PHYSICOCHEMICAL ASSESSMENT OF MICROBIAL COMMUNITIES OF FERMENTED OLIVES IN NAMIBIA USING CULTURE DEPENDENT AND INDEPENDENT METHODS

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ABSTRACT

Wild olives are native to the Mediterranean area, sub-tropical and central Asia, and parts of Africa. Table olives are prepared from the raw fruit of the European or domesticated olive *Olea europaea* L. Hoff. Table olives are one of the lesser known fermented fruits in Namibia with naturally associated lactic acid bacteria (LAB) and yeast. These microorganisms are responsible for the development of aroma and flavour in the final olive product. The aim of this study was to document processing methods of table olive production, determine the physicochemical parameters which affect microbial growth and identify the microbial communities associated with olive fermentation in Namibian table olives using culture dependent and independent methods. Untreated table olives in Namibia are produced mostly by spontaneous fermentation. A total of 30 olive samples were collected from two different sites (Noab and Shalom) from February to October 2018. Total plate count, total phenolic content, total LAB and total yeast and mould count were also determined over 5 months to study the kinetics and dynamics of microbial populations during this fermentation. DNA was extracted using a culture dependent method and the culture independent method of metagenomic analysis was also used. Total phenol content ranged from 2.323×10^{-4} g Gallic Acid Equivalent (GAE) /mL at day 0 to 8.691×10^{-4} GAE/mL on day 150. Olive samples had an alkaline pH (8.9) which was then decreased and stabilized to around 4.5 after day 30 and remained constant up until day 150. Titratable acidity ranged from 0.29 g/L at day 0 to 2.85 g/L at day 150, and combined acidity from 0.008 Mol NaOH/L at day 0 to 0.128 Mol NaOH/L at day 150. NaCl content of both sites ranged from 8.24 to 10.54% NaCl. Total count plate showed that LAB (3.9814 ± 0.25494 $\log_{10}$ CFU/ mL) and yeast (4.1098 ± 0.23534 $\log_{10}$ CFU/ mL) had similar counts for both sites. Phyla including *Rozellomycota, Chytridiomycota, Mucoromycota, Cercozoa, Rotifera, Glomeromycota, Calcarisporiellomycota, Mortierellomycota, Basidiomycota, Ascomycota, Neocallimastigomycota, Fusabacteria, Acidobacteria, Chloroflexi, Deferribacteres, Verrucomicrobia, Actinobacteria, Bacteriodetes, Proteobacteria, Firmicutes* and *Cyanobacteria* for 16S sRNA, 18S rRNA and ITS were identified through metagenomic analysis. This study is the first attempt to use metagenomics analysis on Namibian table olives and confirming the presence of probiotic LAB and yeast which might be responsible for the special organoleptic properties of this product.

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ACRONYMS

ANOVA	One Way Analysis of Variance
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
GAP	Glyceraldehyde Phosphate
GAE	Gallic Acid Equivalent
IOC	International Olive Council
LAB	Lactic Acid Bacteria
MRS	De Man, Rogosa and Sharpe
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide
NaOH	Sodium Hydroxide
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
OTU	Operational Taxonomic Units
PIC	Prior Informed Consent
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
ITS	Internal Transcribed Spacer
SDA	Sabouraud Dextrose Agar
SPSS	Statistical Package for the Social Sciences
TGGE	Temperature Gradient Gel Electrophoresis
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TPC	Total Phenol Content
TTGE	Temporal Temperature Gradient Gel Electrophoresis
YEGC	Yeast Extract Glucose Chloramphenicol Agar

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DECLARATIONS

I, Wynand Zwart, 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 the award of a degree at any other institution.

..... Date: 30.08.2019

Wynand Zwart

CHAPTER 1: INTRODUCTION

1.1 Background of the Study

Namibia has a diverse range of traditional fermented foods and beverages, but a less familiar fermented product in Namibia is table olives (*Olea europaea*). Table olives in Namibia are produced on several sites across the country. Demand for table olives is increasing yearly worldwide, with table olives being one of the most important fermented products in Europe owing to their various known health advantages (Tufariello *et al.* 2015). The fermentation process plays a crucial role in rendering the table olive safe and edible, because table olives first have to be fermented to lower the level of bitter phenolic compounds for human consumption. In addition, studies indicate that the Mediterranean-style diet that uses table olives in large quantities has the potential for reducing oxidative damage associated disease risks (Omar 2008). The phenolic compounds found in table olives are also bioavailable to humans in active components with many enhanced health benefits (Heperkan 2013). Natural black olives in brine is one of three most important processing methods of table olives. The other methods include the Spanish-style green and black oxidized table olive, also known as Californian-style table olive method (Fernández, Adams & Fernández-Díez 1997). Natural black table olive in brine method, also known as Greek-style black table olive method, is a traditional anaerobic method that occurs after harvesting, washing and sorting, when the table olives are placed in 10% brine water. This method is commonly used in Namibia and in many other places across the world. Initially, table olives undergo spontaneous fermentation by native mixed microbiota, including Gram-negative bacteria, lactic acid bacteria (LAB) and yeast in the 10% brine water. After the initial fermentation process where there is extensive competition for nutrients, the process is mostly dominated by LAB and yeast.

Additionally, the microbiota of the table olives in 10% brine depends on different intrinsic factors including pH, phenols, water activity and extrinsic factors like temperature, oxygen and salt to facilitate in the diffusion of salt into the olives and the breakdown of nutrients of the table olives (Romeo 2012). LAB species such as *Lactobacillus plantarum, L. pentosus,* and, *L. paraplantarum* compete for the nutrients of the table olives in 10% salt concentrated brine during olive fermentation (Heperkan 2013). Furthermore, yeast also competes for the nutrients in the 10% brine and can produce desirable volatile compounds and metabolites that improve the organoleptic properties of the final product (Arroyo-López, Querol, Bautista, & Fernández, 2008). Currently, culture dependent and culture independent methods are used to determine the molecular aspect of olive fermentation. Culture independent methods use more modern approaches towards the extraction of the DNA of almost all microorganism within the 10% brine environment of the table olives (Botta & Cocolin, 2012). On the contrary, culture dependent methods rely more on traditional microbiological approaches that use direct isolation of culturable microorganisms from the 10% brine environment of table olives and then elucidates the DNA from those microorganisms to identify the species.

1.2 Statement of the Problem

Fermented table olives are one type of a fermented food in Namibia which is finding more interest from consumers because of its delicacy, health benefits and special organoleptic properties. There is a gap of knowledge on olive fermentation with only a small degree of research and scientific data available for future table olive research and development for this industry (examples include lack of standard starter culture, LAB, and promising probiotic candidates) in Namibia. There is especially an insufficient amount of data on assessing the microbial community associated with

table olive fermentation in Namibia. Knowledge on physicochemical parameters is also important due to their direct impact on the quality of the final product.

1.3 Objectives of the Research

The overall aim was to investigate the main physicochemical parameters, phenolic profiles and to assess, using culture dependent and culture independent approaches, the microbial communities that affect 3 types of olives (Manzanilla, Frantoio and Mission) grown at 2 different plantations (Noab in Khomas Region and Shalom in Erongo Region) in Namibia. The specific objectives were:

1. To assess the physicochemical factors and phenolic content during the fermentation process.
2. To determine and compare the microbial community composition associated with table olives during fermentation, using culture independent and culture dependent methods.

1.4 Significance of the Study

Comparing microbial communities and physicochemical and kinetic factors of Namibian table olives will provide information and valuable data towards the knowledge on table olive microbiota and give feedback to improve the table olive products for farmers on the dominant beneficial microbiota in their olive fermentation. Studying the olive fermentation process and its physicochemical factors will give an insight into the quality of the olive, microbiota responsible for the production of aroma and flavour during fermentation and to provide recommendations on the improvement of olive fermentation in Namibia.

1.5 Limitation of the Study

This study focused intensively on two olive plantations namely; Noab in Khomas Region and Shalom in Erongo Region in Namibia and three types of olives specifically; Manzanilla, Frantoio and Mission. There is no control over cultivar varieties growing on the different olive farms.

1.6 Delimitation of the Study

There are several olive sites in Namibia, therefore the obtained results of the two olive sites may not necessary be representative of and applicable to any other olive sites in Namibia.

CHAPTER 2: LITERATURE REVIEW

2.1 General Background

The International Olive Council (IOC) estimates that the table olives world production sales exceeded over 2.5 million tons per year worldwide in the year 2016/2017 (IOC, 2017). However, in Namibia there are only a few olive plantations that are only selling table olives to the Namibian market on a very small scale (Emvula 2013). Most table olives have to go through some kind of fermentation process because of their peculiar characteristics. This makes almost all table olives except for a few inedible directly from the olive tree. During fermentation, a diverse range of microbial groups are involved, as a microbial community, in the process of breaking down nutrients of the olives. Alternatively, the physicochemical parameters and the availability of substrates strongly influence the ability of microorganisms to ferment table olives in brine (Bleve *et al.* 2015). LAB are the most dominant microorganisms present during this fermentation process, by the production of lactic acid, which results in a lower pH. In addition, yeast plays a significant role in increasing the desirable taste and producing rich aroma of olives due to their metabolites and volatile compounds (Fernández, García, & Balbuena, 1995).

2.2. Olive Tree Classification and History

The olive tree, known by the botanical name of *Olea europaea L.* belongs to the Oleaceae family. Oleaceae are a family of flowering plants that include *Fraxinus, Jasminum, Forsythia* and *Ligustrum.* The olive tree has a unique set of integrated characteristics. It grows as a tree with a large central trunk that can become twisted in older trees with a high capacity to withstand numerous lateral branches to be able to hold multiple olive fruits (Rapoport, Fabbri & Sebastiani 2016). The olive tree is

the only species in the *Olea* genera that is of agricultural importance. Botanical classification of the olive tree is split into two subspecies within the species *Olea europaea* L., with the main difference being morphological (Morrone 2015). *Olea europaea* L. has a unique biodiversity, because of olive species allogamy it has a large genetical inheritance (Angiolillo, Mencuccini & Baldoni 1999). This has caused longevity and preservation of olive variability to be widespread with an estimated 1,200 cultivars worldwide (Bartolini *et al.* 1998). *Olea europaea L.* is one of the oldest cultivated trees known to mankind (Liphschitz *et al.* 1991). The origin of the olive tree has been the topic of much debate (Loukas & Krimbas 1983). It is believed that the olive tree originated in ancient Persia and spread to Syria and Palestine around 5,000 years ago, while others believe that it originated in Africa, mainly Ethiopia and Egypt (Mandel 2000). The Phoenicians and the Greeks are thought to be responsible for the spread of the olive tree to the Mediterranean region (Stamatelatou, Ntaikou & Lyberatos 2012). Later, the Romans discovered olive trees through Greek colonies in Italy (Boskou 2006). In 1492, Christopher Columbus brought the olive tree to America and while wine spread everywhere, olive trees remained restricted to areas similar in climatic conditions of the Mediterranean, such as Chile, Argentina, and California. However, today the olive tree has been dispersed in over 54 countries (Preedy & Watson 2010).

2.3 Characteristics of Table Olives

According to IOC (2017) Standard, the term "table olive" means the product prepared from the sound fruits of varieties of the cultivated olive tree (*Olea europaea L.*). These varieties are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness, and ease of detachment from the stone make them particularly suitable for processing. After that, they are treated to remove

its bitterness and preserved by natural fermentation, or by heat treatment with or without the addition of preservatives; packed with or without covering liquid (IOC,2017). Table olives have to be harvested from the olive tree and processed at the proper stage of ripeness. The main purpose of processing is the removal of bitterness of fruit, then the hydrolysis of the phenolic compounds (Campaniello *et al.* 2005). The peculiar characteristics of table olives to specific cultivars are related to the production area (climate, pedology, edaphology and geographical location) (Rodríguez-Gómez *et al.* 2017) Table olive is a widely fermented drupe fleshy fruit that has a high fat and moisture content, low sugar and phenolic compound concentration and very low ash, fibre and protein concentration. The phenolic compound consists mainly of bitter oleuropein, which prevents direct consumption. Therefore, most olives have to undergo a debittering process to make them edible (McDonald & Sun 1999). The major phenolic compounds, such as oleuropein, phenolic acids, phenolic alcohols, flavonoids, hydroxytyrosol and secoiridoids are among the most important secondary metabolites in olives (Amiot, Fleuriet & Macheix 1986; Vinha *et al.* 2005). Most of the fat in olives are monounsaturated due to the abundance in oleic acid. The protein proportion is low, but it has all the essential amino acids. In addition, table olives are a good source of minerals such as calcium, potassium, magnesium and phosphorous (Arroyo-López *et al.* 2016*)*. Of the three cultivars used in this study, the Spanish Manzanilla has the highest fruit yield while the Frantoio has a medium-sized fruit as well as being an excellent pollinator to surrounding olive trees. The third cultivar, the Mission olives (introduced in California) are one of the primary cultivars for table olives and olive oil in America. It is so widely cultivated that it is listed as the only olive cultivar from the United States (Rejano *et al.* 2010). The table olive fruit of cultivated varieties range from 1

to 4 cm in length, 0.5 to 2 cm in diameter, spherical to elongated forms and can weigh as much as 10 g (Rapoport, Fabbri & Sebastiani 2016).

2.4 Microbial Communities

Microbial communities, even in small niches, can have a rich microbial diversity. Bacteria for instance form the largest domain of species, while most species live alongside many others in interspecies communities (da Silva 2014). Currently, most molecular studies still focus only on pure cultures, rather than the microbial community as a whole. However, the molecular study of table olive fermentation can be performed either by culture-dependent or by culture-independent methods (Botta & Cocolin 2012). The use of methods that rely on the cultivation of microorganism in selective media do not offer a complete profile of the microbial diversity that is present in olive fruit fermentation ecosystem and only a small portion of the true microbial population is detected (Medina *et al.* 2016). It is estimated that above 90% of the microorganisms from natural environments and approximately 25–50% of those present in fermented foods cannot be cultivated, using conventional microbiological techniques (Amann & Kühl 1998; Ampe *et al.* 1999; Cocolin *et al.* 2013). There is a need to better understand microbial interactions, which can be combined with bioengineering to improve biological tasks and safety of products. Metagenomics is such a technique to study the microbial communities by identifying bacterial species present in a DNA sample (Thomas, Gilbert & Meyer 2012). Metagenomics approaches also include ways to functionally isolate DNA from a niche of interest. In a sequence-based analysis, the DNA can be randomly sequenced allowing the determination of the whole metabolic potential of the community from which it was isolated (Sessitsch *et al.* 2012). Therefore, metagenomics has become ubiquitous in the field of microbial ecosystem exploration

and diverse natural environments has been thoroughly explored by this approach (Ercolini 2013; Kergourlay *et al.* 2015). In the case of table olive fermentations, Cocolin *et al.* (2013) and De Angelis *et al.* (2015) have used metagenomics for the study of the bacterial biodiversity adhered to the surface of diverse Italian olive varieties using the 16S rRNA encoding gene as masker. Insight into the bacterial life of table olive fermentation will allow us to obtain valuable information on the fermentation process for the design of new strategies to improve the quality and safety of this fermented vegetable (Medina *et al.* 2016). However, Namibia to date has no evidence of any study done on table olives using the metagenomics approach. Metagenomics will allow detection of new bacterial species in olive fermentations and further studies can help to determine the influence of all the microorganisms in a community on the quality and safety of olives. The decreasing cost in the sequencing techniques has been an essential catalyst for the search of microbial diversity in several environments, such as the ocean (Yooseph *et al.* 2007), stromatolites (Baumgartner *et al.* 2009) and hydrothermal vents (Sogin *et al.* 2006). Post sequencing data storage, processing and analysis have now become relevant to the sequencing costs and are thought to increase in the future (Sboner *et al.* 2011). In the future, the decreasing cost of sequencing will allow more studies to use the metagenomic method.

