absorbance of test.

1,1-diphenyl-2-picrylhydrazyl method is commonly used for the *in vitro* antioxidant activity evaluation due to advantages such as fast, easy to perform, low cost, reproducibility and applicability at room temperature (Munteanu & Apetrei, 2021). Most importantly this assay can be used for both solid and liquid samples (Dontha, 2016). Limitations of DPPH method is the fact that many antioxidants that react quickly with the radical peroxide are almost or entirely inert to DPPH and this method is at times complicated when test compounds have spectra that overlap with DPPH at 515 nm (Dontha, 2016).

2.4.1.2. Reducing power

According to Alam *et al*. (2013) this method is based on the principle of increase in the absorbance of the reaction mixtures. During the reaction, substances which have reduction potential forms a colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured spectrophotometrically at 700 nm (Chanda & Dave, 2009). Alam *et al*. (2013) explained that absorbance and antioxidant activity seem to be directly proportional to each other (an increase in the absorbance indicates an increase in the antioxidant activity). According to Munteanu and Apetrei (2021), Reducing power assay is the simplest spectrophotometric test for antioxidant activity measurement. Another advantage is that it is very fast and lacks the need for calibration according to a standard like Gallic acid.

2.4.1.3. Nitric oxide scavenging assay

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes (Parul, Kundu & Saha, 2012). In addition, Boora, Chirisa and Mukanganyama (2014)

classify NO as a free radical which displays important reactivity with certain types of proteins and other free radicals such as superoxide. In this assay Nitric oxide is generated from sodium nitroprusside in aqueous solution at physiological pH which interact with oxygen to produce nitrite ions, which are measured using the Griess reagent (Chanda & Dave, 2009). In the review, Alam *et al*. (2013) clarified that the reaction is measured spectrophotometrically at 546 nm and the amount of nitric oxide radical inhibition is calculated following this equation:

$$\% \, Inhibition \, of \, NO \, radical = \frac{(A0 - A1)}{A0} \times 100$$

where A0 is the absorbance before reaction and A1 is the absorbance after reaction has taken place with Griess reagent.

2.5. Roles of chemical agents and associated microbes as frog defense mechanism

According to Savitzky *et al*.(2012) animals and plants are defended by an extraordinary array of molecules that render them noxious, and in some cases toxic, to potential predators. The acquisition of noxious or toxic substances can either be endogenous, in which the substances are produced by the organism, or exogenous, in which the substances are produced by another organism and are sequestered (Darst *et al*., 2005). Most plants, animals, and microorganisms make use of chemicals as defensive agents (Jeckel, Grant & Saporito, 2015). For instance, as summarized in Künzler (2018) the defense effectors in microorganisms such as fungi, include secondary metabolites and primary metabolites (peptides and proteins). However, among animals particularly land vertebrates, chemical defenses are restricted to a few monophyletic groups (mostly amphibians and snakes) (Santos, Tarvin & O'Connell, 2016). Nevertheless, secondary metabolites particularly

alkaloids have been reported as amphibians defense mechanisms in numerous articles (Daly *et al*., 1978; Daly, Spande & Garraffo, 2005; Jeckel, Grant & Saporito, 2015). Additionaly, many bacteria have developed physical corporations with other organisms comprising more limited metabolic capabilities, allowing them to interact with them and exploit their resources for mutual benefits (Pérez-Brocal *et al*., 2011). Hence, it is not surprising that the surfaces of animals and plants contain a great abundance and variety of microorganisms, i.e exosymbiotic (Zilber-Rosenberg & Rosenberg, 2008). Equally important, symbionts have been frequently studied (Loudon *et al*., 2014; Becker *et al*., 2015) for their vital role in the host health and survival. In some cases, the symbiont provides toxins, antimicrobials, or other bioactive compounds defending the host directly (Oliver & Russell, 2016). Nonetheless, De Assis, Barreto and Navas (2017) discovered that bacterial communities on the amphibian skin are lineage specific and transported by heredity, but may also be influenced by environmental factors depending on the frog species. It is notorious that frogs can uptake lipophilic alkaloids from arthropod prey items, produced de novo or by symbiotic microorganisms, and store them in skin granular glands as a defensive response (Santos, Tarvin & O'Connell, 2016). This has been reported in poison frogs but it is not clear whether similar cases occur in edible frogs (Santos *et al*., 2016).

2.6. Metagenomics analysis

To effectively comprehend the role of microorganisms in any given environment, it is important to isolate them and study their morphological, physiological, biochemical, and genetic makeup and characteristics. Though, the value of culture based approaches for making discoveries in microbiology is undeniable, the majority of microorganisms in any

given environment have not been cultivated yet even when sophisticated media and new cultivation and isolation methods are applied (Zengler, 2009). Furthermore, cultivation-based approaches are generally not suitable to determine microbial community structure and dynamics over time and often lead to the isolation of microbial weeds, which are well adapted to the conditions offered in the laboratory but not necessarily important in the environment under investigation (Harwood & Merry, 2007).

"Metagenomics" describes the functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample (Loudon *et al*., 2014). Among the methods designed to gain access to the physiology and genetics of uncultured organisms, the genomic analysis of a population of microorganisms, has emerged as a powerful centerpiece (Handelsman, 2005). There are two types of approaches in metagenomics as described by Mande, Mohammed and Ghosh (2012). The first approach is the Shortgun-Sequencing (MGS) in which genomic fragments originating from the entire genomes of organisms are extracted and sequenced. The second approach is the targeted metagenomics approach which involves extraction and sequencing of amplicons corresponding to specific phylogenetic marker genes such as 16S rRNA.

In addition, several Next-Generation Sequencing (NGS) platforms for taxonomic profiling, characterization, and analysis of microbial communities have been developed as discribed in Hozzein (2020). Two common and widely used NGS in metagenomics are the 454 Life Sciences and the Illumina systems (Oulas *et al*., 2015). Additional sequencing technologies are also available and being employed in metagenomic studies which are SOLiD 5500 W Series developed by Applied Biosystems (Waltham, Massachusetts, United States), Single-Molecule Real-Time (SMRT), DNA sequencing from Pacific

Biosciences, and Ion Torrent semiconductor sequencing (Oulas *et al*., 2015). Subsequently to the sequencing process, sequencing data is typically organized into large matrices containing the total observed counts of clustered sequences commonly known as Operational Taxonomic Units (OTUs), that represent bacteria types (Weiss *et al*., 2017).

One of the advantages of metagenomics is that it excludes the use of PCR to amplify gene cassettes or random PCR primers, hence eliminates the restrictions and biases associated with PCR (Harwood & Merry, 2007). In addition, Simon and Daniel (2011) highlighted that metagenomics has revolutionized microbiology by paving the way for a cultivation-independent assessment and exploitation of microbial communities present in complex ecosystems. Furthermore, it has proven to be a powerful tool to isolate new enzymes and drugs of industrial importance (Simon & Daniel, 2011). Equally important, Handelsman (2005) have noted that many bacterial symbionts that have highly specialized and ancient relationships with their hosts do not grow readily in culture. This makes them ideal candidates for metagenomic analysis because the bacteria can be separated readily from host tissue and other microorganisms (Handelsman, 2005). Metagenomics has been successfully used in various studies in different industries including food industry, for instance to determine microbial diversity in fermented food (De Mandal *et al*., 2018). Equally important, metagenomics has been used in achieving some historic milestones such as discovery of novel antibiotics, novel antibiotic synthesis pathways and antibiotic resistance genes among many other house keeping genes (Sukhum, Diorio-Toth & Dantas, 2019).

Nevertheless, there are several challenges concerning the use of metagenomics. Firstly the ideal phylogenetic anchor would be equally represented in all species, however, the 16S

rRNA genes do not meet this standard because microorganisms differ in the number of rrn operons they carry in their genomes (Riesenfeld, Schloss & Handelsman, 2004). Moreover, constructing metagenomic libraries from environmental samples is theoretically simple but technically challenging (Riesenfeld *et al*., 2004). This is due to the fact that, to obtain greater than single sequencing coverage, the size of a metagenomic library would need to be many times the size of the metagenome (Riesenfeld *et al*., 2004). Additionally, Harwood and Merry (2007) noticed that utlilization of any molecular techniques in microbial studies is that it is not always possible to predict the physiology of a microorganism from its phylogenetic relationship to other organisms.

2.6.1. Estimation of microbial diversity

In general, biodiversity has been defined as "the range of significantly different types of organisms and their relative abundance in an assemblage or community" (Fakruddin, 2013). Conversely, microbial diversity describes biodiversity at three levels: within species, species number and within community (Harpole, 2010). Microbial diversity is commonly estimated at two levels using Alpha and Beta diversity. Alpha diversity estimates diversity within a sample, or within a habitat or intra-community whereas Beta diversity give diversity estimation between samples, habitat or inter community diversity (Thukral, 2017). Alpha diversity metrics review the structure of a microbial community with respect to species richness and evenness (Willis, 2019).

Species richness is the absolute number of different species present in a sample or population of interest (Daly, Baetens & De Baets, 2018). In addition, Fakruddin (2013) refers it to as the quantitative variation among species. In contrast, evenness or equability is the distribution of individuals among these species (Fakruddin, 2013). Therefore,

richness measures number of taxonomic groups whereas evenness measures the distribution of abundances of these groups (Willis, 2019). It is for these reasons that species richness and evenness is directly proportional to diversity, such that when richness and evenness increase, diversity increases too (Kim *et al*., 2017).

Several diversity measures exist for Alpha diversity as compiled by Kers and Saccenti (2021) such as Phylogenetic diversity, Observed number of Amplicon Sequence Variants (ASV), Chao1, Simpson and Shannon indices. Whereas, commonly used Beta metrics are Bray-Curtis dissimilarity, Jaccard, unweighted UniFrac and weighted UniFrac (Kers & Saccenti, 2021). Nevertheress, Shannon-Weaver and Simpson diversity indices are the most comomnly used as they provide more inference about the community composition than simple species richness or evenness (Kim *et al*., 2017). Nonetheless, Lemos *et al*. (2011) empasize normalization of the number of sequences in all samples, because diversity index values increase with sample sizes.

2.6.2. Microbial Functional prediction

According to Goswami *et al*. (2017) functional diversity refers to a component of biodiversity that generally covers the range of metabolic traits of microorganisms prevailing in a community and ecosystem. Metagenomics particularly known as functional metagenomics is one powerful experimental approach for studying gene function. Lam *et al*., (2015) enlighten that functional metagenomics involves isolating DNA from microbial communities, cloning DNA fragments, expressing genes in a surrogate host, and screening for enzymatic activities. Lam *et al*., (2015) emphasizes that functional metagenomics approach enable the discovery of novel enzymes whose functions would not be predicted based on DNA sequence only.

Microbial function may be predicted through MGS which sequences entire genomes rather than marker genes, which directly reveals genetic functional potential within microbial communities (Douglas *et al*., 2020). However, MGS may not work well due to possibility of host contaminations such as biopsy, or if there is minimum community biomass (Douglas et al., 2020). Though 16S rRNA amplicon is a commonly used sequencing method, functional profiles cannot be directly identified using 16S rRNA gene sequence data (Douglas *et al*., 2020). Therefore, software tools such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States version 2 (PICRUSt2) and Piphillin among others were developed for prediction of functions from 16S marker sequences data (Tamang, Shangpliang & Rai, 2020).

3. CHAPTER THREE: RESEARCH METHODS

3.1. Sample collection

Frogs were collected from Ondangwa rural constituency in the Oshana region at Okapya village with geographical coordinates of -17.867052,15.933011. Approximately 36 frogs in total were collected between 5 December 2019 and 22 January 2021 in two intervals (18 from the first rain and 18 from the second rain of the rainy season).

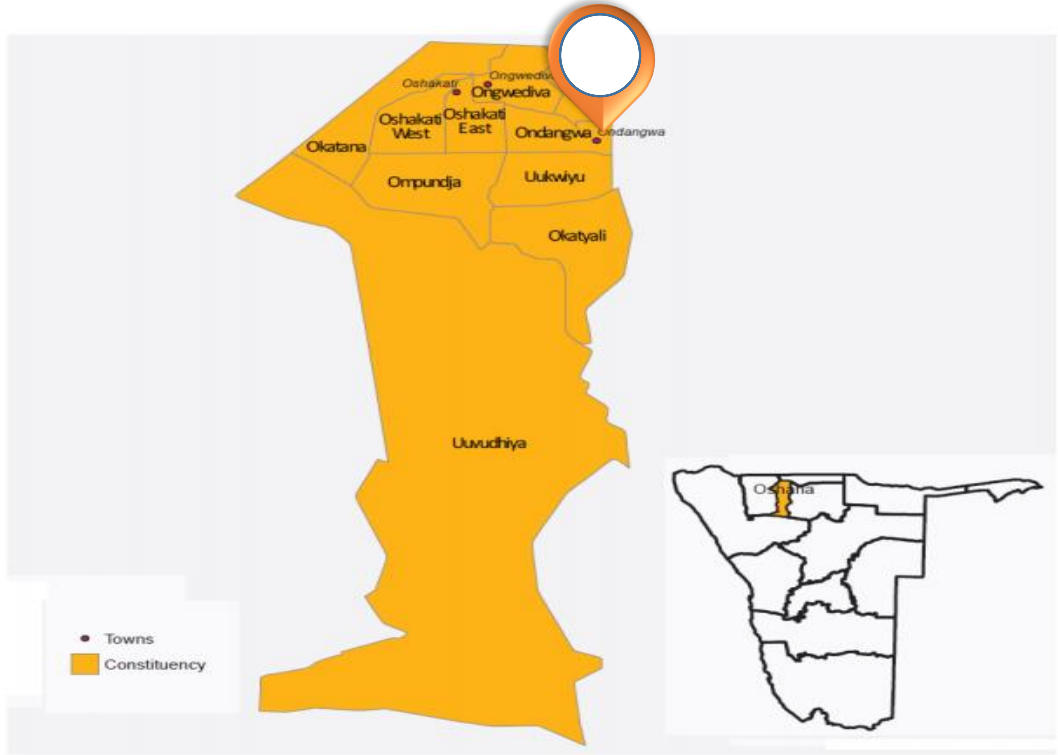


Figure 3: Namibian map showing Ondangwa in Oshana region where sampling occurred adapted from Namibia Statistics Agency (2011).

3.2. Sample preparation

Following the sample collection, frogs were immobilized by a blow on the head as done traditionally (Daniel *et al*., 2016). Individual frogs were washed through running tap water to remove all the dirt before transported in a cooler box to the University of Namibia (UNAM), laboratory. Subsequently, individual frogs were washed again through running

tap water before being deboned using a sterile surgical blade. They were cut in smaller pieces and placed in individual zip seal bags and kept in a -80 ^{0} C freezer until further analysis. Though total collected samples were 36, 1 individual frog was not enough to do even one analysis. Therefore, 30 samples were randomly divided into 5 groups which were homogenized with a blender (Mathew *et al*., 2015). These five homogenized samples where used for all proximate and antioxidant analysis. Only 6 individual frogs were used for DNA extraction (3 from the first rain and 3 from the second rain). Samples used for metagenomics were coded as follows: F1- first sample from first rain, F2-second sample from first rain, F3-third sample from first rain, S1-first sample from second rain, S2-second sample from second rain and S3-third sample from second rain.

Figure 4: a) *Pyxiecephalus adspersus* *b)* deboned *P adspersus meat* in individual zip seal bags.

3.3. Proximate analysis

3.3.1. Crude fat/lipid content

Crude lipid was determined by solvent extraction system (Foss, Soxtec, 2043) following a manual as per manufacturer instructions. Approximately 2g homogenized frog meat samples were placed in individual thimbles and dried in the oven at 103℃ for 2 hours.

Prior to extraction process, fat extraction cups were weighed and the weight was recorded. Subsequent to that about 45 ml of petroleum ether was added to each extraction cup. Samples in corresponding thimbles as well as the extraction cups were placed in a Soxtec 2043 fat extraction system (Foss, Hoganas, Sweden) and fat was extracted with petroleum ether. After the analysis, fat extraction cups were weighed again and the weight was recorded. To calculate the crude fat of each sample, the following equation was used as adapted from the Soxtec 2043 fat extraction system (Foss, Hoganas, Sweden) manufacturer manual. The results were expressed as the percentage of the weight difference of the extraction cups.

$$Crude\ fat = \frac{(W3 - W2)}{W1} \times 100$$

Where W1- thimble weight with sample inside, W2 –fat extraction cup weight before extraction, W3 – fat extraction cup after extraction.