2.5 Metagenomics

Typically, more than 99% of microorganisms from natural ecosystems cannot be cultured under laboratory conditions (Mardanov, Kadnikov & Ravin 2018). Therefore, there is a demand for culture-independent approaches for identification and characterization of unculturable microorganisms and uncovering their role in table olive fermentation. Metagenomics continues to be an invaluable tool for the

improvement of food technology. The low sequencing error rates makes metagenomics the best sequencing technique to deepen the understanding of the microbial community of table olive during fermentation (Kergourlay *et al.* 2015). Exploring the unculturable microbial world will expand the knowledge of what can be possible in biology by exploring new microorganism for food enhancements (De Filippis, Parente & Ercolini 2017). The difficulty on how to gain knowledge on microorganisms that cannot be cultured in the laboratory has been a struggle for decades. The traditional culture dependent method for determining food contamination has proven to be an invaluable tool over the years (Stewart 2012). However, new approaches have been developed since the 1990s, these include culture independent methods such as DGGE, TGGE, T-RFLP, and several other automated PCR-based techniques still widely used today, such as TTGE (Bigot *et al.* 2015). Most of these methods do provide accurate identification of microbial communities through sequencing of ribosomal 16S rDNA targeted gene. However, in the mid to late 1990s two methods stood out, the pyrosequencing and the parallelized ligation-mediated and bead-based sequencing approach (Kergourlay *et al.* 2015). In the 2000s, due to commercially available sequencers, high throughput sequencing of genes of microbial ecosystems appeared more frequent. The use of these sequencing technologies to study food microbial communities is still relatively new, but its popularity is currently booming and its use has become more affordable not only for researchers but also for the food industry (Kergourlay *et al.* 2015). Bacteria is the main aspect focused on by many publications for food systems, targeting the 16S rDNA gene. In addition, yeast and fungi play a key role in deciphering the ecosystem of the microbial community, by targeting the Internal Transcribed Spacer (ITS) region (Kergourlay *et al.* 2015). ITS is a noncoding DNA sequence situated between

the small-subunit ribosomal RNA and large-subunit rRNA genes in the chromosome. Metagenomics, also called environmental genomics, is a modern culture independent method used to isolate bacterial species present in the entire genome of all population members to shed light on the nature and function of the microorganisms present (Riesenfeld, Schloss & Handelsman 2004). Metagenomics has become ubiquitous in the field of ecology for exploring natural environments, however only a few food microbiomes have also been reported in literature (Kergourlay *et al.* 2015). This can be due to the low richness of diversity of microbial communities in food. Metagenomics gives genetic information on potentially novel biocatalyst or enzymes, genomic linkages between function and structure. Nucleic-acid-based techniques, such as metagenomics, coupled with a thorough knowledge of the physicochemical parameters of the environment, can provide important clues to identify potential metabolic pathways employed by the desired organisms (Barton & Northup 2011). The first large-scale metagenomic study was performed by the J. Craig Venter Institute on the Sargasso Sea microbial community (Barton & Northup 2011). However, the Sargasso Sea community proved to be more complex than thought. Although this field of microbiology is quite young, discoveries have already been made that challenge existing paradigms and make substantial contributions to biologists' quest to piece together the puzzle of life (Handelsman 2004). Asparagus, cucumbers, kimchi and table olives in brine have all been analysed using metagenomics. The bacterial community of green asparagus consisted mainly of *Proteobacteria* and *Firmicutes*, kimchi consisted mainly of *Leuconostoc* and *Lactobacillus*, while *Acetobacter*, *Gluconobacter* and *Lactobacillus* were reported in cucumbers (del Árbol *et al.* 2016; Medina *et al.* 2016). Table olives had a high level of halophilic bacteria, mainly *Chromohalobacter*, *Halomonas*, and

Marinilactibacillus genera, in the beginning and then *Lactobacillus* were the main bacterial population as reported by Cocolin *et al.* (2013) using the pyrosequencing analysis for the study of fermentation of directly brined *Nocellare etnea* table olive variety. In addition, De Angelis *et al.* (2015) found the main microbial population to be *Lactobacillus* and *Propionibacterium* on not lye treated table olives during fermentation. The bacterial phylogenetic assignation of all samples showed that two bacterial phyla (*Proteobacteria* and *Firmicutes*) included the genera with the greatest number of sequences using metagenomics for natural green Aloreña de Málaga table olives (Rodríguez-Gómez *et al.* 2017). Metagenomics starts with the extraction of DNA from an olive sample, followed by cloning of the DNA into a vector that is transformed into a host. Clones can be screened for a phylogenetic anchor, such as 16S rRNA, 18S rRNA and 23S rRNA, one of the highly conserved genes (Martinez-Porchas *et al.* 2017). CD Genomics (New York, USA) offers the full-length 16S/18S/ITS rRNA services. The 16S rRNA gene is highly conserved between different species of bacteria and archaea, which contains nine hypervariable regions (V1-V9) ranging from about 30~100 base pairs long, they vary dramatically between bacteria (Chakravorty *et al.* 2007). Highly conserved regions allow researchers to design primer pairs that will accurately and reliably amplify the 16s hypervariable region of their choice to achieve identification or characterization of diverse bacterial communities (Rosselli *et al.* 2016). Similar to the bacterial 16S rRNA genes, the eukaryotic 18S rRNA gene has conserved and variable regions 18S rRNA gene sequences and their associated ITS are used to classify fungi and eukaryotes (Hadziavdic *et al.* 2014). CD Genomics uses PacBio SMRT long sequencing technology, which provides full-length 16S/18S/ITS rRNA sequencing services. Especially for 16S rRNA sequencing, the average read length of the PacBio SMRT

sequences is 10~18 K, which can cover all variable areas of 16S rRNA (the total length is around 1,500 bp), and produce over 250,000 reads per SMRT Cell at QV30 to resolve 16S rDNA sequences (Rhoads & Au 2015). Furthermore, circular consensus sequencing produces high intra-molecular consensus accuracy for full-length 16S rDNA to achieve accurate to species classification and identification. In addition, 18S rRNA (total length 1,500-2,000 bp) and ITS (about 400-900 bp) are also able to be performed by full-length sequencing for microbiome analysis. Long-read sequencing enables species-level resolution and drives functional insights with full-length16S/18S/ITS rRNA (Earl et al. 2018).

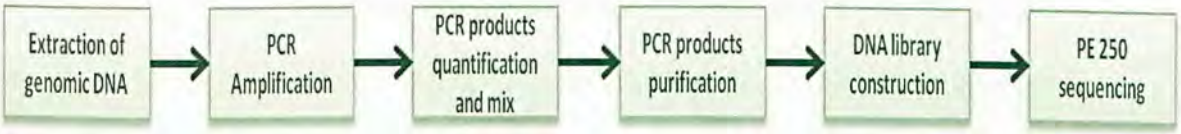

Figure 1. Workflow of Metagenomic Process from CD Genomics

Most metagenomic figures are represented using Operational Taxonomic Units (OUTs) which are cluster of similar sequence variants of the 16S rDNA marker gene sequence (Mysara *et al.* 2017). Each of these cluster is intended to represent a taxonomic unit of a bacteria species or genus depending on the sequence similarity threshold. Typically, OTU clusters are defined by a 97% identity threshold of the 16S gene sequence variants, which was derived from an empirical study that showed most strains had 97% 16S rRNA sequence similarity. From the OTU cluster, a single sequence is selected as a representative sequence. The representative sequence is annotated using a 16S classification method and all sequences within the OTU inherit that same annotation (Nguyen *et al.* 2016).

2.6 Fermentation of Table Olives

The three most commonly used table olives in brine methods are the Spanish style green olives, the Greek style naturally black olives, and the Californian style black ripe olives (Brenes 2004). In brine, table olive fermentations occur spontaneously without having to add any additional starter culture. Olive fruits undergo a fermentation whose characteristics depend on the physicochemical conditions, cultivar, temperature and salt content (Arroyo-López *et al.* 2010). Table olives are produced from specifically cultivated fruit varieties harvested at a pre-determined stage of maturation (Randazzo *et al.* 2012). The process of fermentation uses microorganisms under anaerobic conditions (although industrial fermentation can also be aerobic) to break down sugars (such as carbohydrates) to alcohol or organic acids and produce carbon dioxide as a by-product (Heperkan 2013). Fermentation is usually carried out by the indigenous microbiota (Heperkan 2013). During fermentation, enzymatic degradation of oleuropein results in the debittering of olives. The process provides preservation, enhanced nutritional and technological characteristics, as well as health benefits (such as exhibiting probiotic properties and being lactose and cholesterol free) (Bonatsou *et al.* 2017).

2.7 Lactic Acid Bacteria of Table Olive Fermentation

LAB are Gram-positive, non-spore forming cocci or rods and lack catalyse that produce various compounds such as organic acid, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations (Savadogo *et al.* 2004). The salt concentration in brine assists in rupturing the fruit membranes, allowing the diffusion of various components into the brine solutions used by naturally occurring LAB for growth and metabolic activities. Among the various

technological options, fermentation by LAB may be considered as a simple and valuable biotechnology for maintaining and improving the safety, nutritional, sensory and shelf-life properties of olives (Rodríguez *et al.* 2009). Most LAB are anaerobes, but are capable to grow in the presence of low oxygen. Although LAB lack catalases, they possess superoxide dismutase and have alternative means to detoxify peroxide radicals. LAB fermentation is based on two pathways: homofermentative and heterofermentative (Bassyouni *et al.* 2012).. Firstly, homofermentative LAB ferment hexoses by the Emden- Meyerhof pathway, and catabolize one mole of glucose to yield two moles of pyruvate (Fig. 1). Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles of ATP per glucose consumed (Bassyouni *et al.* 2012). Secondly, heterofermentative LAB utilize the phosphoketolase pathway (pentose phosphate pathway) to dissimilate sugars. One mole of glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole GAP and one mole acetyl phosphate (Bassyouni *et al.* 2012*)*. GAP is further metabolized to lactate as in homofermentative pathway, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. This process produces equal molar amounts of lactate, carbon dioxide and ethanol from lactose from the hexose monophosphate or pentose pathway (Bassyouni *et al.* 2012). The main differences between the two pathways is based on presence or absence of key cleavage enzymes (Rattanachaikunsopon & Phumkhachorn. 2010). The LAB *Lactobacillus plantarum* is the commercial starter culture that is most frequently used in the fermentation of food products of plant origin (Leal-Sánchez *et al.* 2003).

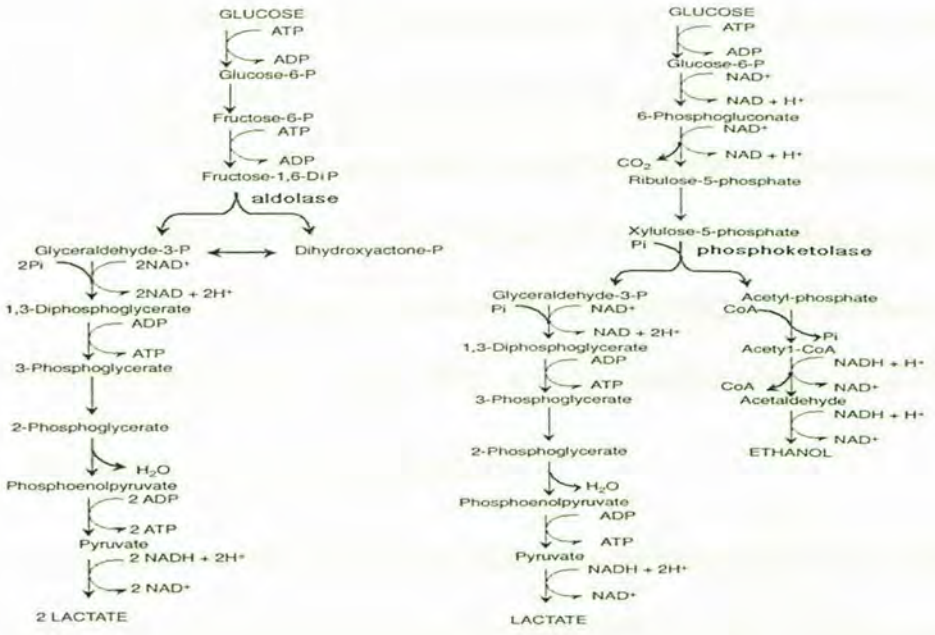

Figure 2. The pathway of homolactic (left) and heterolactic (right) acid fermentation in LAB fermentation in brine.

The composition of the microbiota of the olives, native microorganisms on table olives that causes spontaneous fermentation before adding brine is one of the factors that could affect the dynamics of the fermentation and the quality of the product. In olive fermentation, LAB species such as *Lactobacillus plantarum*, *L. pentosus*, and, *L. paraplantarum* are most frequently present during the whole olive fermentation process in brine. The pH reached at the end of fermentation, originated by the production of lactic acid by LAB must be below 4.5, otherwise growth of undesirable microorganisms could occur (Arroyo-López *et al.* 2010). During the final, third stage of the fermentation all fermentative substrates are exhausted and LAB population declines steadily. LAB generally grow readily in lower pH values ranging from 4.0 to 4.5 and this is considered a good fermentation if the pH can be kept at this level (Lucena-Padrós *et al.* 2014). *Lactobacilli* and *Pediococci* can tolerate a pH

of 3.5, before the growth is inhibited (Parades-Lopez 1992). The pH of both table olive sites was around 4.5 from day 30 till the end of the fermentation period, therefore the study had a good pH fermentation. During the whole fermentation process, *L. plantarum* and *L. pentosus* are the main species, although other LAB species such as *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteriodes*, or *Pediococcus pentosaceus*, among others, are also present (Rodríguez *et al.* 2009).

2.8 Yeast of Table Olive Fermentation

The contribution of yeast in olive processing can be both negative and positive. The positive role yeast play in table olives fermentation is due to the production of desirable volatile compounds and metabolites. This improves the organoleptic properties such as producing flavour compounds such as glycerol, alcohols, esters and carbonyl compounds. In return, it will enhances the growth of lactic acid bacteria which have killer activity and have the ability to biodegrade phenolic compounds. The catalase activity of some yeasts which enables the preservation of the product against peroxide formation and unsaturated fatty acid oxidation (Psani & Kotzekidou 2006) . However, the negative role yeasts play in table olive fermentation is that it may cause gas pocket formation because of excessive carbon dioxide production at the early stage of fermentation. Other negative roles include softening of the olive tissue, clouding of the brines, production of off flavours and odours, resistance to preservatives, and spoilage of the end product (Fadda *et al.* 2014; Hernández *et al.* 2007). During the fermentation of table olive, the yeast species mainly present are from the genera *Saccharomyces, Pichia*, *Candida* and *Wickerhamomyces* (Arroyo-López *et al.* 2008). The most commonly isolated species of table olive fermentation are the species *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida boidinii*, *Candida diddensiae*, *Pichia galeiformis, Pichia membranifaciens* and

Kluyveromyces lactis (Arroyo-López *et al.* 2012; Tofalo *et al.* 2012). Therefore, assessment of the microbial community with focus on yeast populations during fermentation can be of great importance into the improvement of final end product of table olive (Alves, Gonçalves & Quintas 2012). Yeast can survive the initial brine treatments and colonize olives throughout the fermentation process. Although yeast contributes significantly to the flavour content of the final product, yeast can also inhibit the growth of *Lactobacillus* species (Arroyo-López *et al.* 2008). Yeast also consume the sugars present in brines and produce diverse types of organic acids (citric or acetic), vitamins and aromatic compounds (Arroyo-López *et al.* 2010).

2.9 Physicochemical Characterization

The pH, salt concentration, titratable acidity and combined acidity concentration play crucial roles in the microbial community of table olives in brine during the fermentation process (Heperkan 2013). The pH measures the hydronium ion concentration or the negative logarithm of the hydronium concentration in table olive brine (Nielsen 1998) The pH can influence the flavour, colour, microbial stability and maintain the quality of table olives (Sadler & Murphy 2010). pH is one of the most important characteristics during this fermentation process and normally starts off alkaline (pH 8±) and then (Heperkan 2013)drops significantly to around pH 4 after which it stabilises till the end of the fermentation process. If the table olive fermentation processes allow the pH to start off alkaline and continue to be alkaline throughout the fermentation process, then the microbial community will most likely not have a high level of yeast or LAB present in the final product. This can cause an off taste of the final fermented table olive product, as LAB degrades oleuropein in high acidic environments through enzymatic reactions and debittering the olives during fermentation (Ozturk, Guven & Ozturk 2014).

Salt plays a vital role in table olive fermentation. It diffuses into the olive while phenol compounds and nutrients diffuse out of the olive over time (Saúde *et al.* 2017). Salt concentration changes depending on the olive type and processing, and the salt level of olive brines should be kept at a safe level so as not to cause any adverse effects on the high level of salt in the final table olive product (Saúde *et al.* 2017). The initial concentration of NaCl in the brines affects the sodium level in olive flesh, since penetration of NaCl in olives is a matter of diffusion and equilibrium (Bautista-Gallego *et al.* 2011). However, highly concentrated brine can inhibit the growth of potentially beneficial microorganism such as LAB, leading to niche suited to a few viable halophilic microorganisms able to survive in these extreme salt conditions, leading to the production of off flavoured product (Lopez-Lopez *et al.* 2004). Therefore, salt controls the water activity in the olive-surrounding solution and to a certain extent governs the microbial environment by inhibiting microorganisms during fermentation and by the diffusion of soluble constituents into the brine and salt into the olive fruit (Lopez-Lopez *et al.* 2004). Nisiotou stated that mainly yeasts and lactic acid bacteria to a smaller extent dominate the olive fermentations at salt levels >10% NaCl (Nisiotou *et al.* 2010). Therefore, this process leads to a final product with a milder taste and less self-preservation characteristics. Alternatively, reducing the salt level to 6– 8% enables a mixed fermentation by LAB and yeasts that coexist until the end of fermentation, resulting in a product with better characteristics (Nisiotou *et al.* 2010).