3.3.2. Moisture content

Moisture content was determined using the same method as previously described by Efenakpo *et al*. (2015) using a different approach. Instead of using an oven, an ADAM PMB 202 machine was used as per manufacturer instructions. The machine determines the amount of moisture in a sample by weighing it, then drying it and re-weighing it again (Arezou *et al*., 2020). The amount of mass lost can then be used to calculate moisture content. Approximately 2g of the homogenized samples were placed in a moisture analyzer. In the analyzer, individual samples were heated at different temperature ranging from 110° C to 112° C. The initial and final weight as well as the amount of moisture expressed in percentage was displayed on the machine at the end of each sample analysis.

3.3.3. Ash content

Ash content was determined using procedures described in Mathew *et al*. (2015) with little adjustments. Two grams of each sample was weighted into individual pre-weighed crucibles and burned into ashes in the oven at 560°C for 5 hours. The hot crucibles were cooled in a desiccator and weighed. The ash content corresponds to the weight difference between the crucible containing the ash and the empty crucible, expressed as a percentage of the mass of sample used.

$$\% \, Ash = \frac{Ash \; weight \; (g)}{Sample \; weight \; (g)} \times 100$$

3.3.4. Crude protein content

Crude protein was determined in terms of nitrogen using micro Kjeldahl method by Kjeldahl (1983) as demonstrated in (Hussain *et al*., 2011). The nitrogen value was converted to protein by multiplying to a factor of 6.25 (Mariotti *et al*., 2008).

3.4. Determination of antioxidant activities

3.4.1. Sample preparation

Preparation of samples for antioxidant assays followed a procedure similar to that used in Patel, Patel and Kajal (2010) with little adjustment. A total of 5 deboned homogenized frog meat samples were dried in the fume hood for 4 -5 days before blended into powder using a laboratory-based blender. Powdered samples were extracted in 99% methanol on 1:10 ratio in a shaking incubator for 48 hours at 30ºC at 125 rpm. The resulting extracts were then filtered through Whatman's No. 1 filter paper and dried. Extraction was repeated 3 times to get enough extracts. Final dried extracts were reconstituted with the same amount of methanol, kept at room temperature until analysis.

3.4.2. 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging activity was determined following a procedure as described in Chanda and Dave (2009) with little alteration particularly in terms of the amount of volume used. Briefly, 200 µl of the extracts (3.0-20 mg/ml) were mixed with 200 µl of DPPH (0.3 mM in methanol) in a 96 well plate. Plates were incubated at room temperature for 60 minutes in the dark. The absorbance was measured spectrophotometrically at 517 nm. Ascorbic acid was used as a positive control and the absorbance of DPPH (negative control) was also measured. The experiment was done in 3 trials and each trail was done in triplicates. Percentage inhibition was calculated using a formula below:

$$\%\, Inhibition\ = \frac{(A0 - A1)}{A0} \times 100$$

3.4.3. Reducing power assay

Reducing power of the frog meat extracts was determined according to Chanda and Dave (2009). Approximately 1.0 ml extract (3.0 mg/ml – 20 mg/ml) was mixed with 2.5 ml of phosphate buffer (200 mM) and 2.5 ml of potassium ferricyanide (30 mM). The mixture was incubated at 50^0C for 20 minutes before adding 2.5 ml of trichloroacetic acid (600 mM) to the mixture. The resulting mixture was allowed to settle for 10 minutes to separate the layers. About 2.5 ml of the upper layer was added to a mixture of 2.5 ml distilled water and 0.5 ml of $FeCl_3$ (6 mM). Two hundred microliters (200 µl) of the final mixtures were measured spectrophotometrically in a 96 well plate at 700 nm. Ascorbic acid was used as positive control while a blank was used as a negative control. The experiment was done in 3 trials and each trail was done in triplicates.

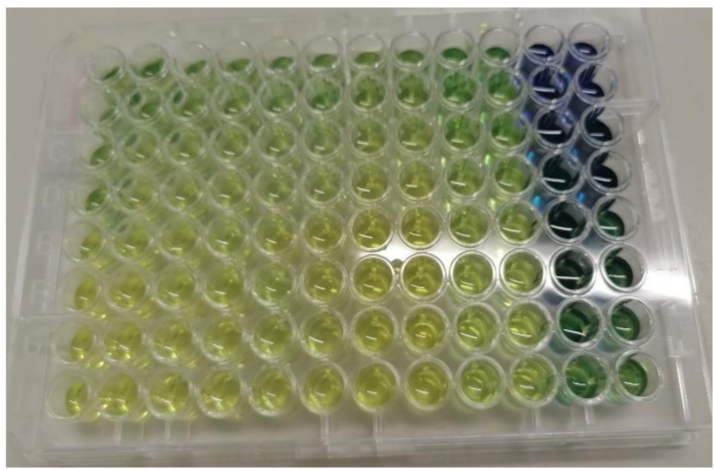


Figure 5: Reducing power assay

3.4.4. Nitric oxide scavenging assay

This assay was carried out following a method as described in Singhal *et al*. (2009) with a little modification from Awah and Verla (2010). Approximately 750 µl of extract or the positive control (ascorbic acid) or the blank (phosphate buffer saline) was mixed with 100 µl sodium nitroprusside (10 mM) before incubation at 25°C for 180 minutes. After incubation 200 µl of Griess reagent was added to the mixture and left for 5 minutes. Two Hundred microliters (200 µl) of the final mixture were transferred to the 96 well plate and measured spectrophotometrically at 546 nm. Experiment was done in triplicates. Percentage inhibition was calculated using the following formula:

$$\% \, Inhibition = \frac{(A0 - A1)}{A0} \times 100$$

where A0 is the absorbance before reaction and A1 is the absorbance after reaction has taken place with Griess reagent.

3.5. Metagenomics analysis

DNA was extracted from 6 different frog meat samples (skin plus tissue). Three were those that were harvested from the first rain, while the other 3 are those that were harvested from the second rain of the rainy season). Extraction was done using a ZymoBIOMICS^{TM} DNA miniprep kit (The epigenetics Company, USA) as per the manufacturer instructions. The extracted genomic DNA was sent for metagenomics analysis to INQABA Biotechnical Industries (South Africa). Samples were sequenced on the Sequel system by Pacific Biosciences (PacBio). Raw subreads were processed through the SMRTlink (v9.0) Circular Consensus Sequences (CCS) algorithm to produce highly accurate reads (>QV40).

3.6. Data analysis

All analysis (proximate and antioxidant) were carried out in triplicates. The results were reported as mean ± standard error of the mean. Statistical analysis was done using Statistics Package for Social Science (SPSS, version 23). Kolmogorov-Smirnov Test and the Shapiro-Wilk Test were used to test for normality whereas One-way ANOVA analog and Kruskal-Wallis test were used as statistic tests depending on whether data were normally distributed or not. Additionally, Tukey post hoc test was used as a follow up test in cases where ANOVA test was significant, to determine which group was different from which other group.

In addition, demultiplexed paired-end sequence reads were trimmed, denoised, merged and clustered into amplicon sequence variants (ASVs) using QIIME2 (Bolyen *et al*., 2019) and DADA2 (Callahan *et al*., 2016) denoised plugin for Pacbio long-read sequences. The resulting representative sequences were assigned taxonomy using a classifier trained on

the SILVA reference database. The potential functions of microbe was predicted via PICRUSt2 (Douglas *et al*., 2020). MetaCyc pathways were used for analyzing predicted functions while statistical differences between samples were determined using the Welch's test (Hwang *et al*., 2020).

3.7. Research ethics

Ethical clearance was obtained from the UNAM Research Ethics Committee (UREC) and research permission was obtained from the UNAM Center for Postgraduate Studies (CPS). Upon completion of the laboratory work, samples were incinerated by City of Windhoek.

4. CHAPTER FOUR: RESULTS

4.1. Proximate analysis

The result showed that *P. adspersus* meat composed abundantly of water and protein. The moisture content ranged from 70.13±6.34 to 78.21±0.38 % whilst crude protein was uniform with averages of about 21.00±0.00%. The result also showed that tested *P. adspersus* meat was low in ash and crude fat content. The crude fat ranged from 0.07±0.06 to 1.65±2.71 % while ash content ranged from 0.53±0.35 to 2.25±1.25% (Table 1). In addition, Kruskal-Wallis test analysis of proximate composition shows no significant difference among crude protein (p value 0.453), moisture (p value 0.065), crude fat (p value 0.308) and ash content (p value 0.136) at 0.05 significant value (Appendix 1).

Table 1*:* Results of the proximate composition of *P. adspersus* meat samples

Samples	Crude protein %	Moisture %	Ash %	Crude fat %
1	21.00±0.00	70.13±6.34	1.19±0.32	0.29±0.13
2	21.00±0.00	72.51±0.35	1.16±0.87	0.15±0.12
3	21.00±0.00	76.53±0.58	0.81±0.44	0.07±0.06
4	21.00±0.00	78.21±0.38	2.25±1.25	0.10±0.09
5	21.00±0.01	72.78±4.06	0.53±0.35	1.65±2.71

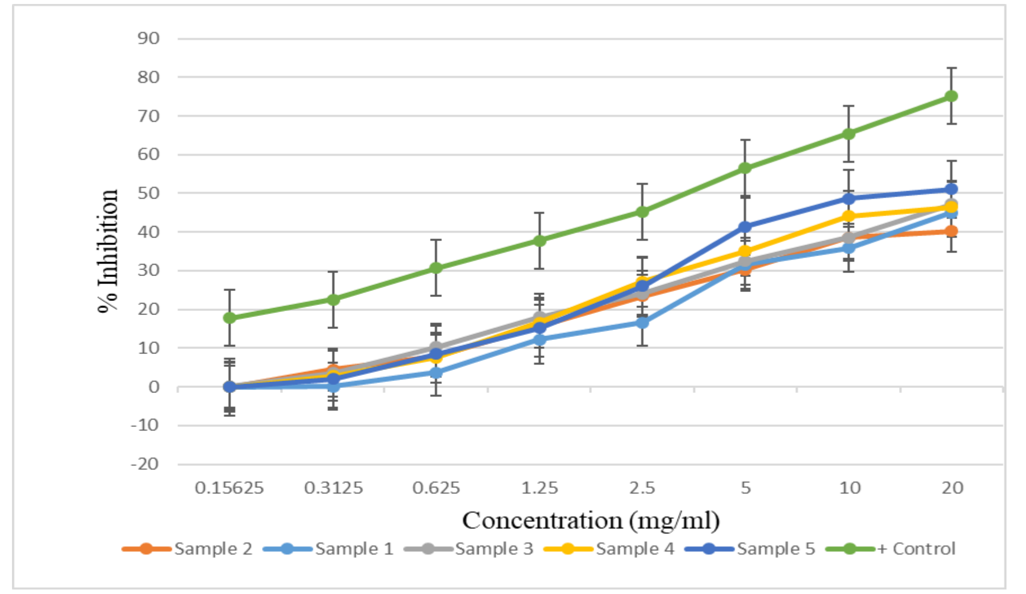
Note: Samples analyses were carried out in triplicate, value represent the mean percentage and standard error of the sample

4.2. Antioxidant activity assay

4.2.1. DPPH free radical scavenging activity

In this study, it has been observed that all 5 samples have significant amounts of radical scavenging activity ranging from 40 to 50 percent though relatively low as compared to the control (Figure 6). The DPPH scavenged activity data were normally distributed by Shapiro-Wilk as p values were greater than 0.05 (Appendix 2). Hence, statistical

significance was determined by One-way ANOVA test. The result shows a statistically significant difference in the mean percentage inhibition of the extracts at different concentrations (Appendix 2). In addition, the Tukey post hoc test reveals significant difference (Appendix 2) between all samples (1-5) against the positive control at all concentrations and no significant difference (Appendix 2) within the samples. Difference was considered to be statistically significant at $p<0.05$.



NB: data are presented as means of three independent experiments and standard error

Figure 6: DPPH radical scavenging activity of the five samples at different concentrations.

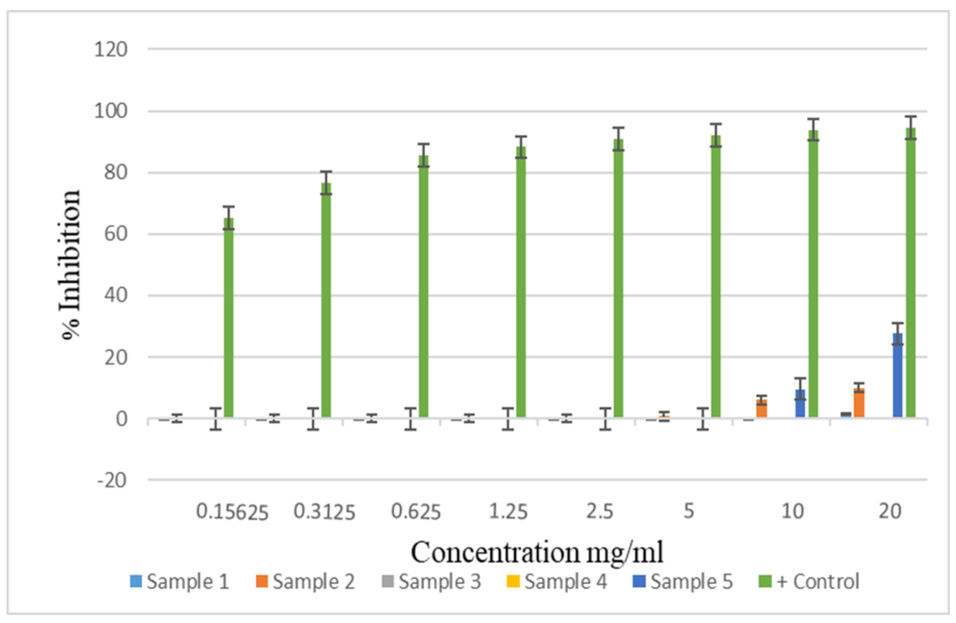
Table 2: IC_{50} values for different sample extracts

Samples	IC_{50} mg/mL
1	-
2	-
3	-
4	-
5	7.6
Positive control	5.25

IC_{50} is calculated as the concentration of antioxidants needed to decrease the initial DPPH concentration by 50% (Rivero-cruz *et al*., 2020).

Rivero-cruz *et al*. (2020) underlined that the lower IC_{50} value the higher antioxidant activity a sample has. IC_{50} value was calculated in excel using the linear regression equation of each trendline from the DPPH graph (Xiao *et al*., 2020). The results showed low IC_{50} value (7.6) for sample 5 and a much lower IC_{50} value (5.25) for ascorbic acid. Sample 5, 50 % inhibition was achieved around 10 mg/ml as seen from the graph (Figure 6). However, the equation produces a lower IC_{50} of 7.6 for sample 5. According to Sebaugh (2011) estimation of IC_{50} via linear regression may be less accurate because graph of these values is not entirely linear. There is usually some scatter in the data points and scatter within the subset of points that is used in the linear calculation which will introduce error into the calculation (Sebaugh, 2011). Nevertheless, 4 of the samples yield less than 50% inhibition which indicated low antioxidant activity and IC_{50} could not be calculated. Therefore, IC_{50} for sample 1,2,3 and 4 can only be achieved once concentration is increased to yield at least 50% inhibition.

4.2.2. Nitric oxide scavenging activity

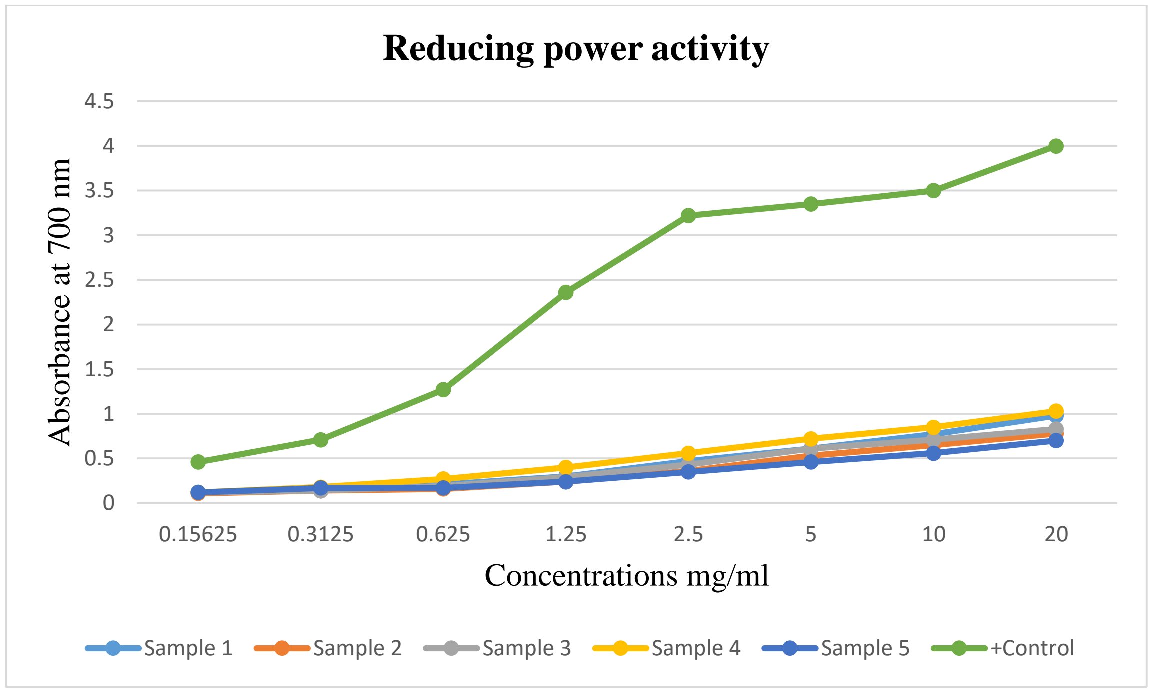


NB: Data are means±SE of three independent experiments.

Figure 7: Nitric oxide scavenging activity of different samples including ascorbic acid.

The tested Nitric oxide scavenging activity was relatively low specifically at low concentration for all tested samples (Figure 7). The highest percentage inhibition among the tested samples was sample 5 with 27.64% at 20mg/ml followed by Sample 2 with 9.9417% at 20mg/ml while the positive control was recorded to have the highest % inhibition of 90.19% at 20mg/ml with a remarkable calculated IC_{50} value of 3.10 mg/ml. In addition, mean difference were found to be significant (p value 0.00) at 0.05 p value using One-way ANOVA test for all concentration and not significant at 0.15625 mg/ml (p value 0.087) (Appendix 3).

4.2.3. Reducing power activity



NB: Data are means±SE of three independent experiments.

Figure 8: Reducing power assays at different concentration.

Sample 4 was recorded to have the highest absorbance of 1.03±0.66 at 20 mg/ml while Sample 5 has the lowest of 0.70±0.54 at the same concentration (Figure 8). Kruskal Wallis test shows a significant difference between the means of samples at lower concentrations

and no significant difference observed between the means rank of the groups at higher concentrations (Appendix 4). Mean difference were found to be significant at 0.05 p value.

4.3. Metagenomics analysis

4.3.1. Bacterial community composition of *P. adspersus* meat

A total of 6 samples were chosen randomly, 3 from first and 3 from second rain. However, among six genomic DNA from six different samples, at least four (Figure 9) were successfully amplified with PCR for 16S. However only three were eligible for the downstream analysis as the other three were not successfully amplified. Sample F2 (sample 2 of the first rain) produced less data than expected, hence was not used for further analysis. It was observed that S1 (sample 1 of the second rain) had high number of polymerase reads which accounts for more bases. It can already be seen from Figure 9 that S1 has higher richness in taxonomic groups. Nonetheless, none of the DNA samples were successfully amplified for ITS. According to Hashim (2016) some of the reasons for PCR failure include degraded DNA or low DNA integrity, insufficient quantity of DNA, or template DNA may contains PCR inhibitors such as ethanol.

Bio Sample Name	Barcode Name	Polymerase Reads	Bases
S1	M13_bc1002_F--M13_bc1055_R	103,363	156,853,408
S2	M13_bc1002_F--M13_bc1056_R	151,153	229,255,681
S3	M13_bc1002_F--M13_bc1057_R	78,537	118,892,357
F2	M13_bc1002_F--M13_bc1058_R	461	585,719

Figure 9: 16S PCR reads of different DNA samples.
NB: S= Samples from the second rain, F=Samples from the first rain.

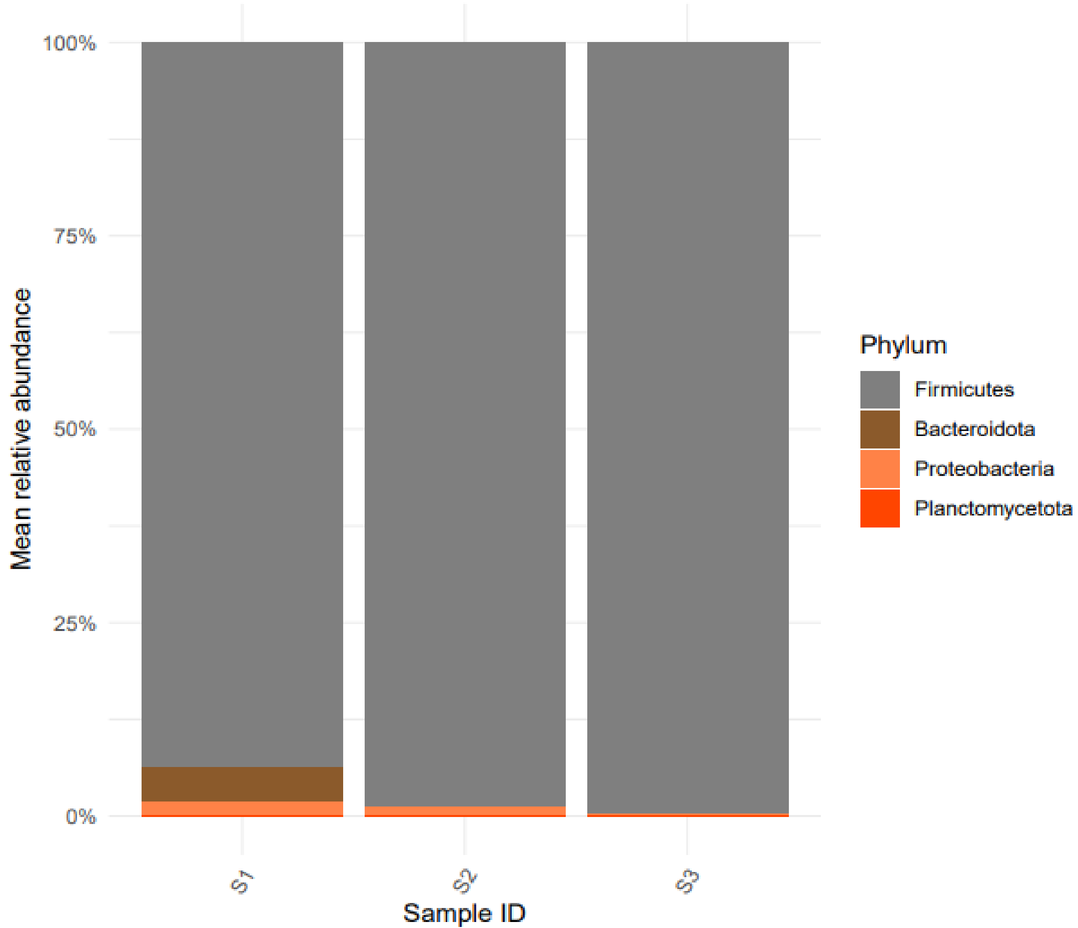


Figure 10: Mean relative abundance of the dominant phylum of 16rRNA sequences classifications in *P. adspersus* meat samples

At phylum level, the dominant phyla in both S1, S2 and S3 is *Firmicutes* accounting for over 80% of the total population. In addition, *Firmicutes* was the most abundant phylum in the 3 samples while *Proteobacteria* was the second abundant phylum in all 3 samples. Nonetheless, *Bacteroidota* phyla and *Planctomycetot*a were the least abundant.

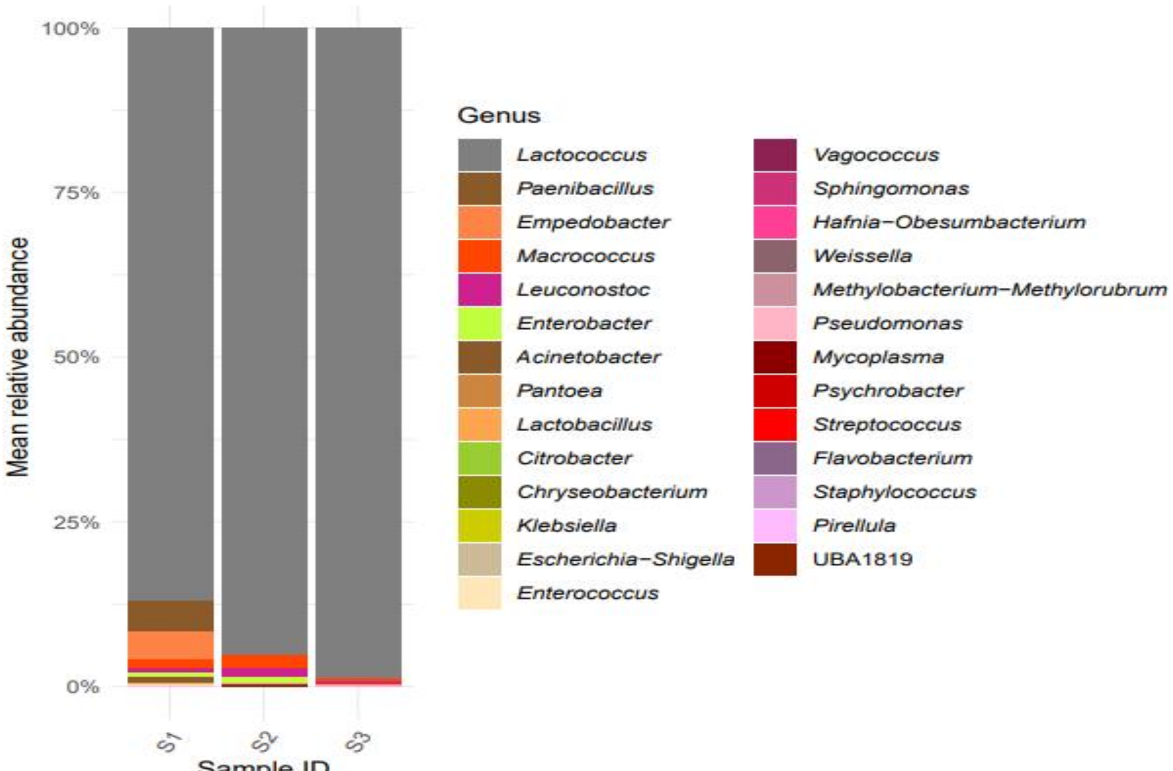


Figure 11: Genera relative abundance using 16S rRNA sequences classification in *P. adspersus* bacteria community.

At the genus level, a total of 27 genera were obtained from the three samples. Among the 27 genera detected, at least 21 genera exist in both samples. However, only *Lactococcus* and *Paenibacillus* have a relative abundance greater than 0.10% of the total bacteria. *Lactococcus* accounts for more than 80% of the entire 27 detected genera. According to the distribution of the *P. adspersus* bacteria at the phylum (Figure 10) and genus (Figure 11) level, it is evident that the abundance of microbial species in S1 was higher than that in S2 and S3.

4.3.2. Bacterial diversity and functional prediction

Only a total of 3 samples were eligible for alpha diversity analysis. As a result of PCR failure for sample F2 and F3 as well as low reads obtained for F1, Beta diversity was not possible for the comparison of bacterial diversity between samples collected after first rain

and those collected after the second rain of the rainy season. The number of the observed ASVs obtained for all three samples were satisfactory, suggesting that a sufficient number of reads had been obtained in the samples to accurately assess bacterial diversity. The ASV table was normalized to a sequencing depth of 36,532.00 counts or sample prior to the determination of Alpha diversity.

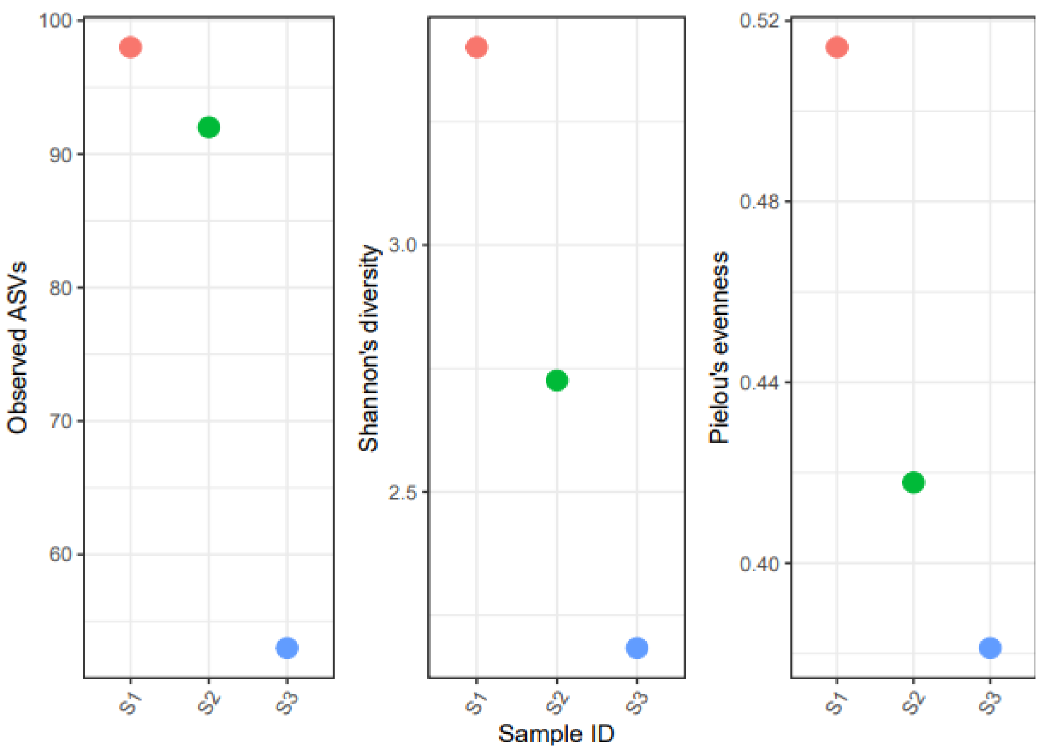


Figure 12: Observed ASVs with corresponding Shannon diversity and Pielou's evenness for sample S1,S2 and S3. Where S1= orange dot, S2=green dot and S3= blue dot.

Shannon and Pielou's indices of bacterial diversity (Figure 12) revealed that sample S1 exhibited the highest level of bacterial diversity, followed by sample S2, while the lowest Alpha diversity was observed in sample S3. Shannon and Pielou's indices are directly correlated with alpha diversity. The Shannon index revealed that sample S1 has the highest species richness and the species richness was found to be more evenly distributed as portrayal by Pielou's indices (Figure 12) than sample S2 and sample S3.

A functional profile of the bacteria that were found to be associated with *P. adspersus* meat sample S1, S2 and S3 was generated using PICRUSt. The metabolic pathways generated from MetaCyc website https://biocyc.org/META/class-tree?object=Pathways were predicted based on bacterial metagenomes by modelling genes from 16S rRNA data derived from the generated ASVs. About 328 bacterial metabolic pathways has been predicted from all the three samples analyzed (Appendix 4). Bacterial metabolic pathways predicted from *P. adspersus* meat samples were found to encode amino acid degradation such as Arg+polyamine-syn (super pathway of arginine and polyamine biosynthesis), carbohydrates degradation such as glycolysis, biosynthesis of nucleoside and nucleotide, aromatic compound degradation and a few vitamins and alcohol degradation pathways among others (Appendix 5). However, there are no significantly different pathways across samples (Appendix 6), though sample S2 and S3 show slight statistical differences (Figure 13).