Total acidity or titratable acidity is one of the physicochemical characteristics that measures the total acid concentration contained in the brine of table olive fermentation. In table olive fermentation, the decrease of pH is also caused by acids in large quantities. The main contributing acids of titratable acidity include lactic,

acetic, malic, citric, tartaric, oxalic, ascorbic and sulphuric acid (Nielsen 1998). However, the combined acidity, also called residue lye, of table olive gives an indication of the buffer capacity of fermenting brines (Lopez-Lopez *et al.* 2004). As a general approach, the higher the combined acidity, the higher the amount of acid needed to reach a specific pH value. If the final pH of 4.0 or lower could not be reached, there will be potential of spoilage during storage. Naturally, black olives presented significantly lower combined acidity, as they are not subjected to lye treatment (Panagou, Tassou & Skandamis 2006).

2.10 Phenolic Compounds of Table Olive Fermentation

The pulp of table olives contains phenolic compounds, which are hydrophilic substances found in the table olives (Benyazza 2010). The class of phenolic compounds includes numerous substances, including caffeic acid, tyrosol and hydroxytyrosol, and more complex compounds such as oleuropein, ligstroside, and the lignans (Benyazza 2010). The main antioxidants of olive oil are the phenolic compounds. Antioxidants may be defined as substances which, when present at low concentrations compared to that of oxidizable substrates, significantly delay or inhibit oxidization of those substrates. Phenolic compounds are important polar compounds in table olives. These compounds are directly related to sensory characteristics of olive such as flavour content. The olive fruit has a high number of phenolic compounds and there has been a growing interest in this group due to their chemo-preventive activities against carcinogenesis and mutagenesis (Ryan *et al.* 2002). The reason for this interest is the recognition of their antioxidant properties, their great abundance in olives, and their probable role in the prevention of various diseases associated with oxidative stress (Manach *et al.* 2004). However, the health effects of phenolic compounds depend on the amount of olives consumed and, on the

bioavailability of those specific olives (Chung, Wei & Johnson 1998; Shen, Ji & Zhang 2007). The contribution of phenolic compounds to aroma is mainly due to the volatile phenols (Rodríguez *et al.* 2009). The most important classes of phenolic compounds in olive fruits include phenolic acids, phenolic alcohols, flavonoids and secoiridoids (Soler-Rivas, Espin & Wichers 2000). The phenolic alcohols of olives are 3,4-dihydroxyphenylethanol (hydroxytyrosol or h-tyrosol) and p hydroxyphenylethanol (tyrosol) (Romero *et al.* 2002; Ryan & Robards 1998). The flavonoids of olives are luteolin 7-*O*-glucoside, rutin and apigenin 7-*O*-glucoside, and the anthocyanins, cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside (Esti, Cinquanta & La Notte 1998; Romani *et al.* 1999). The secoiridoids of olives are mainly oleuropein and ligstroside; some oleuropein derivatives, namely demethyloleuropein, oleuropein aglycone and elenolic acid (Servili *et al.* 2004) and the main hydroxycinnamic acid derivative, verbascoside (Romani *et al.* 1999). There have only been a few works published on the characteristics of olive fruits, and none of them reports on the exact concentrations of phenolic compounds of specific olives (Tanilgan, Özcanb & Ünverb 2007; Ünal & Nergiz 2003). There are also no known published reports on the phenolic content of table olives in Namibia. An *in vitro* study in cells showed that hydroxytyrosol was transported via passive diffusion in a dose dependent manner (Manna *et al.* 2000). Another human study showed that tyrosol and hydroxytyrosol were excreted in urine and that these olive oil phenols were absorbed in the intestine after ingestion, but it was unclear to what extent (Visioli *et al.* 2000).

2.11 Probiotic Olive Future

The term probiotic used to be only restricted to dairy fermented products such as yogurt (Peres *et al.* 2014). However, recently a new trend towards vegetables as

carriers of probiotic strains are becoming the norm. For instance, *Pediococcus pentosaceus* and *Lactobacillus plantarum* have been isolated from pickled vegetables by Chiu *et al.* (2008) and shown to inhibit the growth of *Salmonella* in mice. Artichokes inoculated with *L. plantarum* and *L. casei* survived up to 90 days and improved the overall health benefits of artichokes (Valerio *et al.* 2006). Lactic acid fermentation in brine of table olives is a potential mechanism that can be used for incorporating bacteria into the final product by using starter cultures. Starter cultures can have faster acidification rates, nutritional, health, and safety advantages as well as produce a favourable influence on the organoleptic properties (López-lópez *et al.* 2018). For instance, De Bellis *et al.* (2010) used *L. paracasei* IMPC2.1 to control the green Spanish style fermentation and colonise the olive surface. The use of a LAB probiotic strain on olives has been demonstrated to have been highly dependable on its physicochemical characteristics. As a result, an adequate LAB starter culture can provide a considerable LAB load on fermented table olives. Several reports have emphasized the role of table olives as adequate carriers for delivering probiotic bacteria to humans, particularly when the LAB strains were previously isolated and characterized from olive microbiota (Montoro *et al.* 2016). According to López-lópez *et al.* (2018) there is no sensory difference between olives with a starter culture and olives without, therefore a starter culture can be used successfully without any adverse effect on appearance, odour and taste. In Namibia, however, there is no known evidence of any olive farm or production incorporating starter cultures in brine. It can therefore be used as a potential future process to increase the load of LAB and have associated probiotic olives.

CHAPTER 3: RESEARCH METHODS

3.1 Research Design

The research used both quantitative and qualitative approaches in the research design. The quantitative approach consisted of physicochemical tests (pH, NaCl, titratable and combined acidity), microbial counts and microbial analysis. Microbial counts were performed on each sample on different days during the fermentation process. However, the microbial analysis was only performed on the final fermentation process using culture dependent methods (biochemical and phenotypic identification) and a culture independent method (metagenomics). The qualitative data produced were the documentation of the precise processing methods of each site.

Figure 3. Satellite map of Namibia, showing Noab and Shalom (Close to Swakopmund) Table Olive sites (mapcarta.com)

The processing methods were similar in that both used 10% NaCl brine with no lye treatment. The only difference was that site 1 used a double lid method, two lids with the top lid having a small hole, and the fermentation process was carried out in the laboratory at UNAM, while site 2 used the cloth method, in which a cloth was placed inside to cover the olives with one lid, and the fermentation process was carried out on the farm itself. The two selected sites were Noab (site 1) and Shalom (site 2). Noab Farm is situated in the Khomas Region, close to Remhoogteberge, while Shalom Farm is situated in the Swakopmund River bed in the Erongo Region. Fresh olive brine samples were stored in sterilized beakers and placed in the fridge before analysing.

3.2. Table Olive Processing Methods

The fermentation process for the olive samples from site 1 (Noab) was carried out in the Masters Laboratory of the Department of Biological Sciences at UNAM in a 10 litres container. The table olives used were a combination of Manzanilla and Frantoio, turning black olives.

Figure 4. The 10 litre table olive fermenting container of Noab (left) and Shalom (right)

This processing method used two-lids, the first lid kept the olives submerged and the secondary lid had a small hole to allow the release of carbon dioxide built up, which can be seen from Figure 4. In site 2 (Shalom), situated at Erongo Region, the fermentation was carried out on site in a 10 litre container. The table olives used were all black Mission olives. This processing method used a cloth to submerge the table olives and a closed lid. Both processing methods used 10% NaCl brine and 1 litre additions at the intervals of 0, 15, 30, 60, 90 and 150 Days. The olives were fermented naturally without the addition of lactic acid bacteria starter cultures.

3.3 Sample

The table olives and brine sample were collected at different intervals during the fermentation at Swakopmund and in the lab for Noab, in 400 ml sterilized, screw capped bottles for the brine and 50 ml sterilized beakers. Fresh samples were kept on ice, transported to UNAM and stored in the fridge at 4 °C until analysis. A total of 30 samples were collected and analysed, during February to October 2018.

3.4 Physicochemical characteristics

All physicochemical tests were measured in triplicate at the different fermentation intervals of 0, 15, 30, 60, 90 and 150 days. All tests used the olive brine from the tested samples, except for total phenol content which uses the fleshy part of the olive fruit.

3.4.1 pH determination

The pH of olive brine was measured using a pH meter (Oakton pHTestr 10, Oakton Instruments, Vernon Hills, Illinois, USA). The pH meter was first turned on and allowed to stabilise for 15 minutes. The brine was poured into a clean beaker and the probe was submersed. The digital display reading was taken after 30 seconds. As a

quality control measure, the rapid method of using Merck universal indicator strips for pH was also incorporated.

3.4.2 Combined Acidity

The combined acidity was determined by titrating hydrochloric acid solution (0.1 M) slowly using a burette into 25 mL of brine, while shaking the brine beaker slowly until a pH of 2.6 was reached. Results were expressed in normality, the equivalents of sodium hydroxide per litre of brine (Tan *et al.* 2016).

3.4.3 Total/Titratable Acidity

The total or titratable acidity was calculated by titrating sodium hydroxide (0.1 M) using a burette until the pH reached 8.1 with 25 mL of brine in a 150 ml beaker, while using a magnetic stirrer in the beaker. The results of titratable acidity were express in g/litre (Smyth 2012).

Equation 1:

$$\textbf{Titratable acidity (g/litre)} = \textbf{titration} \times \textbf{0.36032}$$

where titration represents the amount of sodium hydroxide added until the pH reached 8.1.

3.4.4. NaCl or Sodium Chloride (Salt) Content

The Volhard's Titration method was used to determine the salt content as described by Smyth (2012). Brine (1 ml) was added to purified water (50 ml) in a 250 ml Erlenmeyer flask. Then 1 ml of 5% potassium chromate was added. Finally, silver nitrate solution (0.1 M) was titrated using a burette until a permanent red tinge colour appeared which indicated the end point of the titration. The volume of the titrant used was recorded.

Equation 2:

$$\% \text{ NaCl} = \text{silver nitrate (ml)} \times 0.5845$$

where silver nitrate represents the amount of silver nitrate added until the colour reached a permanent red tinge.

3.4.5. Total Phenolic Content

The Folin-Ciocalteu method of Singleton & Rossi (1965) was used to determine the total phenolic content. Table olives were depitted using a sharp clean blade and then place into a blender. The sample was macerated at high speed for 2 minutes and centrifuged for 5 minutes. One hundred microlitre of Folin-Ciocalteu was directly added to 500 µl of the table olive extract and kept in a dark room. After 15 minutes, 2.5 ml of saturated sodium carbonate (1 M) was added and kept for a further 30 min in a dark room. The absorbance at 760 nm was read using a spectrophotometer (GeneSyn 20, Thermo, USA) (Cheikhyoussef, Summers & Kahaka 2015). Gallic acid was used to construct the standard curve. The standard curve was prepared by using 0.025 g of garlic acid and adding 25 ml of water.

Equation 3:

$$Y = mx + c$$

Where Y represents the absorbance of the olive at 760nm, x represents the concentration of phenols.

Equation 4:

$$Y = 3272.7x$$

where 3272.7 represents the gradient of the line, calculated from the gallic acid standard.

The gallic acid in water then had dilutions of 0, 2, 4, 8, 16 and 32. The final volume consisted of 1 ml Folin-Ciocalteu reagent and 3 ml of dilution mixture. The initial dilution factor had a concentration of 9.86×10^{-4}, the dilution factor 2 had a concentration of 4.93×10^{-4}, the dilution factor 4 had a concentration of 2.465×10^{-4}, the dilution factor 8 had a concentration of the 1.233×10^{-4}, the dilution factor 16 had a concentration of 6.1625×10^{-5}, the dilution factor 32 had a concentration of 3.0813×10^{-5}.

3.5. Microbiological Analysis

3.5.1 Enumeration of LAB, Yeast and Moulds of Table Olives

Serial dilutions were prepared using 1% peptone buffered water. The various general cultivation media (Total plate count agar, De Man, Rogosa and Sharpe (MRS) agar, Nutrient agar and Sabouraud dextrose agar (SDA) and selective (Rogosa agar, M17, Yeast Extract Chloramphenicol Agar and MacConkey) were prepared for microbial enumeration, identification, and physiological determination as described by Asehraou *et al.* (2000). The serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were cultured using the pour plate method. Plate count agar were prepared for the enumeration of total mesophilic bacteria at 30 °C for 48 hours in aerobic conditions. MRS agar with 0.17 g/l of cycloheximide were incubated at 30 °C for 4 days in anaerobiosis for LAB. Nutrient Agar was used for the enumeration of a wide range of non-fastidious organisms, as it can support the growth of both fungi and bacteria, at 32 °C for 2 days. SDA (Sabouraud dextrose agar) was prepared with 0.1 g/L of

chloramphenicol and incubated at 25 °C for 3 days for yeasts and moulds. Rogosa agar was prepared for LAB enumeration at 35 °C for 3 days.

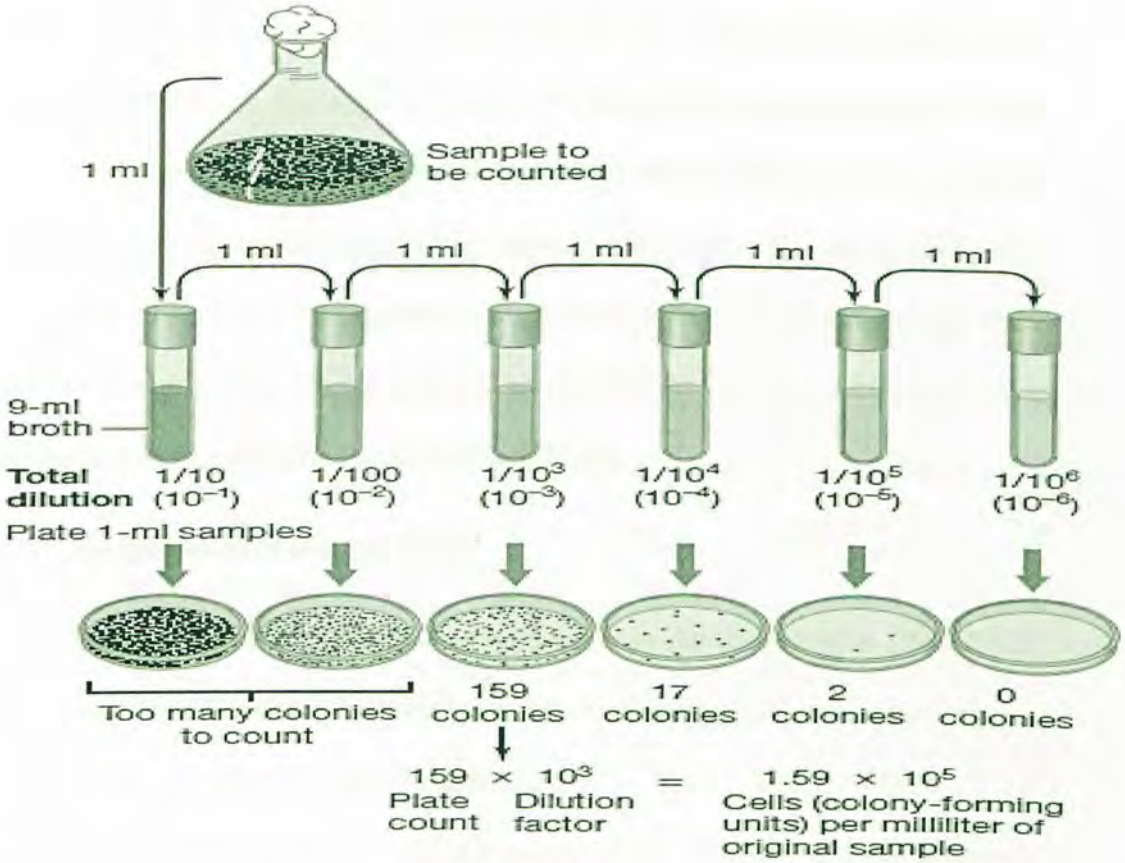

Figure 5. Illustration of the pour plate method (Madigan *et al.* 2012)

M17 were prepared anaerobically at 30°C for 3 days for the enumeration of LAB. Yeast Extract Glucose Chloramphenicol Agar was incubated aerobically for up to 5 days at 25 °C for yeast and moulds (Aponte *et al.* 2010). After enumeration, three colonies were then randomly selected from countable different selective media (MRS, Rogosa and M17) agar plate replica. The colonies were purified by successive streaking onto appropriate agar media (MRS, Rogosa, M17) before being subjected to characterization. In total, 6 colonies from each sample were selected and characterized further. The isolates were transferred to MRS broth, incubated for 24 hours while some were preserved in 25 % of glycerol until further analysis.

3.5.2 Culture Dependent Method

3.5.2.1 Genomic DNA Extraction

DNA was extracted from pure cultures whereby the isolated LAB colonies were grown in MRS broth. The grown cultures were pelleted by centrifugation at 13 000 × g for 5 minutes and washed with double distilled water. DNA was then extracted using the Zymo Bacterial/Fungal DNA Extraction Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions with a few modifications. Then the DNA was run on 1% agarose gel to confirm the presence of DNA, before proceeding to do polymerase chain reaction (PCR).

3.5.3. Amplification of the 16S rRNA

Only 5 isolates were used for PCR amplification in the thermocycler (BIO RAD, Singapore). The 16S rRNA bacterial gene was amplified using the universal primers 27F (5' AGAGTTTGATCTGGCTCAG 3') and 1492R (5' CGGTTACCTTGTTACGACTT 3') (Tajabadi *et al.* 2013). The oligonucleotide primers were manufactured by Inqaba Biotech (South Africa). The amplification of the genes was similar to Randazzo *et al.* (2017) with some modifications performed in a 25 µl PCR reaction mixture containing: 5µl of DNA template, 1 µl of each primer and 18 µl of RNase free water, polymerase enzyme, the dNTPs and the MgCl (Thermo Scientific, USA). The PCR cycling conditions consisted of 1 cycle (initial denaturation at 94 °C for 4 minutes, followed by 23 cycles of: melting at 94°C for 1 minute, annealing at 48 °C for 30 seconds, and extension at 72 °C for 2 minutes, and then a final extension step at 72 °C for 2 minutes. Thereafter the sample was stored at 4 °C until being used for gel electrophoresis and sequencing (Lucena-Padrós *et al.* 2014). After the completion of PCR reaction, amplified products were resolved in 1

% agarose gel in which 5 µl ethidium bromide solution (10 mg/ml) was added. A 1 kb DNA ladder was used to identify the approximate size of each sample run on the gel. Electrophoresis was performed at 150 V for 45 min. The gel was viewed under UV light to confirm amplification and the un-purified PCR products were sent to Inqaba Biotech, South Africa for purification and sequencing.

3.5.4 Pyrosequencing

From both sites, 10 samples were sent for CD Genomics (USA, New York) for metagenomic analysis. The samples were prepared by cutting a small piece of fresh tissue with a sterile blade and placing inside a capped microplate 2 mL tube. Then 1 mL of RNA (Thermo Fisher Scientific, USA) was added to each tube, before being tightly capped. The samples were then labelled and each tube sealed with parafilm to prevent accidental opening, leaking, or unexpected evaporation. Additionally, the tubes were secured in a rack and padded inside a protective box to avoid damage and any cross contamination.

Heatmap is a graphical representation of clustering in terms of the size of the data matrix in terms of color gradients and the similarity of species or sample abundance. According to the taxonomy composition and relative abundance of each sample, the species heatmap analysis was carried out to extract the species at each taxonomic level and plotted using the R language tools, the heatmap clustering analysis was conducted at the level of phylum, class, order, family, genus and species respectively (Hugerth & Andersson 2017).

For phylogenetic analysis the sequence of OTU with the highest abundance level was selected by QIIME software as the representative sequence, and the phylogenetic tree was constructed by multiple sequence alignment, and then the graph was drawn by

Python language tool (Navas-Molina *et al.* 2013). Each branch in the phylogenetic tree represents a species whose length is the evolutionary distance between two species, or the degree of species difference.

Four different metrics were calculated to assess the alpha diversity: "Chao1 and Ace" simply estimate the number of species in a community; "Shannon and Simpson" account for both richness and evenness of a community. The larger the Chao1, Ace and Shannon indices, the smaller the Simpson index value, indicating the more diverse of the species (Grice *et al.* 2009).

3.6 Data Analysis

After each sample was repeated in triplicate, the colony counts were transformed to log CFU (Colony Forming Units)/ mL values before using Microsoft Excel 2016 to compute the means, standard deviation and standard error. The changes in physicochemical properties of fermentation for days 0, 15, 30, 60, 90 and 150 were analysed using the t-test. Initially, an F-test was performed to assess the normal distribution on the tested samples dataset, to assume equal or unequal variances, at 95 % confidence interval.

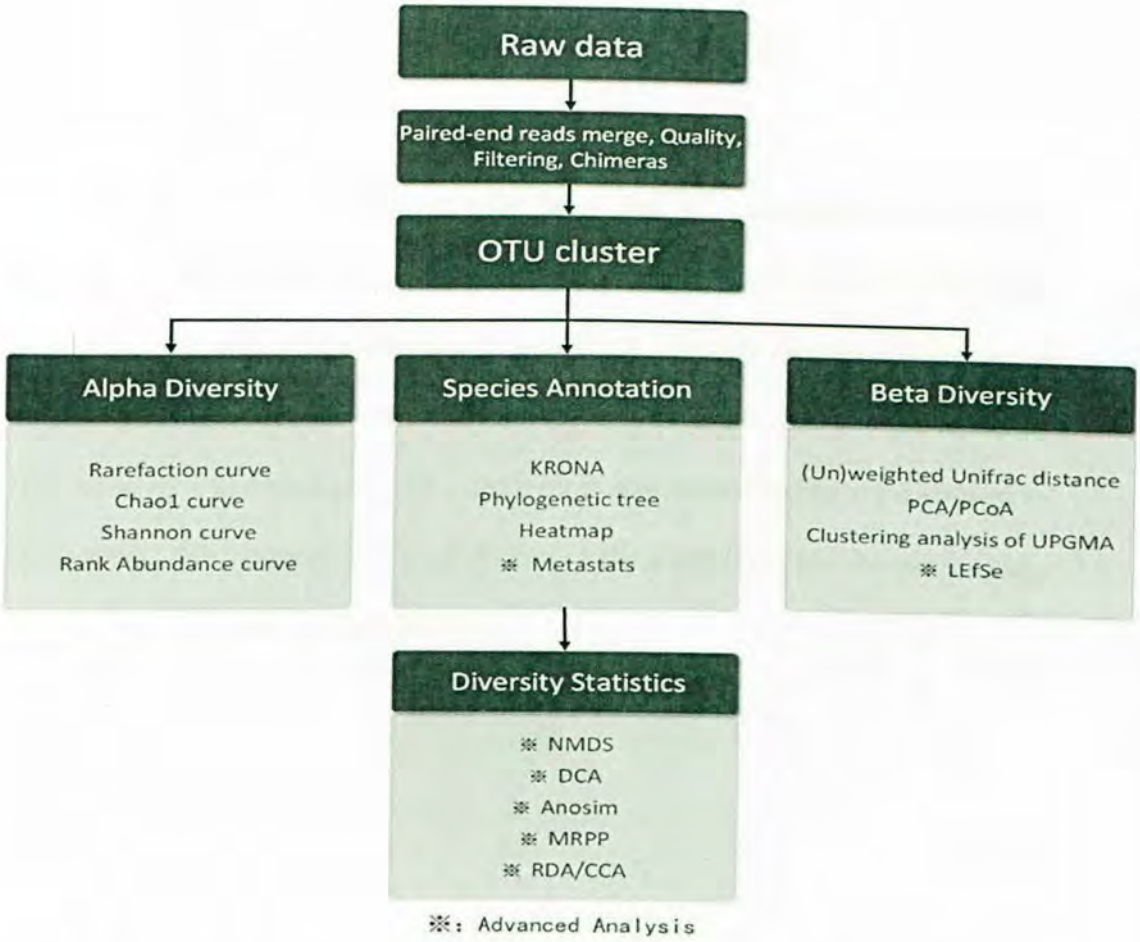

Figure 6. Outline of materials and methods of 16S rRNA,18S rRNA and ITS for Noab and Shalom Table Olives

The final fermentation was compared in terms of microbial profiles, using culture dependent and independent methods. One-way analysis of variance (ANOVA) was used to compare total LAB count values among different olive sites by SPSS with Levene's Test. Finally, Principal Component Analysis was carried out using XLSTAT in Excel 2016 for the total LAB, total Yeast and Mould and pH. Metagenomics data analysis was carried using UCLUST (Caporaso *et al.* 2010) in QIIME (Edgar 2010) (version 1.8.0) to cluster the tags with 97% similarity and acquired the OTUs, then the OTUs were annotated based on Silva taxonomic database.

3.7 Research Ethics

Olive samples were purchased from the farms and the processing methods were documented. Prior Informed Content (PIC) was obtained before the start of the study from every olive site, with permission to send DNA and olive samples for analysis to South Africa and USA respectively. Ethnical clearance was shared with all farms after the study was approved by the Centre for Postgraduate Studies at the University of Namibia. This research was done following the Guidelines and Research Ethics Policy of the University of Namibia (2017).

4.2 Physicochemical and Kinetic Factors

The physicochemical and kinetic factors for site 1 of Noab olive farm contains a mixture variety of two cultivars, Manzanilla and Frantoio, meanwhile , the site 2 of Shalom olive farm contains the cultivar of Mission table olives are presented in table 1 and 2.

Table 1. Physicochemical and microbiological attributes for the olive samples of Site 1

Site 1 - Noab – Manzanilla and Frantoio						
	Day 0	Day 15	Day 30	Day 60	Day 90	Day 150
pH	8.77 ± 0.04^{a}	4.13 ± 0.02^{b}	4.14 ± 0.06^{b}	4.46 ± 0.09^{b}	4.43 ± 0.02^{b}	4.48 ± 0.06^{b}
Combined Acidity (Mol NaOH/L)	0.013 ± 0.0053^{a}	0.061 ± 0.029^{b}	0.077 ± 0.005^{b}	0.10 ± 0.011^{c}	0.12 ± 0.069^{d}	0.12 ± 0.0071^{d}
Titratable Acidity (g/L)	0.35 ± 0.032^{a}	0.82 ± 0.024^{b}	1.14 ± 0.032^{c}	1.67 ± 0.064^{c}	1.69 ± 0.016^{c}	2.64 ± 0.032^{d}
NaCl (%)	10.14 ± 0.20^{a}	9.70 ± 0.31^{b}	9.76 ± 0.12^{b}	9.82 ± 0.08^{b}	9.07 ± 0.20^{c}	8.84 ± 0.08^{d}
TPC (g of GAE / mL)	$6.63 \pm 2.71\times10^{-4\,a}$	$4.46 \pm 1.82\times10^{-4\,b}$	$4.11 \pm 1.67\times10^{-4\,b}$	$3.21 \pm 1.31\times10^{-4\,c}$	$2.48 \pm 1.01\times10^{-4\,d}$	$2.33 \pm 9.50\times10^{-5\,d}$
Total Plate Count ($\log_{10}$ CFU/ mL)	2.61 ± 1.07^{a}	4.64 ± 1.89^{b}	4.74 ± 1.94^{b}	5.12 ± 2.09^{c}	5.23 ± 2.13^{c}	5.67 ± 2.31^{c}
Total LAB Count ($\log_{10}$ CFU/ mL)	1.57 ± 0.53^{a}	2.23 ± 0.74^{b}	3.99 ± 1.33^{c}	4.75 ± 1.58^{d}	4.87 ± 1.62^{d}	5.39 ± 1.80^{e}
Total Yeast and Mould ($\log_{10}$ CFU/ mL)	1.51 ± 0.62^{a}	4.07 ± 1.66^{b}	4.53 ± 1.85^{b}	4.69 ± 1.92^{b}	5.01 ± 2.04^{c}	5.20 ± 2.12^{c}
Total Coliform Count ($\log_{10}$ CFU/ mL)	1.58 ± 0.91^{a}	1.70 ± 0.98^{a}	1.92 ± 1.11^{a}	2.24 ± 1.29^{b}	2.89 ± 1.67^{b}	3.10 ± 1.78^{b}

Mean values of pH and of each microbial group in the same row followed by different lowercase letters are significantly different ($P < 0.01$).

Table 2. Physicochemical and microbiological attributes for the olive samples of Site 2

	Site 2 - Shalom - Mission					
	Day 0	Day 15	Day 30	Day 60	Day 90	Day 150
pH	7.75 ± 0.07^{a}	5.25 ± 0.02^{b}	4.43 ± 0.03^{c}	4.37 ± 0.03^{c}	4.45 ± 0.02^{c}	4.48 ± 0.3^{c}
Combined Acidity (Mol NaOH/L)	0.027 ± 0.002^{a}	0.035 ± 0.009^{a}	0.07 ± 0.009^{b}	0.093 ± 0.005^{c}	0.10 ± 0.0027^{c}	0.12 ± 0.0027^{c}
Titratable Acidity(g/L)	0.42 ± 0.01^{a}	0.77 ± 0.03^{b}	1.27 ± 0.02^{c}	2.21 ± 0.21^{d}	2.71 ± 0.07^{d}	2.76 ± 0.7^{d}
NaCl (%)	10.14 ± 0.02^{a}	9.62 ± 0.20^{b}	9.33 ± 0.29^{b}	9.12 ± 0.07^{b}	9.7 ± 0.41^{b}	8.44 ± 0.12^{c}
TPC (g of GAE / mL)	$8.26 \pm 3.4\times10^{-4\,a}$	$7.15 \pm 2.92\times10^{-4\,b}$	$6.79 \pm 2.7\times10^{-4\,c}$	$6.09 \pm 2.48\times10^{-3\,c}$	$5.45 \pm 2.23\times10^{-4\,d}$	$4.43 \pm 1.81\times10^{-4\,e}$
Total Plate Count ($\log_{10}$ CFU/ mL)	2.60 ± 1.06^{a}	4.36 ± 1.78^{b}	4.64 ± 1.89^{b}	5.24 ± 2.14^{c}	5.44 ± 2.22^{c}	7.14 ± 4.91^{d}
Total LAB Count ($\log_{10}$ CFU/ mL)	1.48 ± 0.49^{a}	2.46 ± 0.82^{b}	3.91 ± 1.30^{c}	4.88 ± 1.63^{d}	5.04 ± 1.68^{e}	5.83 ± 1.94^{e}
Total Yeast and Mould ($\log_{10}$ CFU/ mL)	1.55 ± 0.63^{a}	2.62 ± 1.07^{b}	4.16 ± 1.70^{c}	5.05 ± 2.06^{d}	5.17 ± 2.11^{d}	5.86 ± 2.39^{d}
Total Coliform Count ($\log_{10}$ CFU/ mL)	1.48 ± 0.85^{a}	1.54 ± 0.89^{a}	1.75 ± 1.01^{a}	1.94 ± 1.12^{b}	2.02 ± 1.17^{b}	2.11 ± 1.22^{b}

Mean values of pH and of each microbial group in the same row followed by different lowercase letters are significantly different ($P < 0.01$)

4.2.1 pH

The pH of Noab olive samples had a mean value of 5.08 ± 0.28 with a range of 3.93 to 8.90. Shalom olive samples had a pH mean of 5.12 ± 0.20 with a range of 4.30 to 7.90. In Figure 9, a clear decrease in pH is observed and stabilises between 4-5 for the remainder of the fermentation days for both Noab and Shalom olive samples. An independent samples t-test was performed to compare the pH for Noab and Shalom olive samples (Appendix 1-Table 3). In Table 3, there was no significant difference ($P > 0.05$) in pH between Noab and Shalom olive samples.