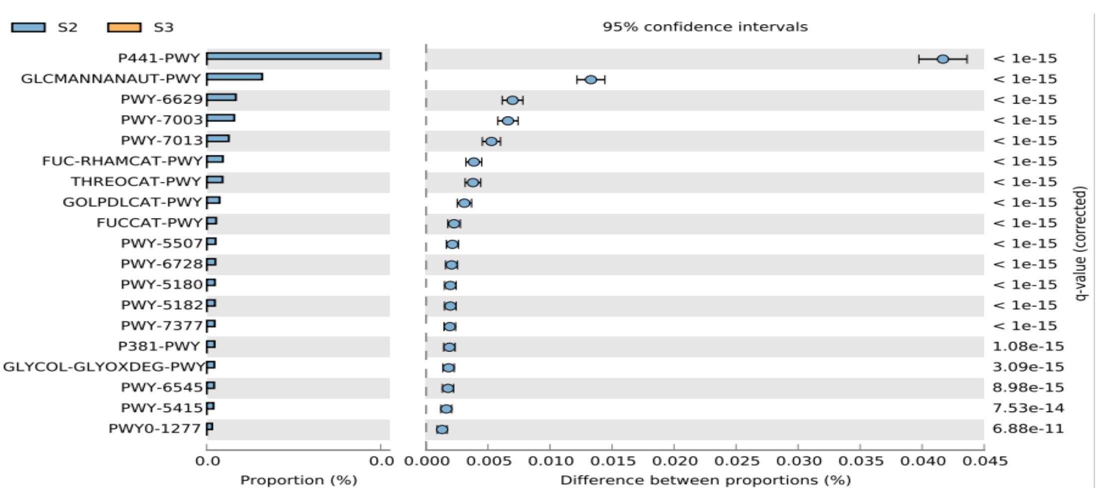


Figure 13:Statistical comparison of bacteria predicted pathways from 16S rRNA derived data of sample S2 and S3 at 95% confidence intervals.

5. CHAPTER FIVE: DISCUSSION

5.1. Nutritional composition of *P. adspersus* meat

Proximate analysis is one of the important criteria for determining the nutritional values and quality of food. According to Charrondiere *et al*.(2013) nutritional values are generally used to address all forms of malnutrition (i.e. undernourishment, micronutrient deficiency and over nutrition) by increasing the availability and affordability of a wide range of diverse foods that are needed for a healthy diet. However, the potential of indigenous, neglected or underutilized food to improve dietary diversity remains largely unknown (FAO, 2019). Therefore, as the first step in determining the nutritional composition of *P. adspersus* meat, proximate analysis was applied.

5.1.1. Crude fat content

The crude fat value in this study ranged from 0.07% to 1.65%. These values were far lower than 9.75%, 7.58% and 8.47% reported for *Hoplobatrachus occipitalis*, *Hildebrandtia ornate* and *Ptychadena pumilio* respectively (Efenakpo *et al*., 2015). Yet, these results were in accordance with 1.20% recorded for *Rana esculenta* (Özogul *et al*., 2008). Though the Soxhlet method is commonly used for fat analysis, there is no single standard method for the determination of fats in different foods (Nielsen, 2010). However, the difference in values from different literature may be influenced by sample preparation, the solvent used for extraction as well as the preservation of the sample prior to analysis (Nielsen, 2010). Additionally, El Oudiani *et al*. (2019) highlighted that the level of fat in aquatic animals depends on diet composition as well as environmental factors and may vary seasonally. According to Jiménez-Colmenero, Carballo and Cofrades, (2001) there is numerous evidence that fat-rich diets are associated with obesity, colon cancer and

cardiovascular diseases in humans. Conversely, de Oliveira *et al*. (2017) stated that the use of frog meat in diets are intended for the treatment of cholesterol, obesity, gastrointestinal diseases, and in diets with fat and calorie restrictions. Hence, low fat content recorded in this study for *P. adspersus* is evident that this meat could be a strong contender in the fat or calories restricted diet.

5.1.2. Moisture content

Moisture content recorded for *P. adspersus* ranged from 70.13±6.34% to 78.21±0.38%. This study reveals a high moisture as compared to 3.49±0.56% recorded for *Pelophylax esculentus* (Mathew *et al*., 2015). However, the results were in agreement with 78.6 ± 0.02% recorded for *Dicroglessus occipitalis* (Burubai, 2016). Besides, moisture content recorded was high as compared to that of different fish species (Ndome, Oriakpono & Ogar, 2010). According to Nielsen (2010) different methods may yield dissimilar results, for instance some methods attempt to remove or quantitate all water present which is often complicated by interference by other food constituents. Nevertheless, meat in general has an average of more than 70 % moisture content, making it part of perishable food (Rabia, Ali & Muhammad, 2018). Furthermore, Rabia *et al*. (2018) elucidated that besides reduction in shelf life, high moisture content have a strong impact on the color, texture and flavor of muscle tissues of meat. Therefore, similar to any other type of meat, high moisture content in *P. adspersus* makes the meat more susceptible to spoilage and preservations measures have to be considered for long term storage. In Nigeria, drying is the easiest and only available traditional form of preserving frog meat (Efenakpo, Ayodele & Ijeomah, 2016).

5.1.3. Ash content

In one of the studies, ash content was reported to be about 25.49%, 19.6% and 17.46% for *Hoplobatrachus occipitalis*, *Hildebrandtia ornate* and *Ptychadena pumilio* respectively (Efenakpo *et al*., 2015). Nonetheless, the present study recorded a very low ash content for *P. adspersus* ranging from 0.53±0.35% to 1.19±0.32%. These results were in agreement with those recorded for wild and cultured *Rana ridibunda* (Cagiltay *et al*., 2014). Park and Bell (2004) described ash content as an estimation of the total mineral content in food. They further explain that, ash content does not necessarily represent the exact composition of minerals present in the original food because there may be losses via volatilization or some interaction between constituents. This, therefore, means that values reported for this study might not be a true reflection of the amount of minerals in *P. adspersus* although the results are in line with those recorded for indigenous chickens in Malawi (Tanganyika, 2017). Nevertheless, in a magazine article, Baker (2015) highlighted that generally any natural food will be less than 5% ash in content and only some processed foods can have ash content of more than 10%.

5.1.4. Protein content

It should be noted that *P. adspersus* has a high protein content of about 21% (Table 1). The results are in line with 18.52 and 22.95 g/100 g recorded for wild and cultured *Rana ridibunda* respectively (Cagiltay *et al*., 2014). However, protein value obtained from the current study is a little lower than those obtained from other frog species. *Hoplobatrachus occipitalis, Hildebrandtia ornate* and *Ptychadena pumilio* have been reported to have a much higher protein content of about 48.23%, 52.83% and 49.22% respectively (Efenakpo *et al*., 2015). According to Nielsen (2010) protein content may be affected by the type of

method used as well as the present of other major food components (such as lipids and carbohydrates) which may interfere physically with the analysis. Nevertheless, as a reference from the above values, frog meat has a much lower protein content than that reported for various fish species (Ndome *et al*., 2010). On the contrary, frog meat protein content has been noted to be higher than that in other meat type such as chicken, beef and rabbit meat (Omotayo *et al*., 2016). Amazingly, Burubai (2016) discovered that *Dicroglossus occipitalis* has a protein content of about 28.68% high than 17.28% reported for acute mudsnail (*Viviparous contectus*).

A review by Halton and Hu (2004) suggested that higher protein diets may significantly increase total weight lost and possibly percentage of fat lost when compared to a lower protein diet in the short term. The current data therefore, would highly recommend frog meat in special diets for weight loss programs. Additionally, frog meat is a good source of protein and may be recommended as part of a balanced diet especially in rural constituencies where they are harvested. Statistically, there is no significance difference between means of the five samples tested at p value 0.453 as portrayed in Appendix 1. The proximate analysis data recorded for this study was a confirmation that frog meat could be used as a functional food and various frog meat products can be produced to increase consumptions of this species.

5.2. Antioxidant activities in *P. adspersus* meat

Aliyu *et al*.(2019) revealed that the search for natural antioxidants would continue to be a dominant research interest due to the increasing understanding on the role of oxidative stress on cells. This occurs as a result of over production of free radicals and ROS in human systems, which are linked to inflammation, cancer and diabetes. Hamid *et al*.

(2010) highlighted that most natural antioxidants are of plant origins and only few have been reported in meats, poultry and fish (Serpen *et al*., 2012). Most research has either focused on using plants derived antioxidants to prevent lipid oxidations in meat (Alvarez-Parrilla *et al*., 2014) or using synthetic antioxidants to enhance antioxidant capacity of meat (Saleh *et al*., 2018). However, a study by Bhouri *et al*. (2011) discovered antioxidant activity in farmed sea bream and farmed fish. Additionally, Martínez *et al*. (2014) findings suggested that the consumption of meat may significantly contribute to the total antioxidant capacity of a standard diet. Regardless of the high consumption rate, protein content as well as variable minerals reported in edible frogs, there is limited or no research that has been conducted to investigate antioxidant activity in frog meat.

5.2.1. DPPH radical scavenging assay

The results of DPPH scavenging experimentation indicated that *P. adspersus* possess scavenging of DPPH radicals in concentration dependent manner (scavenging activity increases with concentration). Though, there was no statistically significance difference between the means of the five tested samples, it was observed that sample 5 has higher percentage of inhibition (Figure 6) while sample 1 has the lowest (Figure 6). Furthermore, IC_{50} value was noted to be lower in sample 5 (Table 2) in response to the higher antioxidant activity. A study by Serpen, Gökmen and Fogliano (2012) divulged a high DPPH radical scavenging activities of more than 20% for meat, chicken, fish and pork. In addition, the present results showed significantly low DPPH values of about 50% at 10 mg/ml as compared to that recorded for raw sea bream (*Sparus aurata* Linnaeus, 1758) of about 60% at the same concentration (Bhouri *et al*., 2011).

5.2.2. Nitric oxide scavenging activity

The methanol extracts of *P. adspersus* showed a low NO scavenging effect. The highest among the samples had 27.64% at 20mg/ml as compared to the positive control ascorbic acid where 90.19% scavenging was observed at similar concentration with IC_{50} value of 3.10 mg/ml. The results were however relatively low as compared to that reported in other studies for other meat types. Hwang, Jang and Huh (2019) reported NO scavenging activity of ethanol extracts of raw Alaska Pollock (*Gadus chalcogrammus*) of 57.9% at 1.0 mg/ml as compared to 0% activity for methanol extract of *P. adspersus* at the same concentration. Adebayo *et al*. (2015) enlightened that the release of NO promotes inflammation, therefore extracts that could act as scavengers of NO could be used to mitigate the propagation of inflammation by NO. Though the current study showed insignificant values of NO scavenging activity, only one solvent was successfully used in the extraction process. Rao, Ahmad and Mohd (2016) affirms that NO scavenging activity could be affected by the type of solvents used in the extraction process.

5.2.3. Reducing power activity

In the reducing power assay, substances which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which then react with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm (Jayanthi & Lalitha, 2011). Moreover, Jayanthi and Lalitha (2011) noted that higher absorbance of the reaction mixture indicates higher reductive potential. It can be noted that *P. adspersus* exhibited good reducing power as demonstrated in Figure 8. The results also showed a direct relationship between reducing power and sample extracts concentration such that reducing power of all samples increased with concentration.

Sample 4 (Figure 8) showed high significant reducing power with about 0.85±0.65 absorbance as comapred to 0.1 recorded for wild raw fish at the same concentration (Bhouri *et al*., 2011). The results served as a significant reflection of the antioxidant activity in frog meat. Chanda and Dave (2009) emphasized that compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes.Though there have been very few or no articles reported for antioxidants activities in frog meat, *P. adspersus* meat retained antioxidants activities specifically DPPH and reducing power that could be compared to those in other meat types (such as chicken, pork and fish).

5.3. Bacterial composition, diversity and functionality associated with Giant African Bullfrog meat.

The present study has endeavored for the first time to determine the bacterial composition, diversity and function of the African Bullfrog meat. From this study, across all 3 samples of the African Bullfrog meat, 4 different phyla were detected: *Firmicutes, Bacteroidota, Proteobacteria* and *Planctomycetota.* Nevertheless, only *Firmicutes* phylum of the 4 detected comprised nearly 90% of the total bacterial accumulation. Previous studies have reported high abundance of *Firmicute*s as well associated with food from both plant and animal sources (Jarvis *et al*., 2018; Guan *et al*., 2021). In addition, from the limited number of studies that have investigated the microbiome of meat and meat products using 16S amplicon sequencing, a few of these showed prevalence of *Firmicutes* (Doster *et al*., 2020; Guan *et al*., 2021). According to Microscopemaster (2022) *Firmicutes* phylum is made up mostly of low G+C content Gram-positive bacteria. Many members of this phylum forms part of the human gut microbiota (Tekere *et al*., 2011). There is evidence

that the gut microbiota belonging to phylum *Firmicutes* are important carbohydrate fermenters and may help in absorption and retrieving of energy from unabsorbed dietary carbohydrates (Flint *et al*., 2012). Furthermore, Młynarska *et al*. (2022) mentioned that gut microbiota including those belonging to *Firmicutes* can affect human behavior and mood. Huang *et al*. (2018) underlined that the defects of the *Firmicutes* may lead to the depression in short-chain fatty acids, which could account for the physiological basis of low-level inflammation of depression. Therefore, Huang *et al*. (2018) recommend that a diet rich in *Firmicutes* may aid in lowering the chances of depression in human. Nonetheless, from the present study, *Firmicute*s were dominated by *Lactococcus* genera, and its abundance was the highest in sample S3. *Lactococcus lactis* is one of the dominant species in this study. It has been reported that *L. lactis* is the most widely studied lactic acid bacterial species and has been exploited in fermented food studies (Kelleher e*t al*., 2017). Moreover, it has been established that *L. lactis* have the ability to preserve meat efficiently due to its antibacterial properties (Akbar & Anal, 2014). Additionally, this bacterium has great potential as a bio-control agent in meats and meat products as it tends to grow rapidly and out-competing with other bacteria including pathogenic ones (Akbar & Anal, 2014).

Meanwhile, *Lactococcus garvieae* which is the second most abundant species in this study has been reported to be an etiologic agent. *L. garvieae* is being associated with several urinary tract infections in human (Woolery, 2015). As explained in Woolery (2015) *L. garvieae* is principally a fish pathogen, however it has recently been isolated from mastitis infections in cows and water buffalos. Nonetheless, the association of *L. garvieae* in human infection is alleged to be primarily through contaminated cow’s milk, cheese, or raw fish products (Woolery, 2015). Though there has been little or no evidence indicating

the presence of *L. garvieae* in frog meat before, symptoms similar to that of urinary tract infection has been reported (Okeyo *et al*., 2015) upon consumption of *P. adspersus* meat harvested after the first rain of the rainy seasons. Additionally, *Acinetobacter bereziniae*, *Chryseobacterium gleum* and *Enterococcus faecalis* were also some of the pathogenic bacteria which were detected in this study. These species were as well implicated in various health illnesses including urinary tract infection (Visca *et al*., 2011; Li *et al*., 2020; Tsouvalas *et al*., 2020). Though this is the first time such pathogens have been associated with frog meat, related human pathogens such as *Salmonella* and *Shigella* have also been detected in edible frog (Kia *et al*., 2018). There is a speculation that pathogens are acquired from the water sources where frogs are harvested or acquired from their feed (Kia *et al*., 2018). Therefore, consumption of improperly cooked infected frogs may serve as a route of transmission of pathogens to human.

Nonetheless, no bacterial biochemical pathway was found to be associated with any sort of microbial toxins. In a review Hernande-Cortez *et al*. (2017) highlighted some bacteria producing toxins associated with food such as Cholera toxin (Ctx) (*Vibrio cholerae*), Thermolabile toxin (LT) Thermostable toxin (ST) (Enterotoxigenic *E. coli)*, Shiga Toxin (*Shigella dysenteriae* and *E. coli* O157:H7) Botulinum toxin (BTX) (*Clostridium botulinum*) including many more. Though detected in low abundance, *Escherichia-Shigella* is one of the detected genera in the present study. Regardless, Hernande-Cortez *et al*. (2017) explained that bacteria toxins may be produced in food or once the pathogen has colonized the digestive tract. Additionally, Oyewusi *et al*. (2021) confirmed that 16S rRNA amplicon sequencing technique may be the key aspect of studies of microbial communities but it does not provide direct evidence of a community's functional

capabilities. This may be one of the reasons why some genes and pathways responsible for toxins production in bacteria were not detected.