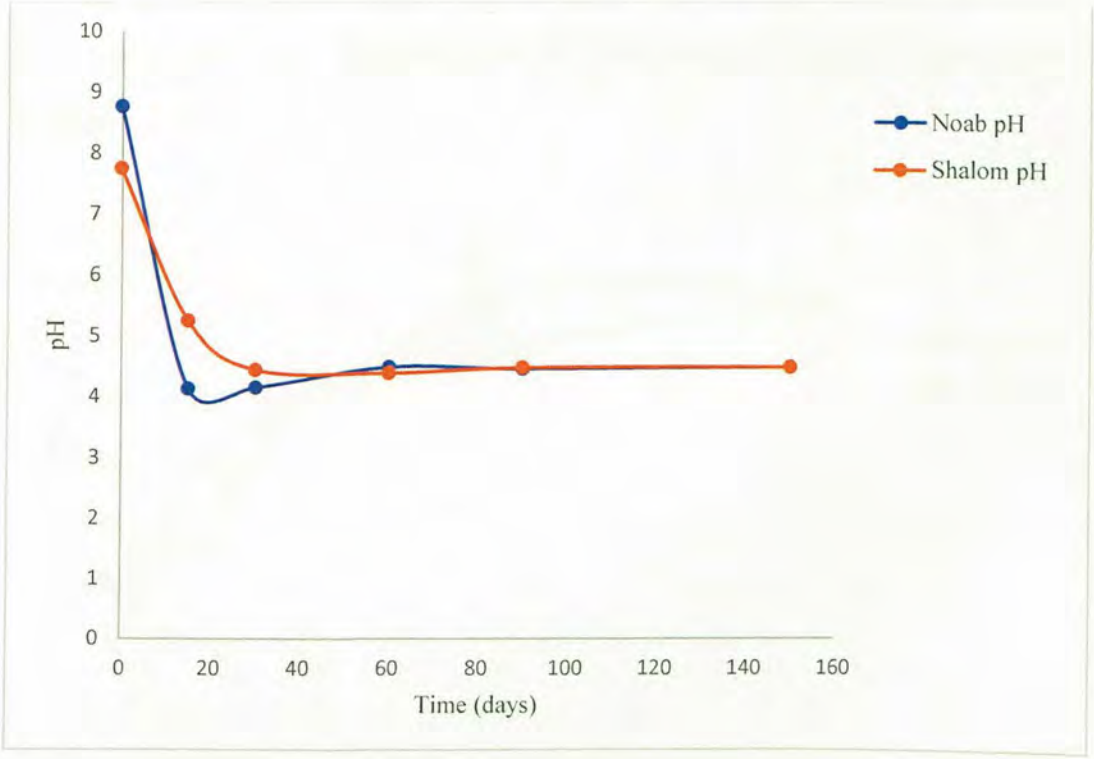

Figure 9. pH of Noab and Shalom Table Olives

4.2.2 Combined Acidity

The combined acidity of Noab olive samples had a mean value of 0.8 ± 0.009 Mol NaOH/L with a minimum of 0.008 Mol NaOH/L and a maximum of 0.128 Mol NaOH/L. Shalom olive samples had a mean combined acidity of 0.073 ± 0.0082 Mol NaOH/L with a minimum of 0.016 Mol NaOH/L and a maximum of 0.120 Mol NaOH/L. In Figure 10, a slight increase for both Noab and Shalom olive samples over time can be observed. An independent samples t-test was performed to compare the combined acidity for Noab and Shalom olive samples (Appendix 1-Table 4). In Table 4, the outcome variable was found to be normally distributed and equal variance are assumed based upon results of Levene's test ($F(34) = 0.73$, $P = 0.545$). There was no significant difference ($P > 0.05$) in pH between Noab and Shalom olive samples.

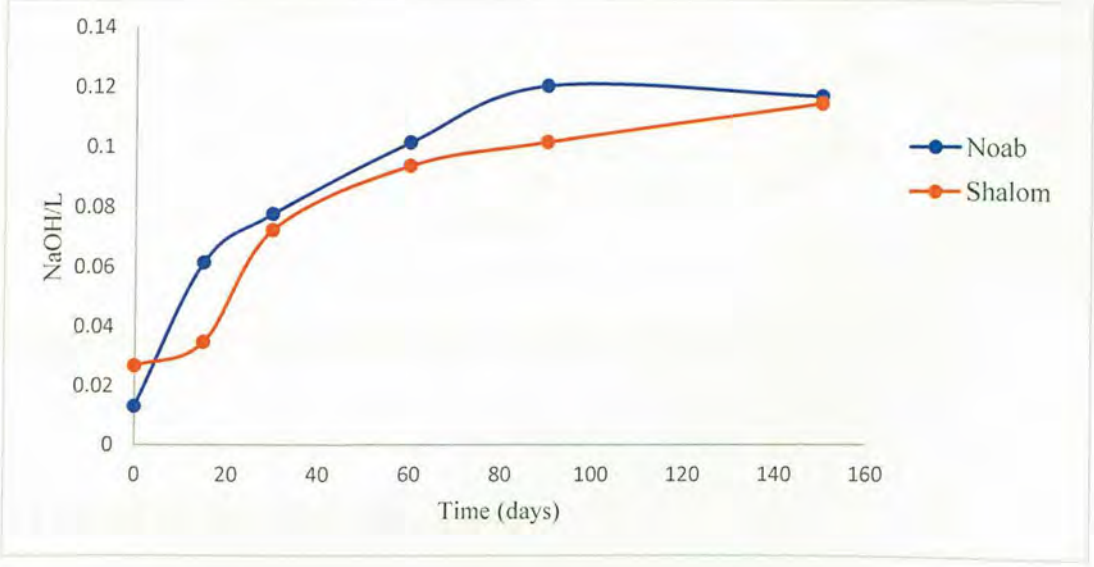

Figure 10. Combined Acidity of Noab and Shalom Table Olives

Figure 11 indicates that as the pH decreased and stabilised to 4-5 range, the combined acidity increased significantly for both Noab and Shalom olive samples. The correlation between pH and combined acidity was -7.774, which shows that there is a significant correlation between pH and combined acidity and the negative sign indicates in Table 5 (Appendix 1-Table 5). shows that as pH increases combined acidity decreases.

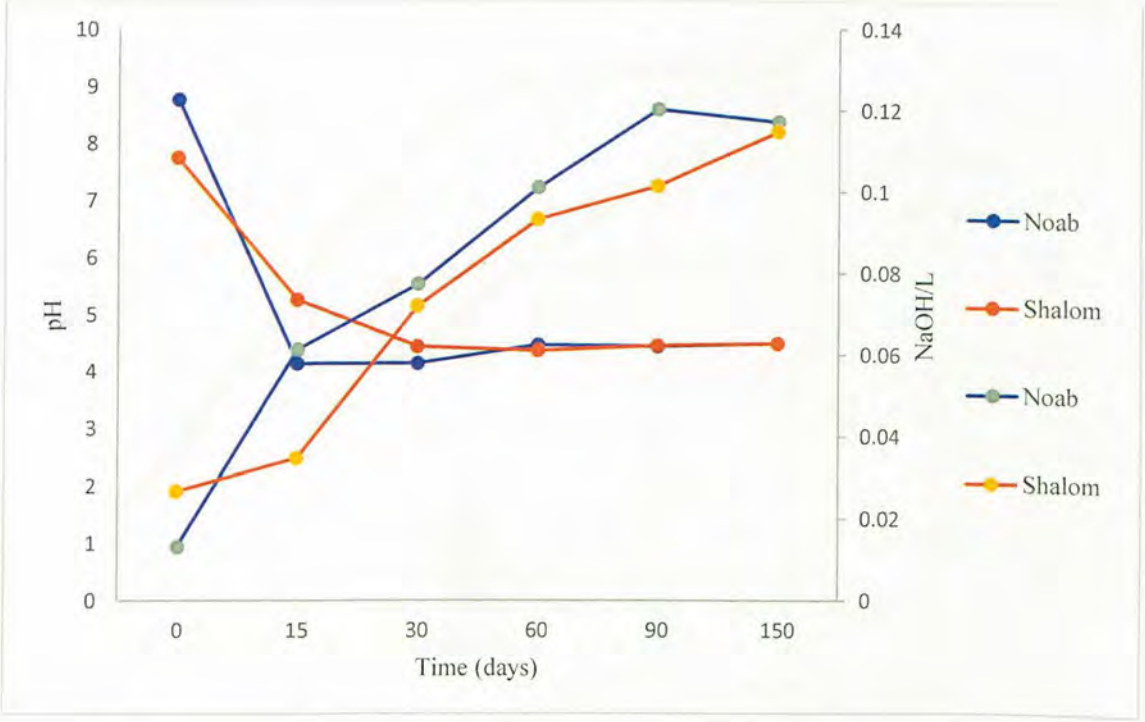

Figure 11. pH and Combined Acidity of Noab and Shalom Table Olives

4.2.3 Total Acidity or Titratable Acidity

The titratable acidity of Noab olive samples had a mean value of 1.38 ± 0.17 g/L with a minimum of 0.29 g/L and a maximum of 2.70 g/L. Shalom olive samples had a mean of 1.69 ± 0.22 g/L with a minimum of 0.40 g/L and a maximum of 2.85 g/L. Figure 12 shows that there is a slight increase in titratable acidity till the end of

fermentation days. An independent samples t-test was performed to compare the titratable acidity for Noab and Shalom olive samples (Appendix 1-Table 6). In Table 6, the outcome variable was found to be normally distributed and equal variance are assumed based upon results of Levene's test (F (34) = 3.965, P = 0.055). There was no significant difference (P> 0.05) in titratable acidity between Noab and Shalom olive samples.

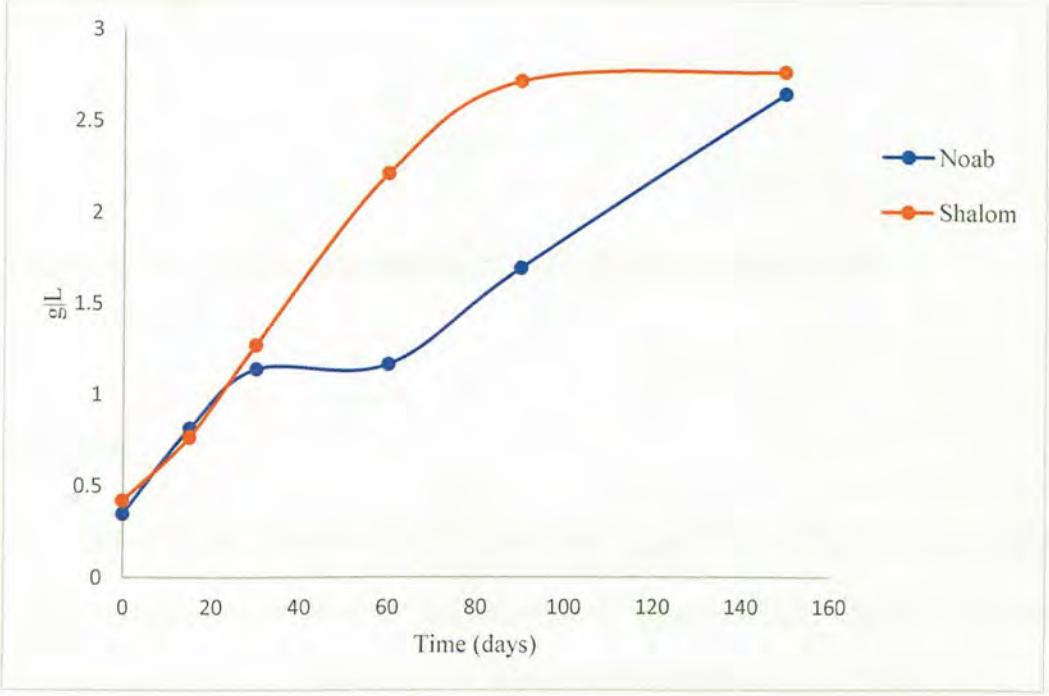

Figure 12. Titratable Acidity of Noab and Shalom Table Olives

The Pearson's Rank correlation showed in Table 7 (Appendix 1-Table 7) shows that there is a significant correlation between pH and combined acidity. The -0.774 showed that as the pH decrease the combined acidity increases.

The pH and titratable acidity are clearly displayed in Figure 13 and it shows that as pH reaches and stabilizes at 4-5, titratable acidity increased significantly for both Noab and Shalom olive samples.

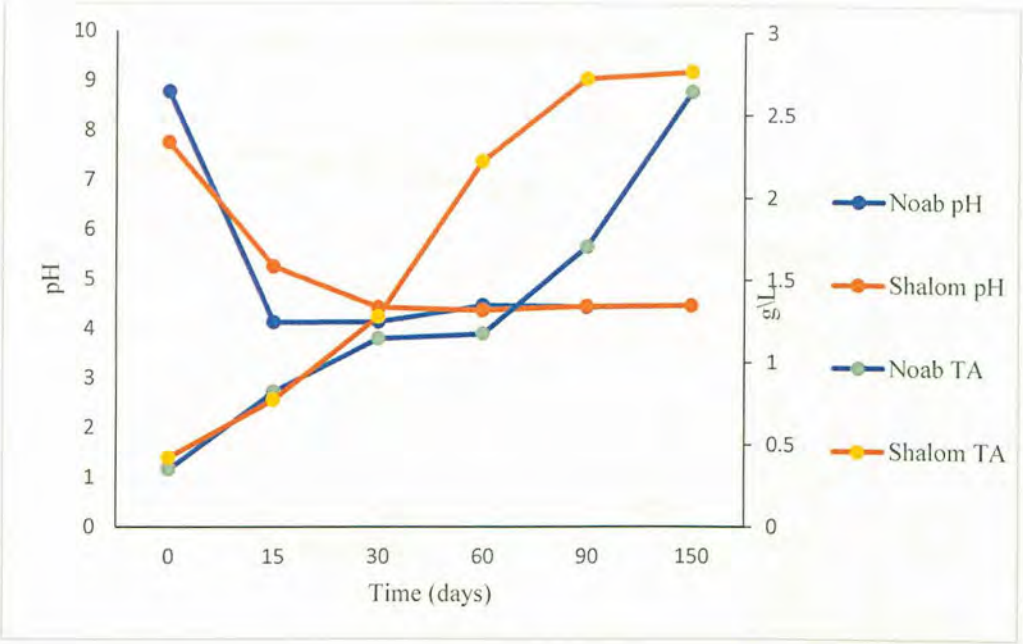

Figure 13. pH and Titratable Acidity of Noab and Shalom Table Olives

4.2.4 NaCl

The NaCl of Noab olive samples had a mean value of 9.57 ± 0.11 % NaCl with a minimum value of 8.82 % NaCl and a maximum value of 10.54 % NaCl. The NaCl of shalom olive samples had a mean of 9.24 ± 0.15 % NaCl with a minimum value of 8.24 % NaCl and a maximum value of 10.18 % NaCl. Figure 14 indicates that there is a slight decrease in NaCl during the fermentation period. An independent samples t-test was performed to compare the NaCl for Noab and Shalom olive samples. There was no significant difference ($P > 0.05$) in pH between Noab and Shalom olive samples (Appendix 1-Table 8).

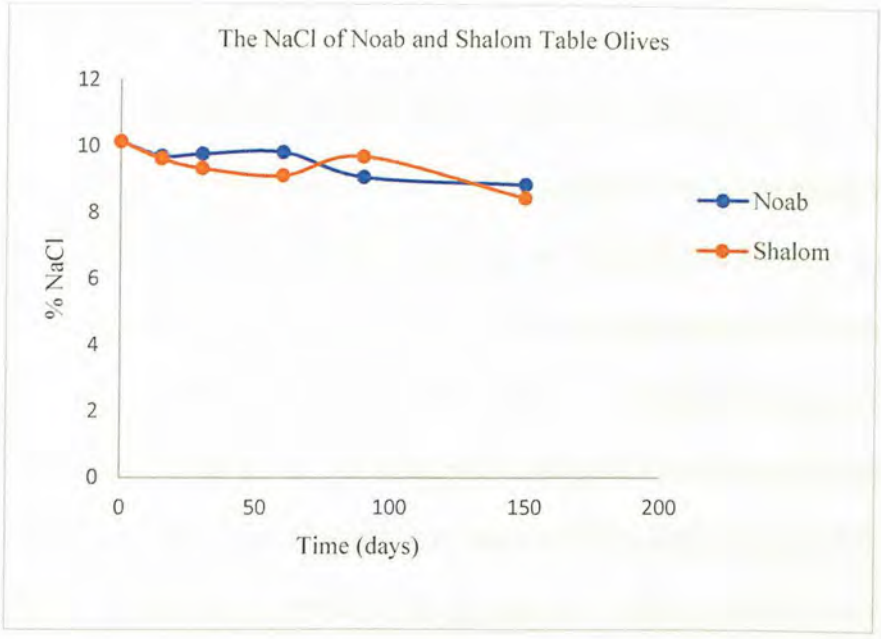

Figure 14. NaCl of Noab and Shalom Table Olives

In Table 9 (Appendix 1-Table 9), the correlation between pH and NaCl is 0.616 which shows that there is a significant correlation between pH and NaCl. The positive sign indicates as pH increases the NaCl increases. Figure 15 shows that both pH and NaCl decreased as the fermentation period increased.

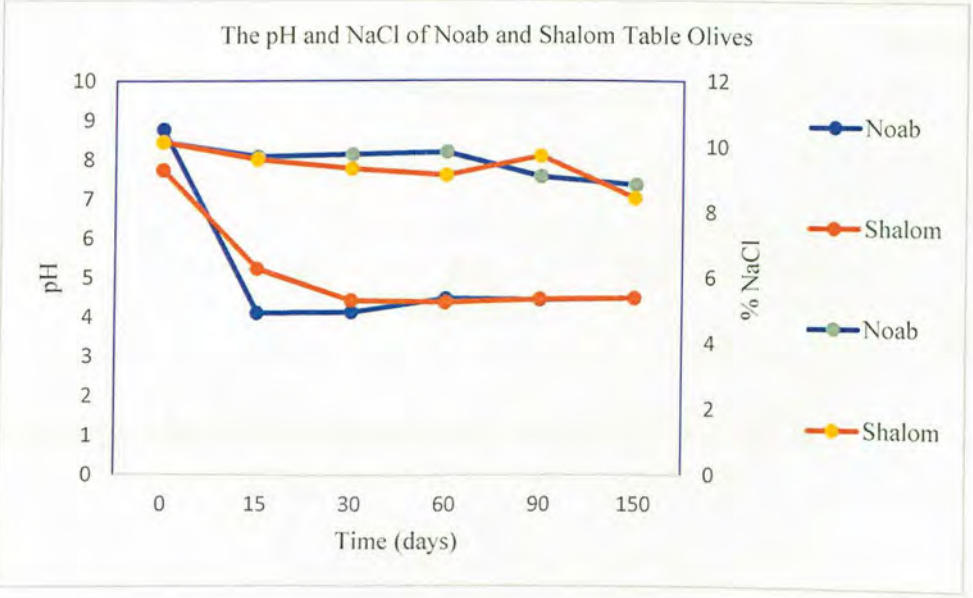

Figure 15. pH and NaCl of Noab and Shalom Table Olives

4.2.5 Total Phenol Content

The TPC of Noab olive samples had a mean value of $3.86 \pm 0.35 \times 10^{-4}$ g of GAE / mL with a minimum value of 2.32×10^{-4} g GAE/mL and a maximum value of 6.65×10^{-4} g GAE/ mL. The TPC of Shalom had a mean of $6.37 \pm 0.30 \times 10^{-4}$ g GAE/ mL with a minimum value of 4.34×10^{-4} g GAE/mL and a maximum value of 8.69×10^{-4} g GAE/mL. Figure 16 shows that there is a slight decrease in TPC as the fermentation days go on. An independent samples t-Test was performed to compare the pH for Noab and Shalom olive samples (Appendix 1-Table 10). There was a significant difference ($p < 0.001$) in pH between Noab and Shalom olive samples.

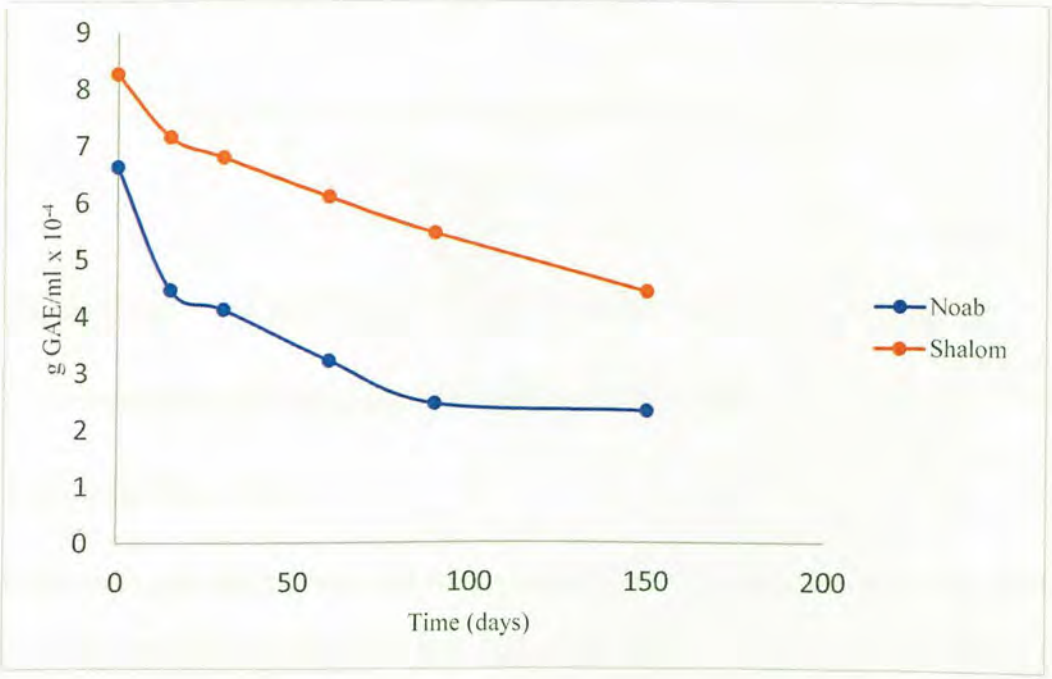

Figure 16. Total Phenol Content of Noab and Shalom Table Olives

The Pearson's r showed that there is a significant correlation between pH and TPC. In Table 11 (Appendix 1-Table 11), as the pH decreases, the TPC also increases.

In Figure 17, the pH and Total Phenol Content decreases over time.

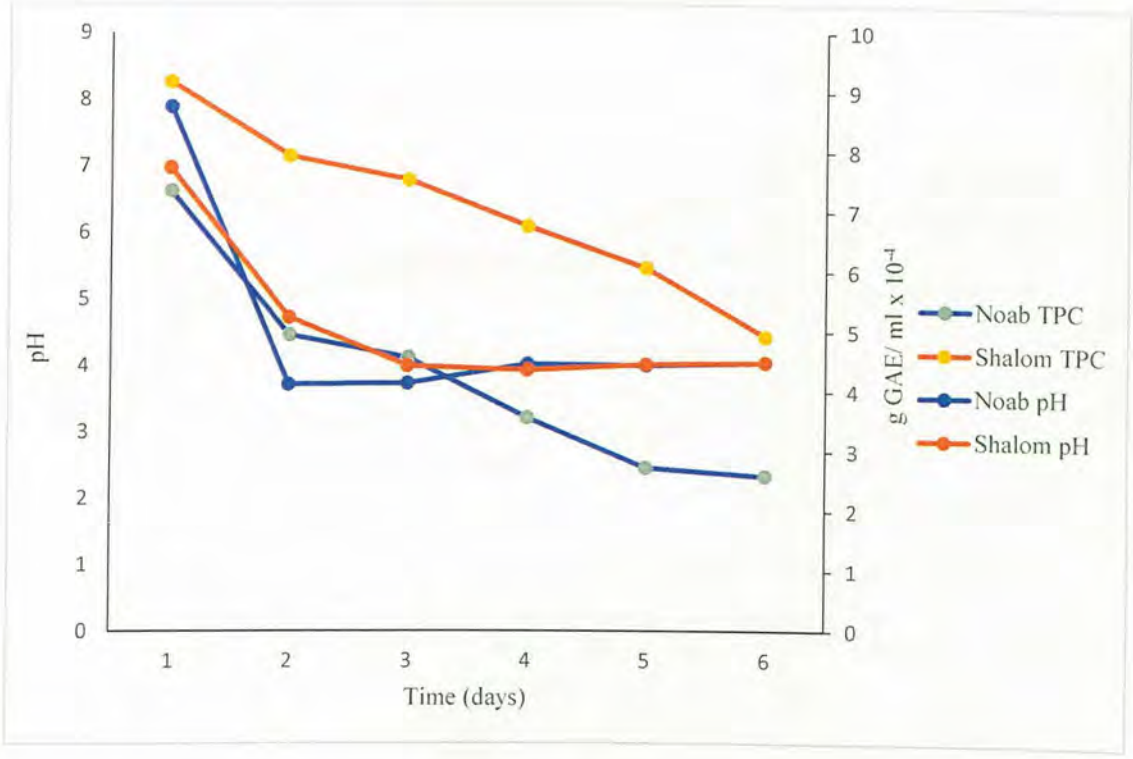

Figure 17. pH and Total Phenol Content of Noab and Shalom Table Olives

4.3 Enumeration of LAB, yeast and moulds of Table Olives

4.3.1 Total Plate Count

Plate count agar and nutrient agar were used to determine total plate count. The plate count agar had a mean of 4.82 ± 0.19 $\log_{10}$ CFU/ mL with a minimum of 2.57 $\log_{10}$ CFU/ mL and a maximum of 7.23 $\log_{10}$ CFU/ mL. Nutrient Agar had a mean of 4.57 ± 0.20 $\log_{10}$ CFU/ mL with a minimum of 2.46 $\log_{10}$ CFU/ mL and a maximum of 7.24 $\log_{10}$ CFU/ mL. Noab olive samples (Figure 18) had a mean of 4.64 ± 0.16 $\log_{10}$ CFU/ mL with a minimum of 2.46 $\log_{10}$ CFU/ mL and a maximum of 5.95 $\log_{10}$ CFU/ mL for total plate count. Shalom olives samples (Figure 18) had a mean of

4.7515 ± 0.43 $\log_{10}$ CFU/ mL with a minimum of 2.49 $\log_{10}$ CFU/ mL and a maximum of 7.24 $\log_{10}$ CFU/ mL for total plate count. An independent samples t-test was performed to compare the total plate count for Noab and Shalom olive samples (Appendix 1-Table 12).

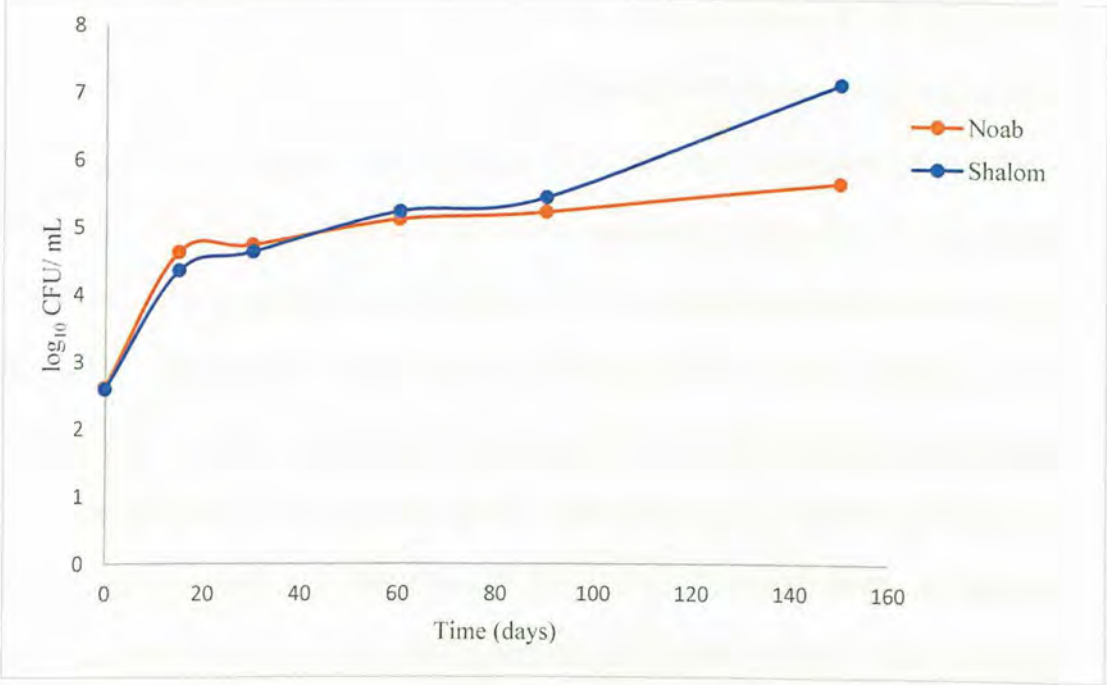

Figure 18. Total Plate Count of Noab and Shalom Table Olives

There was no significant difference (P> 0.05) in total plate count between Noab and Shalom olive samples. In Figure 18, an increase in $\log_{10}$ CFU/ mL can be observed for plate count agar of both Noab and Shalom can be observed.

An independent samples t-test was performed to compare the total plate count agars (nutrient agar and plate count agar) for Noab and Shalom olive samples (Appendix 1-Table 13). There was no significant difference (P> 0.05) in pH between Noab and Shalom olive samples.

4.3.2 Total LAB Count

For total LAB count, three different agars were used, respectively MRS, M17 and Rogosa, to estimate the total LAB count. MRS had a mean of 3.98 ± 0.25 $\log_{10}$ CFU/ mL with a minimum of 1.48 $\log_{10}$ CFU/ mL and a maximum of 6.63 $\log_{10}$ CFU/ mL M17 had a mean of 3.42 ± 0.22 $\log_{10}$ CFU/ mL with a minimum of 1.48 $\log_{10}$ CFU/ mL and a maximum of 5.35 $\log_{10}$ CFU/ mL. Rogosa had a mean of 3.85 ± 0.23 $\log_{10}$ CFU/ mL with a minimum of 1.56 $\log_{10}$ CFU/ mL and a maximum of 5.33 $\log_{10}$ CFU/ mL. For total LAB count, Noab olive samples had a mean of 3.83 ± 0.20 $\log_{10}$ CFU/ mL with a minimum of 1.48 $\log_{10}$ CFU/ mL and a maximum of 6.63 $\log_{10}$ CFU/ mL. Shalom had a mean of 3.67 ± 0.18 $\log_{10}$ CFU/ mL with a minimum of 1. $\log_{10}$ CFU/ mL and a maximum of 5.80 $\log_{10}$ CFU/ mL. An independent samples t-test was performed to compare the pH for Noab and Shalom (Appendix 1-Table 14). There was no significant difference (P> 0.05) in LAB between Noab and Shalom olive samples. Levene's Test was performed (Appendix 1-Table 15) for normally distribute and equal variance are assumed as shown in Table 15 (F (107) = 0.315, P = 0.730) for the total LAB agars (MRS, M17 and Rogosa). An ANOVA test was performed to compare the total LAB agars (MRS, M17 and Rogosa) (Appendix 1-Table 16). In Table 16, there was no significant difference (P> 0.05) between the LAB Agars (MRS, M17 and Rogosa).

From Figure 19, it becomes clear that the LAB count increased during the duration of the fermentation process and Shalom had the highest count at the end of fermentation period.

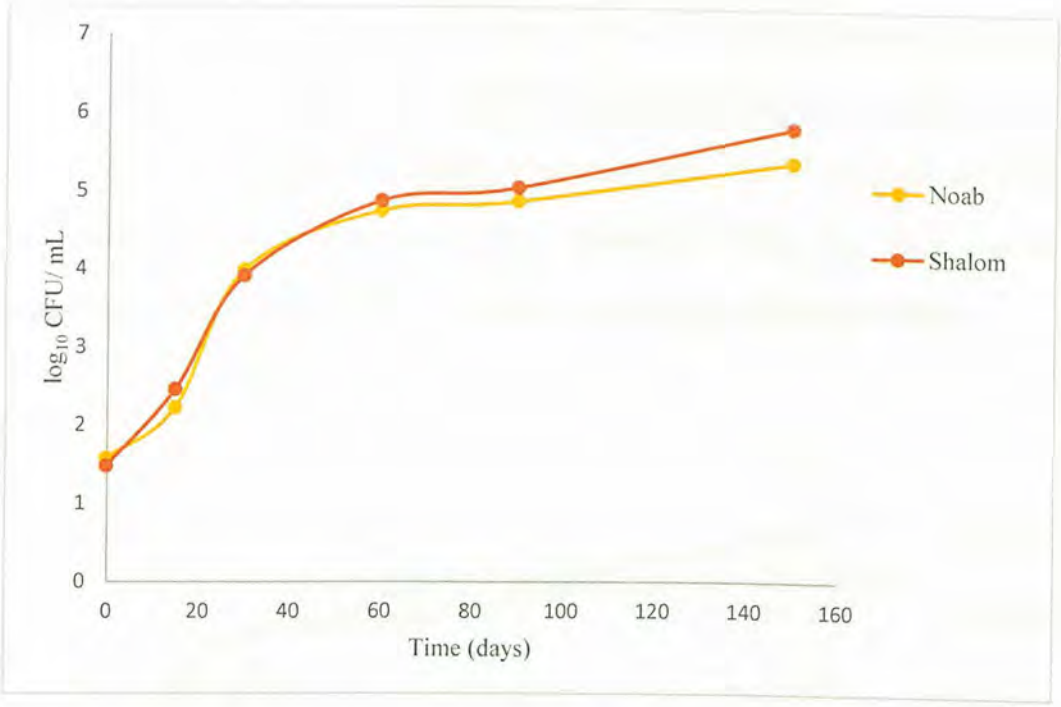

Figure 19. Total LAB Count of Noab and Shalom Table Olives

4.3.3 Total Yeast and Mould Count

Total yeast and mould were performed on two different agars, SDA and YEGC. SDA had a mean of 4.10 ± 0.23 $\log_{10}$ CFU/ mL with a minimum of 1.49 $\log_{10}$ CFU/ mL and a maximum of 6.08 $\log_{10}$ CFU/ mL. YEGC had a mean of 3.99 ± 0.25 $\log_{10}$ CFU/ mL with a minimum of 1.48 $\log_{10}$ CFU/ mL and a maximum of 5.57 $\log_{10}$ CFU/ mL. For Total Yeast and Mould, Noab had a mean of 4.14 ± 0.21 $\log_{10}$ CFU/ mL with a minimum of 1.48 $\log_{10}$ CFU/ mL and a maximum of 5.24 $\log_{10}$ CFU/ mL. Shalom had a mean of 3.96 ± 0.27 $\log_{10}$ CFU/ mL with a minimum of 1.48 $\log_{10}$ CFU/ mL and a maximum of 6.08 $\log_{10}$ CFU/ mL.