The present study also provided information related to metabolic functions as well as those related to aromatic compound degradation such as Toluene degradation super pathway (Appendix 5). Toluene is one of the aromatic hydrocarbon with a serious health effect on the human nervous system (Varshini & Sumathy, 2018). Humans are principally exposed to Toluene through ingestion or inhalation and slightly lethal when absorbed through skin (Varshini & Sumathy, 2018). Consequently, having bacteria capable of degrading such environmental pollutant is essential and can be used as an eco – friendly and efficient bioremediation tool. *Pseudomonas* genus is particularly one that has been studied for its abilities to degrade various aromatic compounds making it a perfect candidate in bioremediation of environmental pollutants by metabolic engineering (Arvind *et al*., 2020). The discovery of Toluene degradation pathways as a predicted function of bacteria isolated from *P. adspersus* meat articulates that the environment where the frogs were harvested from could slightly be contaminated (Appendix 5). Nevertheless, presence of such pathways including other various pathways predicted from this study such as vitamin, carbohydrate, amino acids and alcohol degradation possess unique enzymes that may be of industrial importance if isolated.

6. CHAPTER SIX: CONCLUSION

The present study has demonstrated a comprehensive investigation on the *P. adspersus* meat regarding its nutritional content, antioxidant properties as well as bacteria composition, diversity and function. It has revealed that the meat of *P. adspersus* harvested from Ondangwa rural constituency in the Oshana region at Okapya village comprises nutrition and antioxidant properties. The results divulged *P. adspersus* as a good source of protein in addition to relatively low fat content. As a result, the study encouraged consumption and recommended *P. adspersus* meat as part of a balanced diet especially in rural and vulnerable communities where they are harvested.

Consequently, due to the high moisture content detected, it is recommended to implement different preservative measures to enable consumption of this species throughout the year. The *P. adspersus* meat possess scavenging activities which may help protect and reverse some of the damages caused by free radicals. This make *P. adspersus* one of the few studied source of animal-derived antioxidant. Additionally, the present study provided a clear indication of the bacteria composition associated with *P. adspersus*. A total of 4 bacterial phyla were detected with about 25 corresponding species. A wide range of bacteria functions were detected including aromatic compound degradation. Although alleged toxin producer associated bacteria were not detected, it is interesting to note that several species are implicated as pathogens suspected for urinary tract infections in human beings. This serves as the first attempt in determining the cause of the severe dysuria resulting from consumption of *P. adspersus* as reported by Okeyo *et al*. (2015). This study's findings have provided useful references for future research concerning the African Bullfrog meat.

7. CHAPTER SEVEN: RECOMMENDATIONS

The present study serves as a screening research for the African Bullfrog meat. However, the sample size was really small to draw a concrete conclusion. The study recommends a larger sample size to increase the probability of obtaining more discoveries (such as minerals, amino acid and fatty acid compositions) of the *P. adspersus* meat. Additionally, it is recommended that future studies use different proximate analysis and antioxidants techniques for comparison purposes. Furthermore, since only bacteria sequences were processed, metagenomics must be reconsidered in order to accommodate all microbes including other potential toxin coding genes that may be associated with *P. adspersus.* Moreover, as explained by De Assis *et al*. (2017), microbial composition on the amphibian skin may be influenced by environmental factors. It is highly recommended to sample from diverse habitats. Though, the current study was the first attempt in determining the cause of reported dysuria, it is recommended that these results should be considered preliminary until more studies are undertaken for a more comprehensive comparison especially between frog samples from different rainy seasons in different environments. Additionally, clinical laboratory analysis should be considered for urine samples of infected individuals. Alternatively, records of cases of dysuria need to be obtained from the nearest health centers to see if recorded cases changes during the period when frogs are consumed. Finally, determination of alkaloid toxins in Giant African Bullfrog skin must be considered to provide conclusive analysis on the dysuria condition.

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9. APPENDICES

Appendix 1. Ethical Clearance Certificate issued by the University of Namibia Research Ethics Committee (UREC)

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: EEREC/0008 **Date: 13th March 2020**

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Environment and Engineering Research Ethics Committee (EEREC).

Title of Project: *Assessment of Microbial Diversity of Alkaloids Toxins in Giant African Bullfrog (Pyxicephalus Adspersus) Meat from Oshana and Oshikoto Regions of Namibia*

Nature/Level of Project: *MSc*

Researcher: *Martha N. HATUTALE*

Student Number: *201120366*

Faculty: *Agriculture and Natural Resources*

Supervisor(s): *Dr Jean D. Uzabakiriho, (Main) UNAM*

Take note of the following:

(a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the EEREC. An application to make amendments may be necessary.

(b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the EEREC.

(c) The Principal Researcher must report issues of ethical compliance to the EEREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by EEREC.

(d) The EEREC retains the right to:

i. Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

ii. Request for an ethical compliance report at any point during the course of the research.

REC wishes you the best in your research.

Prof. O. T. Johnson: EEREC Chairperson

Signature

Appendix 2. Proximate analysis statistics tests

Test of normality

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Protein content %	.148	15	.200*	.939	15	.376
Ash content %	.194	15	.132	.847	15	.016
Moisture content %	.210	15	.073	.884	15	.055
Fat content %	.445	15	.00	.364	15	.000

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Kruskal-Wallis test analysis

Test Statistics

	Protein content %	Ash content %	Moisture content %	Fat content %
Kruskal-Wallis H	3.665	7.004	8.831	4.800
Df	4	4	4	4
Asymp. Sig.	.453	.136	.065	.308

The results shows that there is no significant difference among sample means at 0.05 p value.

Appendix 3. DPPH tests of normality and ANOVA test

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Samples IDs	Statistic	df	Sig.	Statistic	df	Sig.
Conc_0.15625	Sample 1	.206	3	.	.993	3	.837
	Sample 2	.287	3	.	.929	3	.486
	Sample 3	.294	3	.	.921	3	.456
	Sample 4	.259	3	.	.959	3	.610
	Sample 5	.355	3	.	.818	3	.159
	Positive control	.282	3	.	.936	3	.511
Conc_0.3125	Sample 1	.237	3	.	.976	3	.706
	Sample 2	.245	3	.	.971	3	.671
	Sample 3	.234	3	.	.978	3	.719

	Sample 4	.249	3	.	.968	3	.654
	Sample 5	.273	3	.	.946	3	.552
	Positive control	.316	3	.	.889	3	.352
Conc_0.625	Sample 1	.276	3	.	.943	3	.538
	Sample 2	.347	3	.	.835	3	.202
	Sample 3	.373	3	.	.780	3	.068
	Sample 4	.244	3	.	.971	3	.675
	Sample 5	.197	3	.	.996	3	.873
	Positive control	.350	3	.	.828	3	.185
Conc_1.25	Sample 1	.311	3	.	.897	3	.375
	Sample 2	.203	3	.	.994	3	.851
	Sample 3	.255	3	.	.963	3	.628
	Sample 4	.178	3	.	1.000	3	.957
	Sample 5	.343	3	.	.843	3	.222
	Positive control	.220	3	.	.987	3	.779
Conc_2.5	Sample 1	.294	3	.	.921	3	.456
	Sample 2	.248	3	.	.968	3	.657
	Sample 3	.240	3	.	.975	3	.695
	Sample 4	.175	3	.	1.000	3	.993
	Sample 5	.274	3	.	.945	3	.546
	Positive control	.300	3	.	.913	3	.429
Conc_5	Sample 1	.266	3	.	.952	3	.579
	Sample 2	.359	3	.	.811	3	.140
	Sample 3	.259	3	.	.959	3	.609
	Sample 4	.320	3	.	.883	3	.333
	Sample 5	.342	3	.	.844	3	.225
	Positive control	.259	3	.	.959	3	.609
Conc_10	Sample 1	.221	3	.	.986	3	.772
	Sample 2	.308	3	.	.901	3	.390
	Sample 3	.283	3	.	.935	3	.506
	Sample 4	.375	3	.	.775	3	.056
	Sample 5	.382	3	.	.756	3	.014
	Positive control	.242	3	.	.973	3	.683
Conc_20	Sample 1	.289	3	.	.927	3	.476
	Sample 2	.233	3	.	.979	3	.723
	Sample 3	.337	3	.	.854	3	.251

Sample 4	.194	3	.	.997	3	.888
Sample 5	.296	3	.	.919	3	.448
Positive control	.356	3	.	.816	3	.153

a. Lilliefors Significance Correction

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Conc_0.15625	Between Groups	2953.378	5	590.676	6.397	.004
	Within Groups	1108.098	12	92.342		
	Total	4061.477	17			
Conc_0.3125	Between Groups	3047.624	5	609.525	11.335	.000
	Within Groups	645.262	12	53.772		
	Total	3692.886	17			
Conc_0.625	Between Groups	2550.351	5	510.070	25.000	.000
	Within Groups	244.833	12	20.403		
	Total	2795.184	17			
Conc_1.25	Between Groups	2688.168	5	537.634	24.448	.000
	Within Groups	263.896	12	21.991		
	Total	2952.065	17			
Conc_2.5	Between Groups	3477.351	5	695.470	20.132	.000
	Within Groups	414.556	12	34.546		
	Total	3891.907	17			
Conc_5	Between Groups	2466.985	5	493.397	10.086	.001
	Within Groups	587.026	12	48.919		
	Total	3054.011	17			
Conc_10	Between Groups	4967.680	5	993.536	17.525	.000
	Within Groups	680.296	12	56.691		
	Total	5647.976	17			
Conc_20	Between Groups	4634.694	5	926.939	10.000	.001
	Within Groups	1112.376	12	92.698		
	Total	5747.070	17			

Appendix 4. Nitric oxide test of normality, ANOVA, Multiple Comparisons (Tukey test)

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Sample_IDs	Statistic	df	Sig.	Statistic	df	Sig.
Con_0.15625	Sample1	.385	3	.	.750	3	.000
	Sample 2	.	3	.	.	3	.
	Sample 3	.	3	.	.	3	.
	Sample 4	.351	3	.	.827	3	.179
	Sample 5	.	3	.	.	3	.
	+ Control	.373	3	.	.778	3	.063
Con_0.3125	Sample1	.385	3	.	.750	3	.000
	Sample 2	.	3	.	.	3	.
	Sample 3	.	3	.	.	3	.
	Sample 4	.317	3	.	.889	3	.350
	Sample 5	.	3	.	.	3	.
	+ Control	.237	3	.	.977	3	.706
Con_0.625	Sample1	.385	3	.	.750	3	.000
	Sample 2	.	3	.	.	3	.
	Sample 3	.	3	.	.	3	.
	Sample 4	.	3	.	.	3	.
	Sample 5	.	3	.	.	3	.
	+ Control	.357	3	.	.814	3	.149
Con_1.25	Sample1	.385	3	.	.750	3	.000
	Sample 2	.	3	.	.	3	.
	Sample 3	.	3	.	.	3	.
	Sample 4	.	3	.	.	3	.
	Sample 5	.	3	.	.	3	.
	+ Control	.376	3	.	.772	3	.049
Con_2.5	Sample1	.	3	.	.	3	.
	Sample 2	.	3	.	.	3	.
	Sample 3	.	3	.	.	3	.
	Sample 4	.	3	.	.	3	.
	Sample 5	.	3	.	.	3	.
	+ Control	.319	3	.	.886	3	.341
Con_5	Sample1	.	3	.	.	3	.
	Sample 2	.385	3	.	.750	3	.000

	Sample 3	.	3	.	.	3	.
	Sample 4	.	3	.	.	3	.
	Sample 5	.	3	.	.	3	.
	+ Control	.202	3	.	.994	3	.853
Con_10	Sample1	.	3	.	.	3	.
	Sample 2	.385	3	.	.750	3	.000
	Sample 3	.	3	.	.	3	.
	Sample 4	.	3	.	.	3	.
	Sample 5	.385	3	.	.750	3	.000
	+ Control	.375	3	.	.775	3	.056
Con_20	Sample1	.385	3	.	.750	3	.000
	Sample 2	.296	3	.	.918	3	.447
	Sample 3	.	3	.	.	3	.
	Sample 4	.	3	.	.	3	.
	Sample 5	.260	3	.	.958	3	.607
	+ Control	.184	3	.	.999	3	.928

a. Lilliefors Significance Correction

Normally distributed at 0.05 p value

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Con_0.15625	Between Groups	8803.804	5	1760.761	2.531	.087
	Within Groups	8348.621	12	695.718		
	Total	17152.425	17			
Con_0.3125	Between Groups	6184.772	5	1236.954	12.177	.000
	Within Groups	1218.942	12	101.578		
	Total	7403.714	17			
Con_0.625	Between Groups	8972.466	5	1794.493	18.308	.000
	Within Groups	1176.179	12	98.015		
	Total	10148.645	17			
Con_1.25	Between Groups	10864.594	5	2172.919	171.797	.000
	Within Groups	151.778	12	12.648		
	Total	11016.372	17			
Con_2.5	Between Groups	14107.435	5	2821.487	992.158	.000
	Within Groups	34.125	12	2.844		

	Total	14141.561	17			
Con_5	Between Groups	16725.157	5	3345.031	3531.864	.000
	Within Groups	11.365	12	.947		
	Total	16736.523	17			
Con_10	Between Groups	20222.617	5	4044.523	430.777	.000
	Within Groups	112.667	12	9.389		
	Total	20335.283	17			
Con_20	Between Groups	19479.034	5	3895.807	37.444	.000
	Within Groups	1248.508	12	104.042		
	Total	20727.543	17			

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Sample_IDs	(J) Sample_IDs	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Con_0.15625	Sample1	Sample 2	-32.41052	21.53630	.668	-104.7493	39.9282
		Sample 3	-32.41052	21.53630	.668	-104.7493	39.9282
		Sample 4	-11.71261	21.53630	.993	-84.0513	60.6261
		Sample 5	-32.41052	21.53630	.668	-104.7493	39.9282
		+ Control	-71.02123	21.53630	.055	-143.3600	1.3175
	Sample 2	Sample1	32.41052	21.53630	.668	-39.9282	104.7493
		Sample 3	.00000	21.53630	1.000	-72.3387	72.3387
		Sample 4	20.69791	21.53630	.922	-51.6408	93.0366
		Sample 5	.00000	21.53630	1.000	-72.3387	72.3387
		+ Control	-38.61070	21.53630	.504	-110.9494	33.7280
	Sample 3	Sample1	32.41052	21.53630	.668	-39.9282	104.7493
		Sample 2	.00000	21.53630	1.000	-72.3387	72.3387
		Sample 4	20.69791	21.53630	.922	-51.6408	93.0366
		Sample 5	.00000	21.53630	1.000	-72.3387	72.3387
		+ Control	-38.61070	21.53630	.504	-110.9494	33.7280
	Sample 4	Sample1	11.71261	21.53630	.993	-60.6261	84.0513
		Sample 2	-20.69791	21.53630	.922	-93.0366	51.6408
		Sample 3	-20.69791	21.53630	.922	-93.0366	51.6408
		Sample 5	-20.69791	21.53630	.922	-93.0366	51.6408
		+ Control	-59.30861	21.53630	.134	-131.6473	13.0301
	Sample 5	Sample1	32.41052	21.53630	.668	-39.9282	104.7493
		Sample 2	.00000	21.53630	1.000	-72.3387	72.3387
		Sample 3	.00000	21.53630	1.000	-72.3387	72.3387