An independent samples t-test was performed to compare the total yeast and mould count between Noab and Shalom olive samples (Appendix 1-Table 17). There was no significant difference ($P > 0.05$) in total yeast and mould count between Noab and Shalom olive samples.

From Figure 20, it is clear that the yeast and mould count increased during the duration of the fermentation process and Shalom had the highest count at the end of fermentation. An independent samples t-test was performed to compare the total yeast and mould agars (SDA and YEGC) (Appendix 1-Table 18). There was no significant difference ($P > 0.05$) total yeast and mould agars (SDA and YEGC).

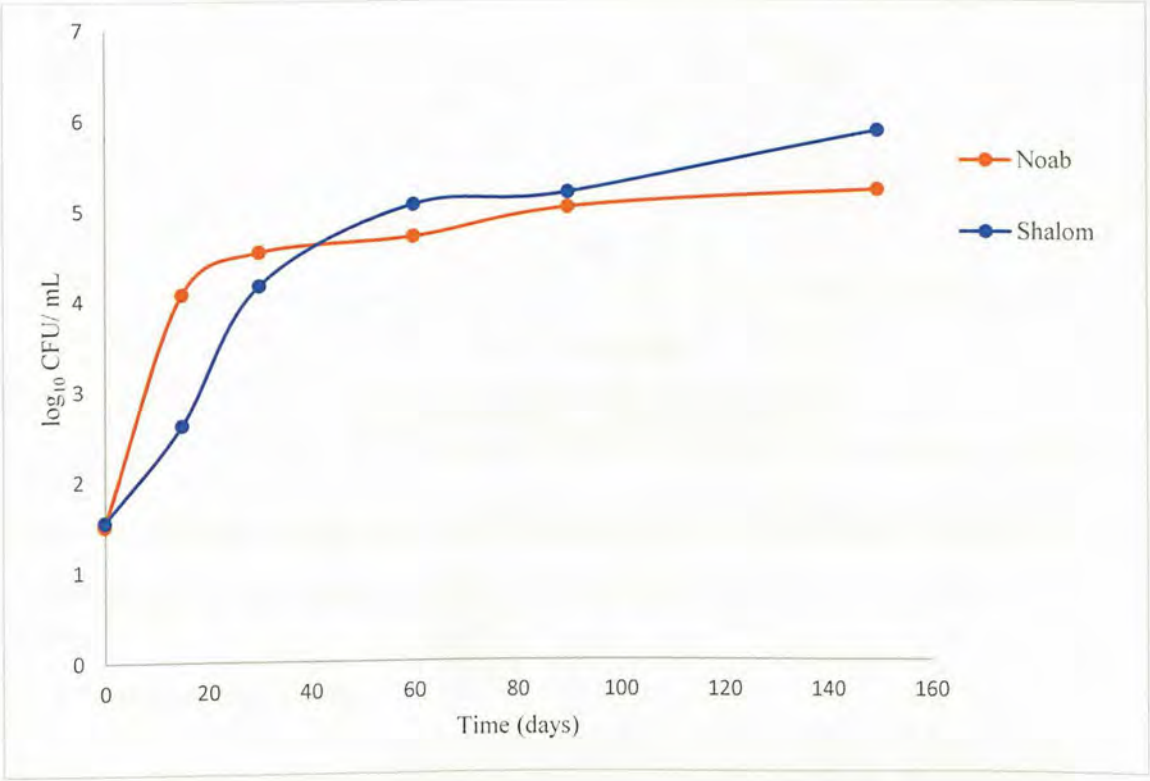

Figure 20. Total Yeast and Mould Count for Noab and Shalom Table Olives

As seen from Figure 21, there was a difference between the Days 0 and Day 15, while Day 60, 90 and 150 show similarity and the pH is inversely to the plate counts.

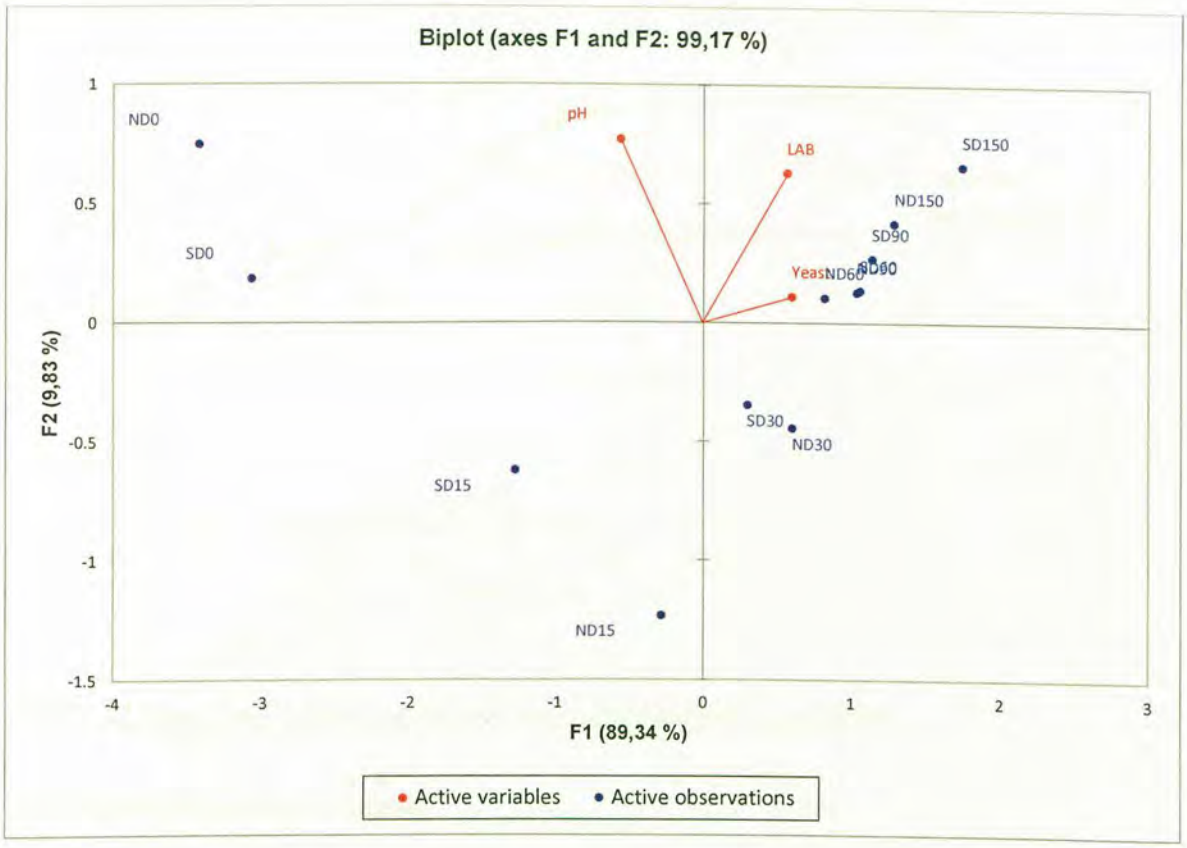

Figure 21. Principal Component Analysis of the LAB, Yeast and mould and pH of Noab and Shalom Table Olives

4.3.4 Total Coliform Count

The total coliform count was determined by using MacConkey Agar. Noab had a mean value of 2.23 ± 0.13 $\log_{10}$ CFU/ mL with a minimum value 1.56 $\log_{10}$ CFU/ mL and a maximum value of 3.15 $\log_{10}$ CFU/ mL. Shalom had a mean of 1.55 ± 0.17 $\log_{10}$ CFU/ mL with a minimum value of 0 $\log_{10}$ CFU/ mL and a maximum value of 3.15 $\log_{10}$ CFU/ mL. Figure 22 shows that total coliform count increases as days go on. An independent samples t-test was performed to compare the total coliform count for Noab and Shalom olive samples (Appendix 1-Table 19). There was a significant difference ($P< 0.005$) in total coliform count between Noab and Shalom olive samples.

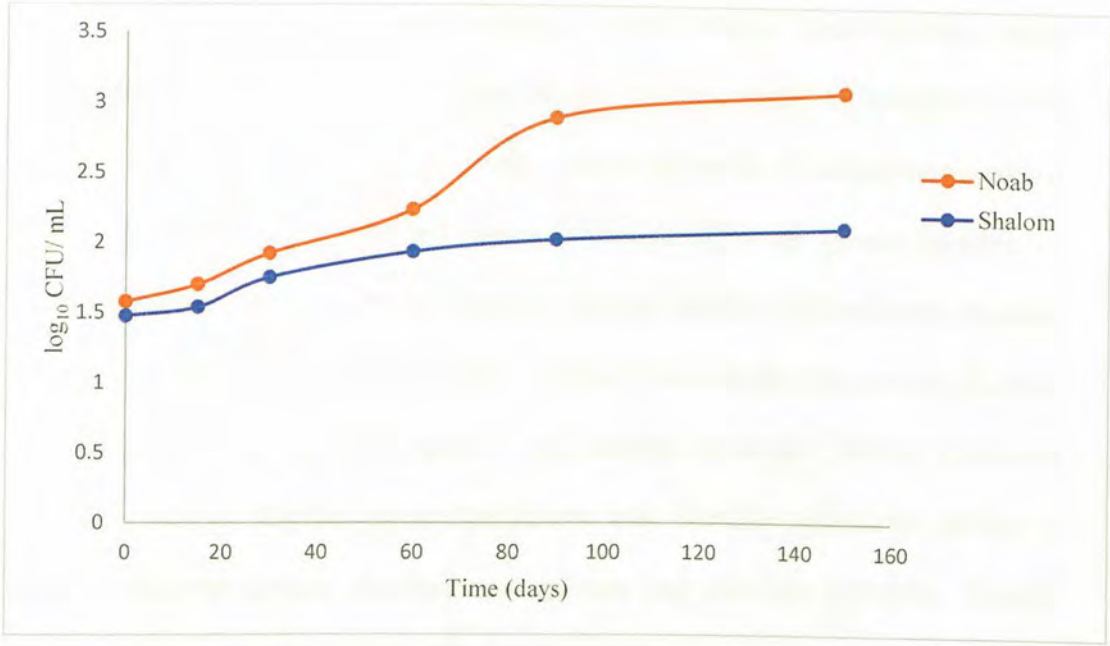

Figure 22. The Total Coliform Count of Noab and Shalom Table Olives

4.4. Culture Dependant Method

Isolates 3-5 had 1.5 kbp and 1 and 2 had After DNA isolation the 16S rDNA region was amplified by PCR protocol. The molecular weight of amplified products was around 50 bp.

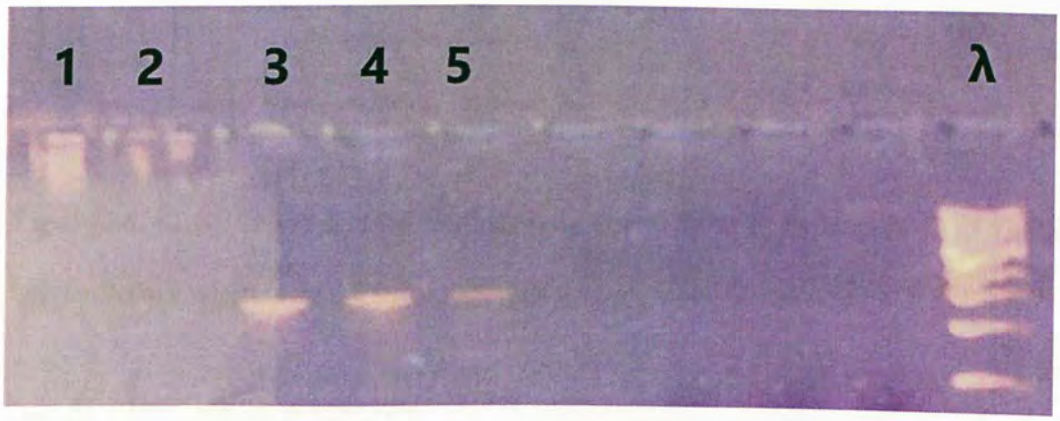

Figure 23. Amplified products and DNA ladder of table olives (amplification products 1-5 and DNA ladder λ)

After cleaning up the isolates received from Inqaba South Africa using MEGABLAST only sequences derived from 5 isolates could be searched in the NCBI Nucleotide BLAST. Isolate 1 was closely related to *B. licheniformis* with a 99% similarity. All the searched sequenced, came from the genus *Bacillus*. In particular a 99% identification for isolate 1 showed *Bacillus licheniformis*, *Bacillus haynesii* and *Bacillus paralicheniformis*. Isolate 2 showed *Bacillus cereus*, *Bacillus nitratireducens*, *Bacillus thuringiensis* and *Bacillus mycoides*. Isolate 3 showed *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus velezensis*. Isolate 4 showed *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides*. Finally, isolate 5 showed *Bacillus cereus*, *Bacillus velezensis* and *Bacillus thuringiensis* to be species closest related to the sequence after the BLAST search. The identification at the species level showed that on the genus level *Bacillus* was the predominant species in the brine of table olives from both sites.

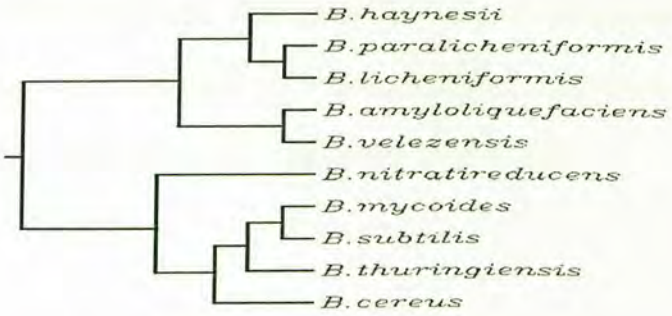

Figure 24. CLUSTALW Rooted Phylogenetic tree of 99% ID isolates (1-3) of Table Olives from Shalom farm.

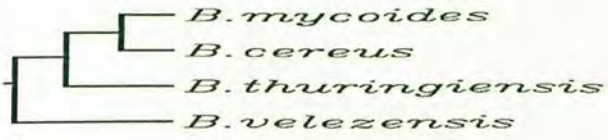

Figure 25. CLUSTALW Rooted Phylogenetic tree of 99% ID isolates (4-5) of Table Olives from Noab farm

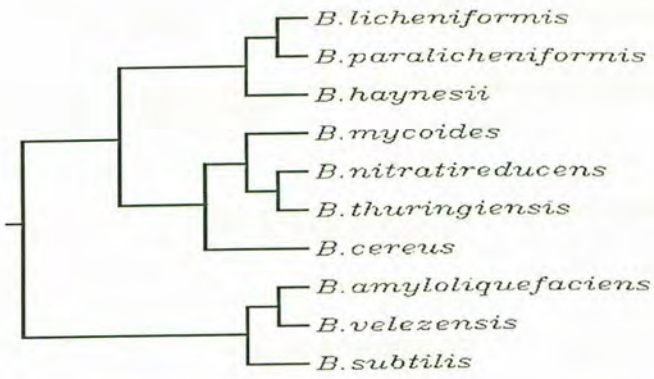

Figure 26. CLUSTALW Rooted Phylogenetic Tree (UPGMA) of 99% ID isolates of Table Olives for both sites

4.5. Microbial Community Associated with Fermented Olives as Determined by Metagenomic Analysis

Fresh tissue samples (Table 20) from various table olive was sent to the CD Genomics (USA, New York) for bacterial metagenomic analysis. Site comparison between Noab table olive farm and Shalom table olive farm was carried out by CD Genomics using 16S rRNA, 18S rRNA and ITS.

Table 20. Sample and Sequencing information for Noab and Shalom Table Olives

Sample name in data analysis	Sample	Comparison	Sequencing Zone
SHA01	Shalom Day 150 Mission	Group 1	ITS
NOA02	Noab Day 150 Mixed	Group 2	ITS
SHA03	Shalom Black Mission 2017	Group 1	ITS
SHA04	Shalom Spanish Queen Mission Green 2017	Group 1	ITS
NOA05	Noab Manzanilla 2017	Group 2	ITS
SHA06	Shalom Day 150 Mission	Group 1	16S rRNA
NOA07	Noab Day 150 Mixed	Group 2	16S rRNA
SHA08	Shalom Black Mission 2017	Group 1	16S rRNA
SHA09	Shalom Spanish Queen Mission Green 2017	Group 1	16S rRNA
NOA10	Noab Manzanilla 2017	Group 2	16S rRNA
NOA11	Noab Mission 2017	Group 2	ITS
NOA12	Noab Manzanilla 2016 Feb	Group 2	ITS
NOA15	Noab Mission 2017	Group 2	16S rRNA
NOA16	Noab Manzanilla 2016 Feb	Group 2	16S rRNA
SHA18	Shalom Day 150 Mission	Group 1	18S rRNA
NOA19	Noab Day 150 Mixed	Group 2	18S rRNA

For ITS, SHA04 had the most PE reads (the PE reads obtained from the sequence platform) of 80273, but NOA11 had the highest clean tag percentage (83.97%) (Table 25). Overall, all samples had PE reads above 70000 and clean percentage above 70%. For 16S rRNA the species of SHA09 was the most dominant (14362) and the species of NOA16 the least dominant (640) (Table 22). For 18S rRNA, the species of SHA18 was the most dominant (21144) and the species of NOA19 the least dominant (9627) (Table 24). For ITS, the species of SHA03 was the most dominant (65044) and the species of NOA02 the least dominant (1801) (Table 26).