		Sample 4	20.69791	21.53630	.922	-51.6408	93.0366
		+ Control	-38.61070	21.53630	.504	-110.9494	33.7280
	+ Control	Sample1	71.02123	21.53630	.055	-1.3175	143.3600
		Sample 2	38.61070	21.53630	.504	-33.7280	110.9494
		Sample 3	38.61070	21.53630	.504	-33.7280	110.9494
		Sample 4	59.30861	21.53630	.134	-13.0301	131.6473
		Sample 5	38.61070	21.53630	.504	-33.7280	110.9494
Con_0.3125	Sample1	Sample 2	-14.05000	8.22915	.552	-41.6911	13.5911
		Sample 3	-14.05000	8.22915	.552	-41.6911	13.5911
		Sample 4	-6.12139	8.22915	.972	-33.7625	21.5197
		Sample 5	-14.05000	8.22915	.552	-41.6911	13.5911
		+ Control	-57.37770*	8.22915	.000	-85.0188	-29.7366
	Sample 2	Sample1	14.05000	8.22915	.552	-13.5911	41.6911
		Sample 3	.00000	8.22915	1.000	-27.6411	27.6411
		Sample 4	7.92861	8.22915	.921	-19.7125	35.5697
		Sample 5	.00000	8.22915	1.000	-27.6411	27.6411
		+ Control	-43.32770*	8.22915	.002	-70.9688	-15.6866
	Sample 3	Sample1	14.05000	8.22915	.552	-13.5911	41.6911
		Sample 2	.00000	8.22915	1.000	-27.6411	27.6411
		Sample 4	7.92861	8.22915	.921	-19.7125	35.5697
		Sample 5	.00000	8.22915	1.000	-27.6411	27.6411
		+ Control	-43.32770*	8.22915	.002	-70.9688	-15.6866
	Sample 4	Sample1	6.12139	8.22915	.972	-21.5197	33.7625
		Sample 2	-7.92861	8.22915	.921	-35.5697	19.7125
		Sample 3	-7.92861	8.22915	.921	-35.5697	19.7125
		Sample 5	-7.92861	8.22915	.921	-35.5697	19.7125
		+ Control	-51.25630*	8.22915	.000	-78.8974	-23.6152

	Sample 5	Sample1	14.05000	8.22915	.552	-13.5911	41.6911
		Sample 2	.00000	8.22915	1.000	-27.6411	27.6411
		Sample 3	.00000	8.22915	1.000	-27.6411	27.6411
		Sample 4	7.92861	8.22915	.921	-19.7125	35.5697
		+ Control	-43.32770*	8.22915	.002	-70.9688	-15.6866
	+ Control	Sample1	57.37770*	8.22915	.000	29.7366	85.0188
		Sample 2	43.32770*	8.22915	.002	15.6866	70.9688
		Sample 3	43.32770*	8.22915	.002	15.6866	70.9688
		Sample 4	51.25630*	8.22915	.000	23.6152	78.8974
		Sample 5	43.32770*	8.22915	.002	15.6866	70.9688
Con_0.625	Sample1	Sample 2	-13.98175	8.08352	.539	-41.1336	13.1702
		Sample 3	-13.98175	8.08352	.539	-41.1336	13.1702
		Sample 4	-13.98175	8.08352	.539	-41.1336	13.1702
		Sample 5	-13.98175	8.08352	.539	-41.1336	13.1702
		+ Control	-69.50621*	8.08352	.000	-96.6581	-42.3543
	Sample 2	Sample1	13.98175	8.08352	.539	-13.1702	41.1336
		Sample 3	.00000	8.08352	1.000	-27.1519	27.1519
		Sample 4	.00000	8.08352	1.000	-27.1519	27.1519
		Sample 5	.00000	8.08352	1.000	-27.1519	27.1519
		+ Control	-55.52446*	8.08352	.000	-82.6764	-28.3726
	Sample 3	Sample1	13.98175	8.08352	.539	-13.1702	41.1336
		Sample 2	.00000	8.08352	1.000	-27.1519	27.1519
		Sample 4	.00000	8.08352	1.000	-27.1519	27.1519
		Sample 5	.00000	8.08352	1.000	-27.1519	27.1519
		+ Control	-55.52446*	8.08352	.000	-82.6764	-28.3726
	Sample 4	Sample1	13.98175	8.08352	.539	-13.1702	41.1336
		Sample 2	.00000	8.08352	1.000	-27.1519	27.1519

		Sample 3	.00000	8.08352	1.000	-27.1519	27.1519
		Sample 5	.00000	8.08352	1.000	-27.1519	27.1519
		+ Control	-55.52446*	8.08352	.000	-82.6764	-28.3726
	Sample 5	Sample1	13.98175	8.08352	.539	-13.1702	41.1336
		Sample 2	.00000	8.08352	1.000	-27.1519	27.1519
		Sample 3	.00000	8.08352	1.000	-27.1519	27.1519
		Sample 4	.00000	8.08352	1.000	-27.1519	27.1519
		+ Control	-55.52446*	8.08352	.000	-82.6764	-28.3726
	+ Control	Sample1	69.50621*	8.08352	.000	42.3543	96.6581
		Sample 2	55.52446*	8.08352	.000	28.3726	82.6764
		Sample 3	55.52446*	8.08352	.000	28.3726	82.6764
		Sample 4	55.52446*	8.08352	.000	28.3726	82.6764
		Sample 5	55.52446*	8.08352	.000	28.3726	82.6764
Con_1.25	Sample1	Sample 2	-4.31291	2.90381	.679	-14.0666	5.4408
		Sample 3	-4.31291	2.90381	.679	-14.0666	5.4408
		Sample 4	-4.31291	2.90381	.679	-14.0666	5.4408
		Sample 5	-4.31291	2.90381	.679	-14.0666	5.4408
		+ Control	-69.23771*	2.90381	.000	-78.9914	-59.4840
	Sample 2	Sample1	4.31291	2.90381	.679	-5.4408	14.0666
		Sample 3	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 4	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 5	.00000	2.90381	1.000	-9.7537	9.7537
		+ Control	-64.92481*	2.90381	.000	-74.6785	-55.1711
	Sample 3	Sample1	4.31291	2.90381	.679	-5.4408	14.0666
		Sample 2	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 4	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 5	.00000	2.90381	1.000	-9.7537	9.7537

		+ Control	-64.92481*	2.90381	.000	-74.6785	-55.1711
	Sample 4	Sample1	4.31291	2.90381	.679	-5.4408	14.0666
		Sample 2	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 3	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 5	.00000	2.90381	1.000	-9.7537	9.7537
		+ Control	-64.92481*	2.90381	.000	-74.6785	-55.1711
	Sample 5	Sample1	4.31291	2.90381	.679	-5.4408	14.0666
		Sample 2	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 3	.00000	2.90381	1.000	-9.7537	9.7537
		Sample 4	.00000	2.90381	1.000	-9.7537	9.7537
		+ Control	-64.92481*	2.90381	.000	-74.6785	-55.1711
	+ Control	Sample1	69.23771*	2.90381	.000	59.4840	78.9914
		Sample 2	64.92481*	2.90381	.000	55.1711	74.6785
		Sample 3	64.92481*	2.90381	.000	55.1711	74.6785
		Sample 4	64.92481*	2.90381	.000	55.1711	74.6785
		Sample 5	64.92481*	2.90381	.000	55.1711	74.6785
Con_2.5	Sample1	Sample 2	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 3	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 4	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 5	.00000	1.37690	1.000	-4.6249	4.6249
		+ Control	-75.11973*	1.37690	.000	-79.7446	-70.4948
	Sample 2	Sample1	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 3	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 4	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 5	.00000	1.37690	1.000	-4.6249	4.6249
		+ Control	-75.11973*	1.37690	.000	-79.7446	-70.4948
	Sample 3	Sample1	.00000	1.37690	1.000	-4.6249	4.6249

		Sample 2	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 4	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 5	.00000	1.37690	1.000	-4.6249	4.6249
		+ Control	-75.11973*	1.37690	.000	-79.7446	-70.4948
	Sample 4	Sample1	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 2	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 3	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 5	.00000	1.37690	1.000	-4.6249	4.6249
		+ Control	-75.11973*	1.37690	.000	-79.7446	-70.4948
	Sample 5	Sample1	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 2	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 3	.00000	1.37690	1.000	-4.6249	4.6249
		Sample 4	.00000	1.37690	1.000	-4.6249	4.6249
		+ Control	-75.11973*	1.37690	.000	-79.7446	-70.4948
	+ Control	Sample1	75.11973*	1.37690	.000	70.4948	79.7446
		Sample 2	75.11973*	1.37690	.000	70.4948	79.7446
		Sample 3	75.11973*	1.37690	.000	70.4948	79.7446
		Sample 4	75.11973*	1.37690	.000	70.4948	79.7446
		Sample 5	75.11973*	1.37690	.000	70.4948	79.7446
Con_5	Sample1	Sample 2	-1.12548	.79461	.718	-3.7945	1.5435
		Sample 3	.00000	.79461	1.000	-2.6690	2.6690
		Sample 4	.00000	.79461	1.000	-2.6690	2.6690
		Sample 5	.00000	.79461	1.000	-2.6690	2.6690
		+ Control	-82.01047*	.79461	.000	-84.6795	-79.3414
	Sample 2	Sample1	1.12548	.79461	.718	-1.5435	3.7945
		Sample 3	1.12548	.79461	.718	-1.5435	3.7945
		Sample 4	1.12548	.79461	.718	-1.5435	3.7945

		Sample 5	1.12548	.79461	.718	-1.5435	3.7945
		+ Control	-80.88498*	.79461	.000	-83.5540	-78.2160
	Sample 3	Sample1	.00000	.79461	1.000	-2.6690	2.6690
		Sample 2	-1.12548	.79461	.718	-3.7945	1.5435
		Sample 4	.00000	.79461	1.000	-2.6690	2.6690
		Sample 5	.00000	.79461	1.000	-2.6690	2.6690
		+ Control	-82.01047*	.79461	.000	-84.6795	-79.3414
	Sample 4	Sample1	.00000	.79461	1.000	-2.6690	2.6690
		Sample 2	-1.12548	.79461	.718	-3.7945	1.5435
		Sample 3	.00000	.79461	1.000	-2.6690	2.6690
		Sample 5	.00000	.79461	1.000	-2.6690	2.6690
		+ Control	-82.01047*	.79461	.000	-84.6795	-79.3414
	Sample 5	Sample1	.00000	.79461	1.000	-2.6690	2.6690
		Sample 2	-1.12548	.79461	.718	-3.7945	1.5435
		Sample 3	.00000	.79461	1.000	-2.6690	2.6690
		Sample 4	.00000	.79461	1.000	-2.6690	2.6690
		+ Control	-82.01047*	.79461	.000	-84.6795	-79.3414
	+ Control	Sample1	82.01047*	.79461	.000	79.3414	84.6795
		Sample 2	80.88498*	.79461	.000	78.2160	83.5540
		Sample 3	82.01047*	.79461	.000	79.3414	84.6795
		Sample 4	82.01047*	.79461	.000	79.3414	84.6795
		Sample 5	82.01047*	.79461	.000	79.3414	84.6795
Con_10	Sample1	Sample 2	-.25197	2.50185	1.000	-8.6555	8.1515
		Sample 3	.00000	2.50185	1.000	-8.4035	8.4035
		Sample 4	.00000	2.50185	1.000	-8.4035	8.4035
		Sample 5	-2.89061	2.50185	.849	-11.2941	5.5129
		+ Control	-90.52464*	2.50185	.000	-98.9282	-82.1211

	Sample 2	Sample1	.25197	2.50185	1.000	-8.1515	8.6555
		Sample 3	.25197	2.50185	1.000	-8.1515	8.6555
		Sample 4	.25197	2.50185	1.000	-8.1515	8.6555
		Sample 5	-2.63864	2.50185	.890	-11.0422	5.7649
		+ Control	-90.27267*	2.50185	.000	-98.6762	-81.8691
	Sample 3	Sample1	.00000	2.50185	1.000	-8.4035	8.4035
		Sample 2	-.25197	2.50185	1.000	-8.6555	8.1515
		Sample 4	.00000	2.50185	1.000	-8.4035	8.4035
		Sample 5	-2.89061	2.50185	.849	-11.2941	5.5129
		+ Control	-90.52464*	2.50185	.000	-98.9282	-82.1211
	Sample 4	Sample1	.00000	2.50185	1.000	-8.4035	8.4035
		Sample 2	-.25197	2.50185	1.000	-8.6555	8.1515
		Sample 3	.00000	2.50185	1.000	-8.4035	8.4035
		Sample 5	-2.89061	2.50185	.849	-11.2941	5.5129
		+ Control	-90.52464*	2.50185	.000	-98.9282	-82.1211
	Sample 5	Sample1	2.89061	2.50185	.849	-5.5129	11.2941
		Sample 2	2.63864	2.50185	.890	-5.7649	11.0422
		Sample 3	2.89061	2.50185	.849	-5.5129	11.2941
		Sample 4	2.89061	2.50185	.849	-5.5129	11.2941
		+ Control	-87.63404*	2.50185	.000	-96.0376	-79.2305
	+ Control	Sample1	90.52464*	2.50185	.000	82.1211	98.9282
		Sample 2	90.27267*	2.50185	.000	81.8691	98.6762
		Sample 3	90.52464*	2.50185	.000	82.1211	98.9282
		Sample 4	90.52464*	2.50185	.000	82.1211	98.9282
		Sample 5	87.63404*	2.50185	.000	79.2305	96.0376
Con_20	Sample1	Sample 2	-5.27245	8.32836	.986	-33.2468	22.7018
		Sample 3	.50787	8.32836	1.000	-27.4664	28.4822

	Sample 4	.50787	8.32836	1.000	-27.4664	28.4822
	Sample 5	-33.87069*	8.32836	.015	-61.8450	-5.8964
	+ Control	-89.66118*	8.32836	.000	-117.6355	-61.6869
Sample 2	Sample1	5.27245	8.32836	.986	-22.7018	33.2468
	Sample 3	5.78032	8.32836	.979	-22.1940	33.7546
	Sample 4	5.78032	8.32836	.979	-22.1940	33.7546
	Sample 5	-28.59823*	8.32836	.044	-56.5725	-.6239
	+ Control	-84.38873*	8.32836	.000	-112.3630	-56.4144
Sample 3	Sample1	-.50787	8.32836	1.000	-28.4822	27.4664
	Sample 2	-5.78032	8.32836	.979	-33.7546	22.1940
	Sample 4	.00000	8.32836	1.000	-27.9743	27.9743
	Sample 5	-34.37855*	8.32836	.014	-62.3529	-6.4043
	+ Control	-90.16905*	8.32836	.000	-118.1434	-62.1947
Sample 4	Sample1	-.50787	8.32836	1.000	-28.4822	27.4664
	Sample 2	-5.78032	8.32836	.979	-33.7546	22.1940
	Sample 3	.00000	8.32836	1.000	-27.9743	27.9743
	Sample 5	-34.37855*	8.32836	.014	-62.3529	-6.4043
	+ Control	-90.16905*	8.32836	.000	-118.1434	-62.1947
Sample 5	Sample1	33.87069*	8.32836	.015	5.8964	61.8450
	Sample 2	28.59823*	8.32836	.044	.6239	56.5725
	Sample 3	34.37855*	8.32836	.014	6.4043	62.3529
	Sample 4	34.37855*	8.32836	.014	6.4043	62.3529
	+ Control	-55.79049*	8.32836	.000	-83.7648	-27.8162
+ Control	Sample1	89.66118*	8.32836	.000	61.6869	117.6355
	Sample 2	84.38873*	8.32836	.000	56.4144	112.3630
	Sample 3	90.16905*	8.32836	.000	62.1947	118.1434
	Sample 4	90.16905*	8.32836	.000	62.1947	118.1434

Sample 5	55.79049*	8.32836	.000	27.8162	83.7648

*. The mean difference is significant at the 0.05 level.

Appendix 5. Reducing power test of normality and test of statistics

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Sample_IDs	Statistic	df	Sig.	Statistic	df	Sig.
Con_0.15625	Sample 1	.384	3	.	.753	3	.006
	Sample 2	.313	3	.	.894	3	.365
	Sample 3	.202	3	.	.994	3	.853
	Sample 4	.375	3	.	.774	3	.053
	Sample 5	.368	3	.	.790	3	.090
	+ Control	.216	3	.	.988	3	.794
Conc_0.3125	Sample 1	.376	3	.	.772	3	.050
	Sample 2	.301	3	.	.912	3	.425
	Sample 3	.226	3	.	.983	3	.751
	Sample 4	.384	3	.	.751	3	.002
	Sample 5	.373	3	.	.779	3	.064
	+ Control	.351	3	.	.827	3	.181
Conc_0.625	Sample 1	.385	3	.	.750	3	.000
	Sample 2	.355	3	.	.820	3	.163
	Sample 3	.366	3	.	.795	3	.102
	Sample 4	.362	3	.	.803	3	.122
	Sample 5	.356	3	.	.816	3	.154
	+ Control	.257	3	.	.961	3	.618
Conc_1.25	Sample 1	.380	3	.	.762	3	.028
	Sample 2	.327	3	.	.871	3	.299
	Sample 3	.175	3	.	1.000	3	.997
	Sample 4	.384	3	.	.751	3	.003
	Sample 5	.375	3	.	.774	3	.054
	+ Control	.233	3	.	.979	3	.722
Conc_2.5	Sample 1	.383	3	.	.756	3	.012
	Sample 2	.371	3	.	.783	3	.075
	Sample 3	.277	3	.	.941	3	.531
	Sample 4	.384	3	.	.753	3	.006
	Sample 5	.381	3	.	.761	3	.024
	+ Control	.254	3	.	.964	3	.634
Conc_5	Sample 1	.366	3	.	.796	3	.104
	Sample 2	.368	3	.	.791	3	.094
	Sample 3	.224	3	.	.984	3	.761
	Sample 4	.383	3	.	.756	3	.012

	Sample 5	.378	3	.	.767	3	.038
	+ Control	.175	3	.	1.000	3	.994
Conc_10	Sample 1	.369	3	.	.788	3	.085
	Sample 2	.378	3	.	.766	3	.036
	Sample 3	.272	3	.	.947	3	.555
	Sample 4	.384	3	.	.752	3	.004
	Sample 5	.308	3	.	.901	3	.390
	+ Control	.385	3	.	.750	3	.000
Conc_20	Sample 1	.255	3	.	.963	3	.629
	Sample 2	.375	3	.	.775	3	.056
	Sample 3	.355	3	.	.818	3	.159
	Sample 4	.383	3	.	.754	3	.009
	Sample 5	.349	3	.	.831	3	.191
	+ Control	.230	3	.	.981	3	.737

a. Lilliefors Significance Correction

Not normally distributed

Test Statistics[a,b]

	Con_0.156 25	Conc_0.31 25	Conc_0.6 25	Conc_1. 25	Conc_2. 5	Conc_5	Conc_1 0	Conc_2 0
Kruskal-Wallis H	11.105	11.854	10.461	11.620	10.357	8.813	8.213	8.205
df	5	5	5	5	5	5	5	5
Asymp. Sig.	.049	.037	.063	.040	.066	.117	.145	.145

a. Kruskal Wallis Test

b. Grouping Variable: Sample_IDs

H_o: means rank of the group are the same

H_1: means rank of the group are not the same

Decision rule: Reject null hypothesis if sig difference is less than p value (0.05)

Appendix 6. Abundance of bacteria metabolic pathways presents for the three different samples.

Pathways	S1	S2	S3
1CMET2-PWY	23610.35	35701.78	20213.07
3-HYDROXYPHENYLACETATE-DEGRADATION-PWY	374.0491	642.8115	59.50978
AEROBACTINSYN-PWY	255.5014	436.0507	43.64846
ALL-CHORISMATE-PWY	4677.45	2411.375	256.1255
ANAEROFRUCAT-PWY	33785.98	49378.06	26937.43
ANAGLYCOLYSIS-PWY	31610.66	46092.09	25109.11
ARG+POLYAMINE-SYN	2421.91	1119.799	99.38966
ARGDEG-PWY	606.4486	721.0592	63.61556
ARGORNPROST-PWY	8568.898	7259.002	2579.356
ARGSYN-PWY	24421.79	37490.46	22336.03
ARGSYNBSUB-PWY	24754.35	37670.36	22495.4
ARO-PWY	27450.33	41099.97	22432.41
ASPASN-PWY	5670.815	1669.663	176.8378
AST-PWY	429.0562	533.0312	51.14341
BIOTIN-BIOSYNTHESIS-PWY	5130.373	1356.197	142.2574
BRANCHED-CHAIN-AA-SYN-PWY	27250.76	39274.66	22676.52
CALVIN-PWY	27508.81	39981.78	21270.47
CATECHOL-ORTHO-CLEAVAGE-PWY	199.6497	73.20803	3.733333
CENTFERM-PWY	175.1459	193.1883	40.67402
COA-PWY	25807.25	38413.23	20914.92
COBALSYN-PWY	4815.575	297.1085	31.55259
COLANSYN-PWY	784.2048	1045.722	109.2653
COMPLETE-ARO-PWY	28549.31	42969.22	23628.88
CRNFORCAT-PWY	34.19507	29.63454	6.674641
DAPLYSINESYN-PWY	11554.07	2861.793	329.1001
DENOVOPURINE2-PWY	23391.52	28858.62	10774.47

DHGLUCONATE-PYR-CAT-PWY	**16.69609**	**18.60658**	**5.687926**
DTDPRHAMSYN-PWY	**28860.7**	**38460.33**	**21141.78**
ECASYN-PWY	**526.4562**	**845.9502**	**78.63776**
ENTBACSYN-PWY	**8667.461**	**2404.514**	**229.9295**
FAO-PWY	**2953.608**	**1117.426**	**104.0726**
FASYN-ELONG-PWY	**40409.77**	**63635.66**	**35240.88**
FASYN-INITIAL-PWY	**12427.44**	**2716.604**	**250.101**
FERMENTATION-PWY	**11434.82**	**6817.687**	**1037.16**
FOLSYN-PWY	**21594.85**	**20999.82**	**4639.792**
FUC-RHAMCAT-PWY	**394.1802**	**175.4516**	**0**
FUCCAT-PWY	**1904.393**	**103.0544**	**0**
GALACT-GLUCUROCAT-PWY	**8812.839**	**1982.016**	**470.6238**
GALACTARDEG-PWY	**313.0994**	**484.7271**	**46.95855**
GALACTUROCAT-PWY	**8126.903**	**1731.246**	**403.8329**
GALLATE-DEGRADATION-I-PWY	**24.5**	**7.27**	**0**
GALLATE-DEGRADATION-II-PWY	**24.5**	**7.27**	**0**
GLCMANNANAUT-PWY	**179.7113**	**604.3921**	**0**
GLUCARDEG-PWY	**312.5958**	**483.4367**	**46.95855**
GLUCARGALACTSUPER-PWY	**313.0994**	**484.7271**	**46.95855**
GLUCONEO-PWY	**14162.01**	**12456.96**	**2023.669**
GLUCOSE1PMETAB-PWY	**613.4041**	**939.4476**	**91.19543**
GLUCUROCAT-PWY	**20595.29**	**32646.27**	**19393.45**
GLUTORN-PWY	**20583.88**	**30550.62**	**18781.52**
GLYCOCAT-PWY	**13885.47**	**14506.44**	**4224.669**
GLYCOGENSYNTH-PWY	**16303.78**	**17260.32**	**5149.099**
GLYCOL-GLYOXDEG-PWY	**95.4807**	**83.02043**	**0**
GLYCOLYSIS	**31686.22**	**47028.02**	**25625.75**
GLYCOLYSIS-E-D	**10928.48**	**3230.462**	**400.6507**
GLYCOLYSIS-TCA-GLYOX-BYPASS	**3141.929**	**2571.016**	**262.4566**

GLYOXYLATE-BYPASS	901.645	1003.521	97.52979
GOLPDLCAT-PWY	110.1369	140.7091	0
HCAMHPDEG-PWY	31.53968	43.66819	0
HEME-BIOSYNTHESIS-II	3730.018	2043.95	300.9192
HEMESYN2-PWY	8032.637	3861.729	537.1919
HEXITOLDEGSUPER-PWY	11506.61	3859.375	427.6961
HISDEG-PWY	3027.163	2008.274	256.1
HISTSYN-PWY	21924.09	31729.6	18967.85
HOMOSER-METSYN-PWY	20221.58	32000.62	18951.51
HSERMETANA-PWY	11687.12	2007.519	205.8056
ILEUSYN-PWY	27774.38	40565.31	23548.47
KDO-NAGLIPASYN-PWY	1298.615	615.5151	58.22118
KETOGLUCONMET-PWY	40.74926	41.16398	0
LACTOSECAT-PWY	7320.274	14846.47	7827.037
LEU-DEG2-PWY	508.0726	112.6582	7.023918
LPSSYN-PWY	0	10.80552	0
MET-SAM-PWY	21817.63	33236.22	19328.42
METHGLYUT-PWY	251.2743	214.0356	6.999384
METHYLGALLATE-DEGRADATION-PWY	30.61785	9.087077	0
NAD-BIOSYNTHESIS-II	577.6939	706.9944	65.30382
NADSYN-PWY	9.130292	0	0
NAGLIPASYN-PWY	2872.736	600.146	57.1
NONMEVIPP-PWY	3610.914	2542.294	377.0647
NONOXIPENT-PWY	27339.88	39568.24	21068.76
OANTIGEN-PWY	25917.95	38237.87	20979.56
ORNARGDEG-PWY	606.4486	721.0592	63.61556
ORNDEG-PWY	422.905	653.4207	66.38769
P105-PWY	3394.332	2808.149	328.2639
P108-PWY	3265.574	203.1692	35.3714

P122-PWY	7974.89	16134.43	2866.117
P124-PWY	25344.64	40411.4	23068
P125-PWY	25209.99	39573.12	23637.7
P161-PWY	35164.09	54944.55	30826.54
P164-PWY	720.2063	955.5189	209.5849
P221-PWY	382.9846	244.2409	13.62202
P23-PWY	2606.466	2092.131	220.953
P281-PWY	20.8902	46.72687	0
P381-PWY	51.25743	85.92564	0
P4-PWY	12206.07	3399.387	371.1301
P42-PWY	6388.423	5291.379	762.3362
P441-PWY	601.1342	1893.778	0
P461-PWY	7997.753	2055.958	216.8354
P562-PWY	2227.038	361.7128	37.24795
PANTO-PWY	7984.742	3815.864	536.8647
PANTOSYN-PWY	10358.59	5460.709	795.2611
PENTOSE-P-PWY	16227.89	8405.9	1212.261
PEPTIDOGLYCANSYN-PWY	29610.59	42626.23	23219.34
PHOSLIPSYN-PWY	29414.48	43030.21	23282.62
POLYAMINSYN3-PWY	1335.737	120.5549	15.27561
POLYAMSYN-PWY	1274.515	568.3921	49.80565
POLYISOPRENSYN-PWY	26246.68	38389.94	20912.76
PPGPPMET-PWY	851.6412	973.0813	88.90053
PROTOCATECHUATE-ORTHO-CLEAVAGE-PWY	351.3494	149.8266	6.502941
PRPP-PWY	18704.01	7811.474	983.469
PWY-1269	2316.076	664.6933	64.31704
PWY-1622	22.17183	49.08358	42.52199
PWY-181	37.14995	52.06773	32.21627
PWY-1861	7002.273	4073.743	616.7296

PWY-2941	**10631.8**	**5081.46**	**850.6655**
PWY-2942	**22531.36**	**32751.96**	**19214.19**
PWY-3001	**24186.78**	**35258.89**	**20628.93**
PWY-3781	**12367**	**5198.169**	**669.2912**
PWY-4361	**2091.933**	**0**	**0**
PWY-4984	**8735.557**	**3477.5**	**539.7728**
PWY-5022	**841.5766**	**1276.837**	**155.3841**
PWY-5028	**185.7064**	**90.61386**	**7.370491**
PWY-5097	**10135.13**	**2265.346**	**256.8755**
PWY-5100	**34032.78**	**54991.61**	**30251.81**
PWY-5101	**29807.66**	**43788.89**	**25488.57**
PWY-5103	**25800.19**	**38275.47**	**22377.98**
PWY-5104	**26061.99**	**39653**	**22858.87**
PWY-5121	**4894.351**	**3549.133**	**534.7737**
PWY-5154	**20976.12**	**32945.2**	**19902.88**
PWY-5178	**44.99448**	**38.15692**	**0**
PWY-5180	**3392.178**	**89.49389**	**0**
PWY-5181	**218.3736**	**124.4621**	**5.98795**
PWY-5182	**3392.178**	**89.49389**	**0**
PWY-5183	**52.39787**	**0**	**0**
PWY-5188	**5550.575**	**2161.093**	**286.2937**
PWY-5189	**5289.321**	**2029.653**	**272.1**
PWY-5265	**4875.957**	**12233.16**	**2279.601**
PWY-5304	**4334.5**	**367.25**	**34.25**
PWY-5345	**8214.766**	**1267.622**	**128.0563**
PWY-5347	**15818.08**	**4489.665**	**473.4191**
PWY-5384	**8287.443**	**11930.79**	**3417.605**
PWY-5415	**97.4441**	**74.66308**	**0**
PWY-5417	**241.0607**	**100.0032**	**5.01211**

PWY-5431	241.0607	100.0032	5.01211
PWY-5484	33822.91	49991.79	27381.14
PWY-5505	3924.264	328.7115	3.568527
PWY-5507	54.31244	96.80385	0
PWY-5509	4609.762	258.5649	30.4973
PWY-5531	11.63265	28.39391	24.31028
PWY-5651	5.752942	0	0
PWY-5659	11929.72	1492.627	142.9102
PWY-5667	31214.25	44381.81	23966.8
PWY-5676	43.00075	119.647	16.66972
PWY-5686	27551.39	42014.28	22822.57
PWY-5695	25395.42	35811.49	20281.76
PWY-5705	208.2596	153.8722	25.16349
PWY-5741	8.490395	22.15242	16.84121
PWY-5747	198.003	102.6126	6.118921
PWY-5837	22953.87	38161.72	20787.58
PWY-5838	13513.1	6148.699	732.7418
PWY-5840	25359.14	39236.34	21383.25
PWY-5845	3596.061	5492.233	578.8431
PWY-5850	3596.061	5492.233	578.8431
PWY-5855	3859.354	874.6509	94.07861
PWY-5856	3859.354	874.6509	94.07861
PWY-5857	3859.354	874.6509	94.07861
PWY-5860	2504.268	3845.283	389.4956
PWY-5861	10729.77	4330.88	494.2779
PWY-5862	2504.268	3845.283	389.4956
PWY-5863	23236.39	38113.12	20797.2
PWY-5896	3596.061	5492.233	578.8431
PWY-5897	24611.31	38168.68	20790.7

PWY-5898	24611.31	38168.68	20790.7
PWY-5899	24611.31	38168.68	20790.7
PWY-5910	24632.48	38979.57	21784.69
PWY-5913	7150.696	1772.727	213.4838
PWY-5918	4151.947	1531.168	196.6756
PWY-5920	1441.002	1274.153	156.1616
PWY-5971	19806	4756.026	446.0303
PWY-5973	40119.78	56904.8	31122.58
PWY-5989	8710.86	1631.526	159.9319
PWY-6071	597.0246	590.9455	53.992
PWY-6107	30.97142	18.87879	0
PWY-6121	30590.23	44383.83	24134.96
PWY-6122	30754.87	45985.87	25084.19
PWY-6123	25805.25	38391.8	20906.25
PWY-6125	18963.74	20585.89	6535.461
PWY-6126	30971.95	45988.72	24758.91
PWY-6147	8329.702	4486.68	640.7239
PWY-6151	24919.37	38347.75	20920.08
PWY-6163	25921.51	38527.13	20921.11
PWY-6182	220.1076	92.04536	5.396652
PWY-6185	238.8925	101.7837	5.438786
PWY-621	18458.83	25494.69	14740.46
PWY-6263	4420.41	0	0
PWY-6269	4665.163	265.9803	30.85268
PWY-6277	30754.87	45985.87	25084.19
PWY-6282	15099.92	3279.261	300.4844
PWY-6317	30468.43	45616.86	25495.69
PWY-6353	1262.525	1271.341	390.5694
PWY-6385	29609.4	42619.41	23217

PWY-6386	29395.99	41891.09	22806.56
PWY-6387	30451.09	43828.11	23877.04
PWY-6396	21168.21	36635.64	22128.45
PWY-6467	2260.18	587.47	57.1
PWY-6470	24866.15	37863.88	21342.42
PWY-6471	28430.37	41426.47	22737.31
PWY-6507	13245.72	11598.3	3355.194
PWY-6519	6390.278	1527.858	158.2521
PWY-6545	0	80.7056	0
PWY-6562	767.0243	14.92973	4.426938
PWY-6588	3518.889	103.607	19.99259
PWY-6590	224.8283	248.1146	52.27332
PWY-6608	741.6386	982.9359	214.8797
PWY-6609	37565.04	48847.71	25562.38
PWY-6612	19203.05	17095.78	3340.078
PWY-6628	4041.842	4803.877	490.8703
PWY-6629	0	317.1835	0
PWY-6630	4041.372	4804.341	490.8723
PWY-6690	31.53968	43.66819	0
PWY-6700	14030.71	13955.74	4119.156
PWY-6703	4557.16	2687.201	372.8719
PWY-6708	3859.354	874.6509	94.07861
PWY-6728	27.01164	93.95103	0
PWY-6737	24244.39	42017.59	25513.96
PWY-6749	0	3.999792	0
PWY-6876	3518.889	25.49316	19.99259
PWY-6891	3448.843	2127.049	310.1479
PWY-6892	757.3382	1224.234	112.4251
PWY-6895	3946.121	1285.595	139.0945

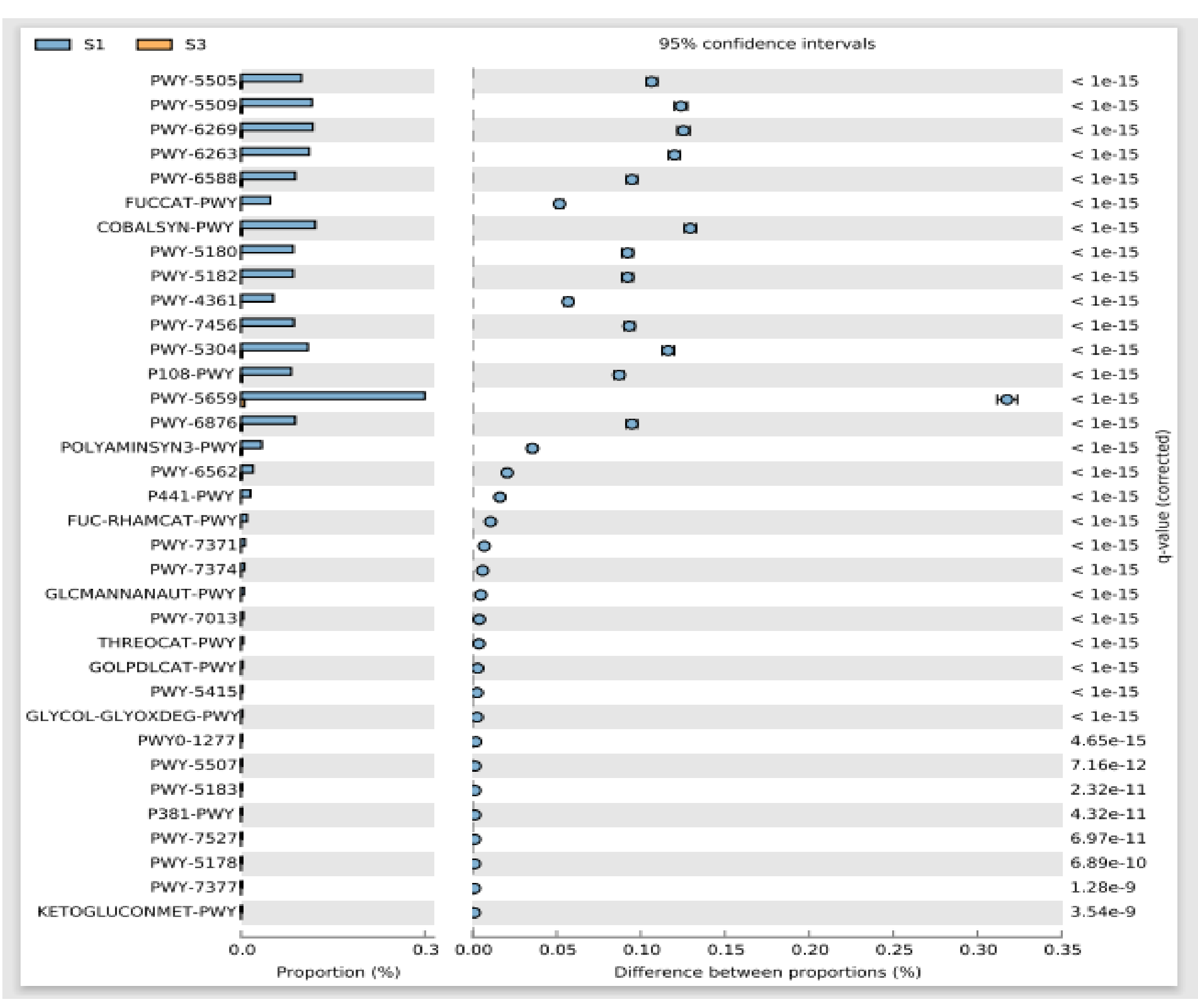
PWY-6897	19228.01	31986.83	18986.86
PWY-6901	12881.87	14358.11	2325.304
PWY-6969	5067.246	4021.361	549.775
PWY-6992	15.92182	0	0
PWY-7003	0	300.4556	0
PWY-7007	7.989086	0	0
PWY-7013	146.847	239.8516	0
PWY-7090	14.25632	11.16437	0
PWY-7094	859.376	408.8576	52.23481
PWY-7111	32758.66	48823.81	28876.14
PWY-7159	11.63265	28.39391	24.31028
PWY-7184	16370.52	16516.35	4772.514
PWY-7187	20823.53	24887.33	8452.881
PWY-7196	18059.62	19616.86	6165.593
PWY-7197	13973.62	14326.16	4184.653
PWY-7199	26652.1	38343.42	20899.08
PWY-7200	17489.97	19583.88	6367.048
PWY-7208	34187.53	51123.23	27860.21
PWY-7211	14312.86	4530.249	531.4701
PWY-7219	29713.69	43974.19	23910.18
PWY-7220	34318.71	52103.61	26965.62
PWY-7221	26798.65	38545.96	20925.58
PWY-7222	34318.71	52103.61	26965.62
PWY-7228	17945.79	18887.18	5840.206
PWY-7229	32040.39	47551.29	25546.02
PWY-7234	25805.25	38390.94	20906.25
PWY-7237	2361.5	408.46	41.5
PWY-7242	23825.7	34206.61	19826.41
PWY-7254	7860.016	5041.466	678.4944

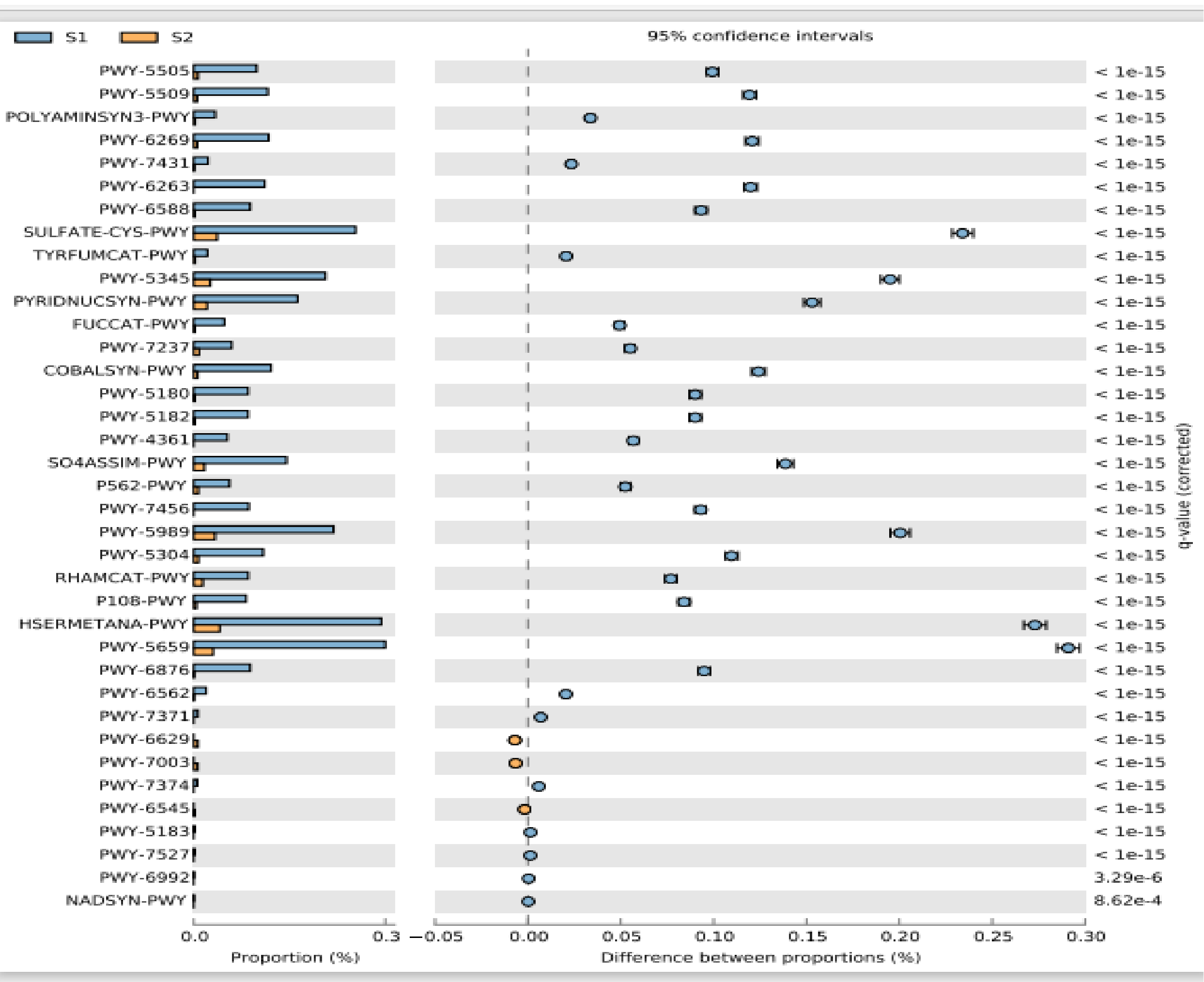
PWY-7315	**613.5788**	**954.0169**	**86.81776**
PWY-7323	**636.5036**	**814.1363**	**82.964**
PWY-7328	**4543.837**	**1400.248**	**147.9498**
PWY-7332	**3.499583**	**0**	**0**
PWY-7347	**8.977547**	**14.95848**	**14.59807**
PWY-7371	**257.3184**	**0**	**0**
PWY-7374	**219.2239**	**0**	**0**
PWY-7376	**32.45851**	**57.09738**	**16.55527**
PWY-7377	**44.12581**	**87.36686**	**0**
PWY-7392	**4625.051**	**3325.741**	**494.9563**
PWY-7400	**24316.22**	**37325.77**	**22249.57**
PWY-7431	**891.4745**	**35.85718**	**26.71985**
PWY-7446	**189**	**365.8889**	**43.5**
PWY-7456	**3429.141**	**0**	**0**
PWY-7527	**49.29301**	**0**	**0**
PWY-7539	**8204.95**	**4460.499**	**640.2058**
PWY-7560	**3610.914**	**2542.294**	**377.0647**
PWY-7663	**52526.36**	**78089.27**	**44160.95**
PWY-7664	**16823.76**	**3799.341**	**350.1634**
PWY-841	**21757.51**	**25420.19**	**8880.754**
PWY-922	**24192.25**	**39303.82**	**22184.85**
PWY0-1061	**25317.6**	**40091.15**	**22678.11**
PWY0-1241	**383.7714**	**602.0405**	**58.02454**
PWY0-1261	**19406.05**	**5047.323**	**595.9424**
PWY0-1277	**69.4856**	**59.28519**	**0**
PWY0-1296	**29597.52**	**41498.01**	**21959.58**
PWY0-1297	**27061.84**	**39569.68**	**21418.38**
PWY0-1298	**26973.49**	**41656.35**	**23025.18**
PWY0-1319	**31214.25**	**44381.81**	**23966.8**

PWY0-1338	**215**	**372.02**	**36.5**
PWY0-1415	**1190.091**	**1242.375**	**149.0631**
PWY0-1479	**1111.966**	**1102.279**	**124.8652**
PWY0-1533	**524.0223**	**639.4794**	**69.49276**
PWY0-1586	**36313.92**	**50030.24**	**27682.07**
PWY0-162	**20455.18**	**23503.82**	**7846.198**
PWY0-166	**21584.95**	**25490.7**	**8597.527**
PWY0-321	**921.5701**	**642.1712**	**57.04981**
PWY0-41	**49.12696**	**136.622**	**11.91843**
PWY0-42	**106.0097**	**92.54498**	**5.367757**
PWY0-781	**5185.478**	**2990.617**	**323.7556**
PWY0-845	**3851.612**	**1279.299**	**127.7706**
PWY0-862	**15029.53**	**3277.297**	**300.4499**
PWY490-3	**5.999569**	**17.9972**	**14.99642**
PWY4FS-7	**28712.21**	**42175.9**	**22847.68**
PWY4FS-8	**28712.21**	**42175.9**	**22847.68**
PWYG-321	**13253.27**	**3706.475**	**345.2797**
PYRIDNUCSAL-PWY	**900.6331**	**939.3013**	**96.61549**
PYRIDNUCSYN-PWY	**6474.131**	**1030.767**	**105.0075**
PYRIDOXSYN-PWY	**2062.835**	**781.9162**	**77.33048**
REDCITCYC	**7292.152**	**5085.729**	**741.1567**
RHAMCAT-PWY	**3397.889**	**690.7381**	**88.93603**
RIBOSYN2-PWY	**22910.05**	**34178.22**	**19644.67**
RUMP-PWY	**6291.115**	**3926.438**	**616.1911**
SALVADEHYPOX-PWY	**852.0597**	**767.9943**	**267.8982**
SER-GLYSYN-PWY	**22424.19**	**33547.65**	**19503.85**
SO4ASSIM-PWY	**5777.053**	**824.2918**	**85.55725**
SUCSYN-PWY	**29.69161**	**49.42946**	**45.00633**
SULFATE-CYS-PWY	**10087.36**	**1804.424**	**191.5577**

TCA	**8839.661**	**5237.915**	**724.9411**
TCA-GLYOX-BYPASS	**1776.194**	**1764.795**	**182.791**
TEICHOICACID-PWY	**27537.85**	**46413.56**	**26525.12**
THISYN-PWY	**2178.669**	**1202.511**	**122.864**
THREOCAT-PWY	**138.994**	**172.1944**	**0**
THRESYN-PWY	**22470.04**	**32549.08**	**19094.05**
TRNA-CHARGING-PWY	**18227.97**	**6019.276**	**803.2108**
TRPSYN-PWY	**22985.29**	**35020.53**	**20948.34**
TYRFUMCAT-PWY	**858.2388**	**121.3929**	**15.0791**
UBISYN-PWY	**3446.39**	**806.3483**	**86.96295**
UDPNAGSYN-PWY	**24692.68**	**38385.33**	**20911.01**
VALSYN-PWY	**27774.38**	**40565.31**	**23548.47**

Appendix 7. Statistical comparison of bacteria predicted pathways from 16S rRNA derived data of sample S1 and S3, S1 and S2 at 95% confidence intervals




S1
S2
95% confidence intervals
PWY-5505
PWY-5509
POLYAMINSYN3-PWY
PWY-6269
PWY-7431
PWY-6263
PWY-6588
SULFATE-CYS-PWY
TYRFUMCAT-PWY
PWY-5345
PYRIDNUCSYN-PWY
FUCCAT-PWY
PWY-7237
COBALSYN-PWY
PWY-5180
PWY-5182
PWY-4361
SO4ASSIM-PWY
P562-PWY
PWY-7456
PWY-5989
PWY-5304
RHAMCAT-PWY
P108-PWY
HSERMETANA-PWY
PWY-5659
PWY-6876
PWY-6562
PWY-7371
PWY-6629
PWY-7003
PWY-7374
PWY-6545
PWY-5183
PWY-7527
PWY-6992
NADSYN-PWY
< 1e-15
3.29e-6
8.62e-4
q-value (corrected)
0.0
0.3
−0.05
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Proportion (%)
Difference between proportions (%)