For 16S rRNA, NOA10 had the most PE reads (80098), but SHA08 had the highest clean tag percentage (74.85%) (Table 21). For 18S rRNA, NOA19 had the most PE reads (79970), but SHA18 had the highest clean tag percentage (87.45%) (Table 23). Overall, Shalom table olives had more dominant species in all samples compared to Noab table olive samples.

Table 22. Number of annotated tags in each classification level of each sample for 16S rRNA of Noab and Shalom Table Olives

Sample	Clean Tags	Avg Len(bp)	Kingdom	Phylum	Class	Order	Family	Genus	Species
SHA06	58230	417	52839	52616	52446	51998	36761	22229	7505
SHA08	58639	413	57420	57410	57390	57342	28841	4038	1568
SHA09	59833	418	56415	56412	56405	56383	52442	34560	14362
NOA07	58524	428	54728	54672	54640	54522	25016	12271	3903
NOA10	59739	412	55010	55000	54984	54939	20876	10073	2960
NOA15	59400	417	55806	55701	55667	55222	31141	24809	15936
NOA16	57633	411	54285	54254	54229	54117	10248	6281	640

Table 23. Data statistics of quality control for 18S rRNA of Noab and Shalom Table Olives

Sample	PE	Raw	Clean	AvgLen(bp)	GC(%)	Q20(%)	Q30(%)	Clean(%)
SHA18	79247	74460	69300	371	46.6	97.08	94.12	87.45
NOA19	79970	74848	69727	372	47.6	97.09	94.12	87.19

PE Reads: the PE reads obtained from the sequence platform. Raw Tags: the merged tags. Clean Tags: the tags after QC. Effective Tags: the tags without the chimeric sequences and can be used by the subsequent analysis. AvgLen: the average length of the Effective Tags. GC (%): the percentage of G and C. Q20%&Q30%: the percentage of bases with a quality score equal to or higher than 20(error rate <1%) and 30(error rate <0.1%). Clean (%): the number of CleanTags take up of the number of Raw PE.

Table 24. The numbers of annotated tags in each classification level of each sample for 18S rRNA of Noab and Shalom Table Olives

Sample	Kingdom	Phylum	Class	Order	Family	Genus	Species
NOA19	67135	10780	10780	10486	10486	9658	9627
SHA18	67761	22485	22485	22481	22481	22470	21144

Table 26. The numbers of annotated tags in each classification level of each sample for ITS

Sample	Clean	AvgLen (bp)	Kingdom	Phylum	Class	Order	Family	Genus	Species
SHA01	65623	287	64797	42942	39445	39285	39151	38720	36109
SHA03	60961	398	66520	65633	65490	65408	65324	65273	65044
SHA04	67002	267	62619	45320	43543	43143	42313	40978	38315
NOA02	63975	248	59863	2194	2020	1990	1969	1897	1801
NOA05	64787	284	63969	38908	36702	36688	36674	36629	36580
NOA11	67125	261	66912	65172	64731	64685	64623	64557	64054
NOA12	63656	264	63200	59340	58036	57119	56749	56484	54768

The phylum for 16S rRNA (Figure 27) represents the OTUs of both sites using the 16S rRNA sequencing to determine the relative abundance of each sample.

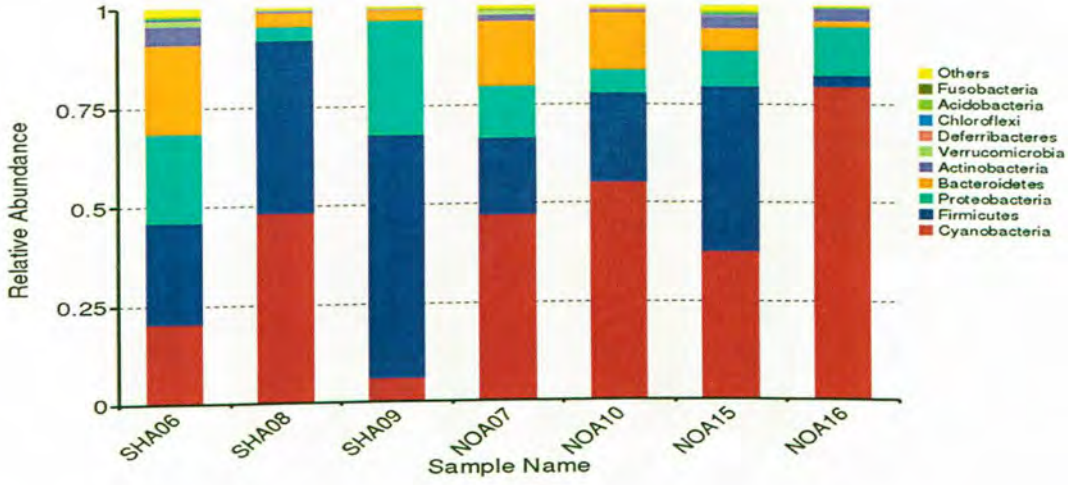

Figure 27. Relative abundance of phylum for Noab and Shalom table olives as determined by 16S rRNA metagenomic analysis.

The class for 16S rRNA (Figure 28) represents the OTUs of both sites using the 16S rRNA sequencing to determine the relative abundance of each sample.

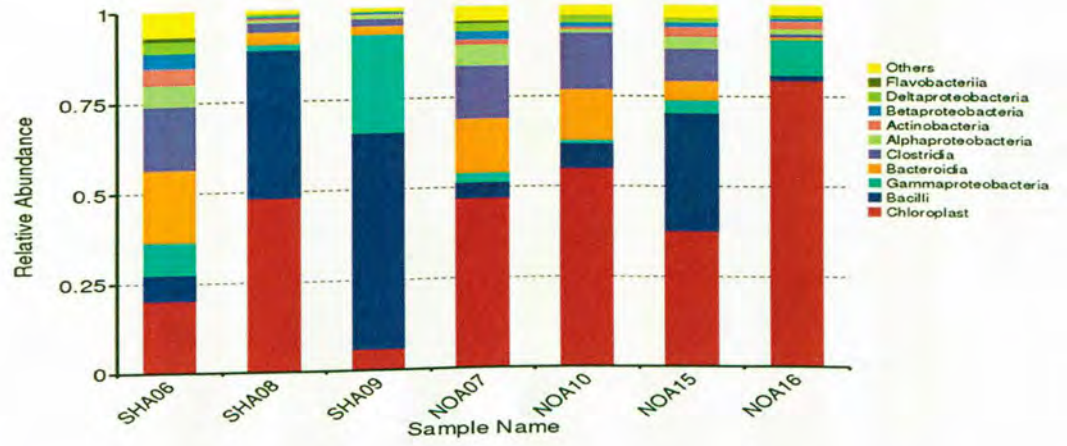

Figure 28. Relative abundance for classes for Noab and Shalom table olives as determined by 16S rRNA metagenomic analysis.

The order for 16S rRNA (Figure 29) represents the OTUs of both sites using the 16S rRNA sequencing to determine the relative abundance of each sample.

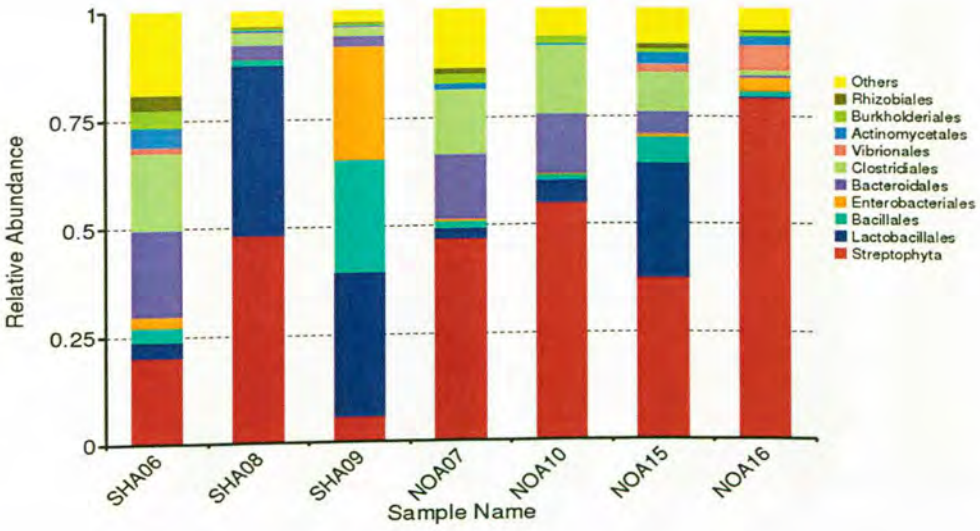

Figure 29. Order for 16S rRNA for Noab and Shalom Table Olives

The family for 16S rRNA (Figure 30) represents the OTUs of both sites using the 16S rRNA sequencing to determine the relative abundance of each sample.

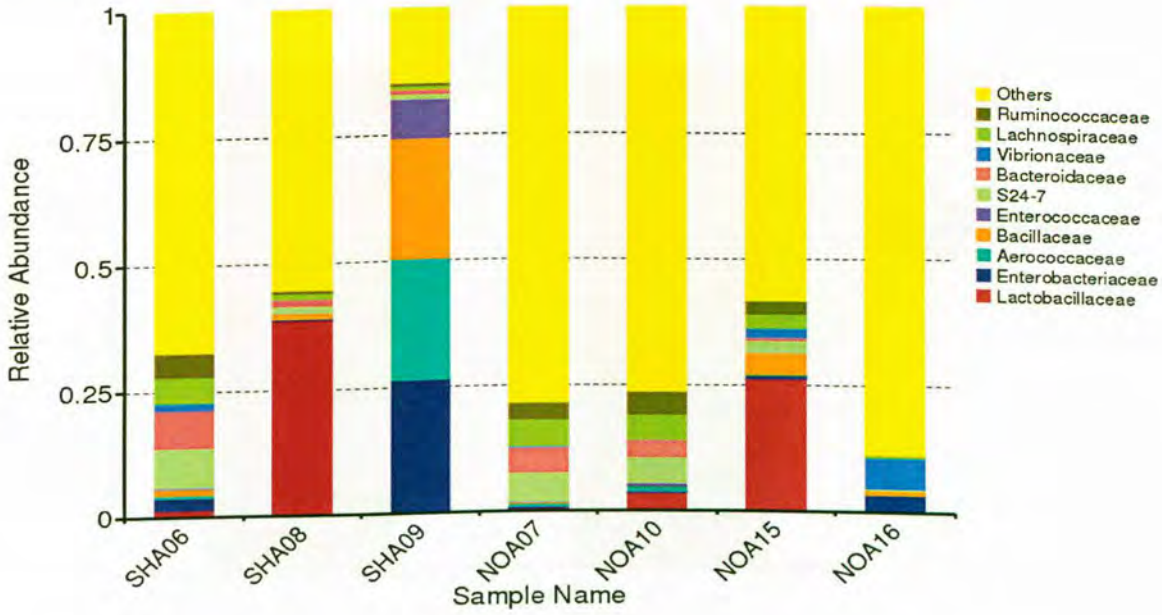

Figure 30. Family for 16S rRNA for Noab and Shalom Table Olives

The genus for 16S rRNA (Figure 31) represents the OTUs of both sites using the 16S rRNA sequencing to determine the relative abundance of each sample.

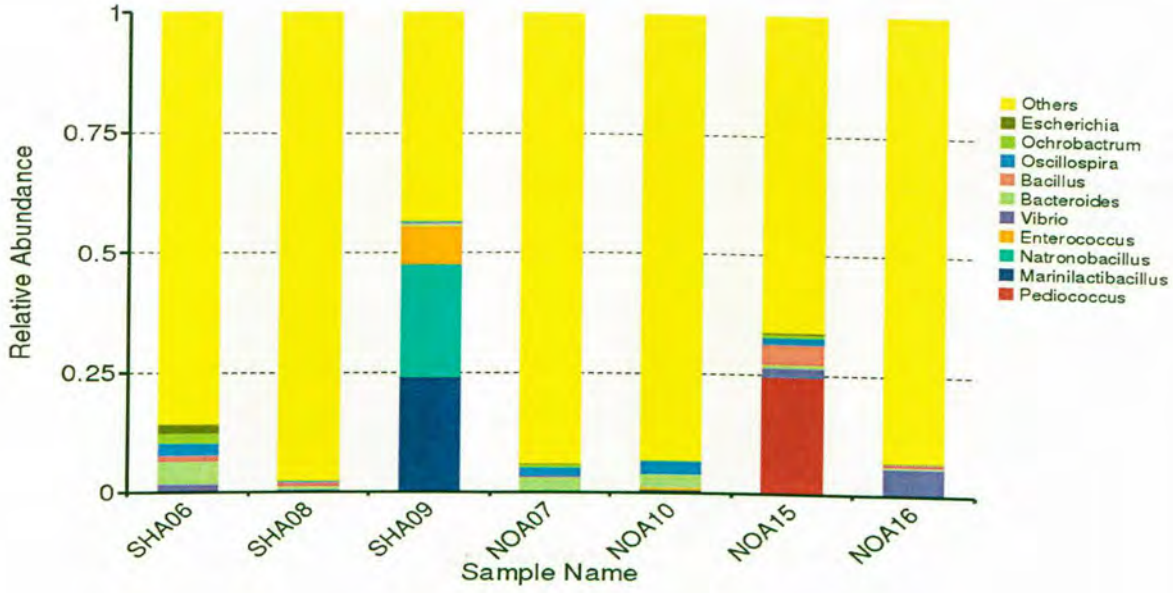

Figure 31. Genus for 16S rRNA for Noab and Shalom Table Olives

The phylum for 18S rRNA (Figure 32) represents the OTUs of both sites using the 18S rRNA sequencing to determine the relative abundance of each sample.

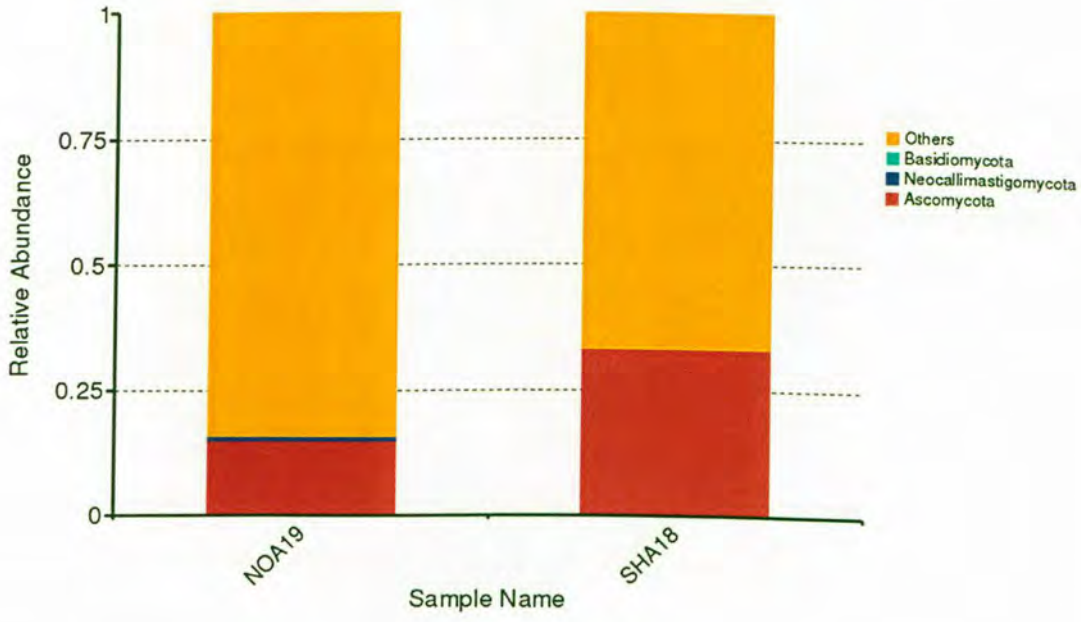

Figure 32. Phylum for 18S rRNA for Noab and Shalom Table Olives

The class for 18S rRNA (Figure 33) represents the OTUs of both sites using the 18S rRNA sequencing to determine the relative abundance of each sample.

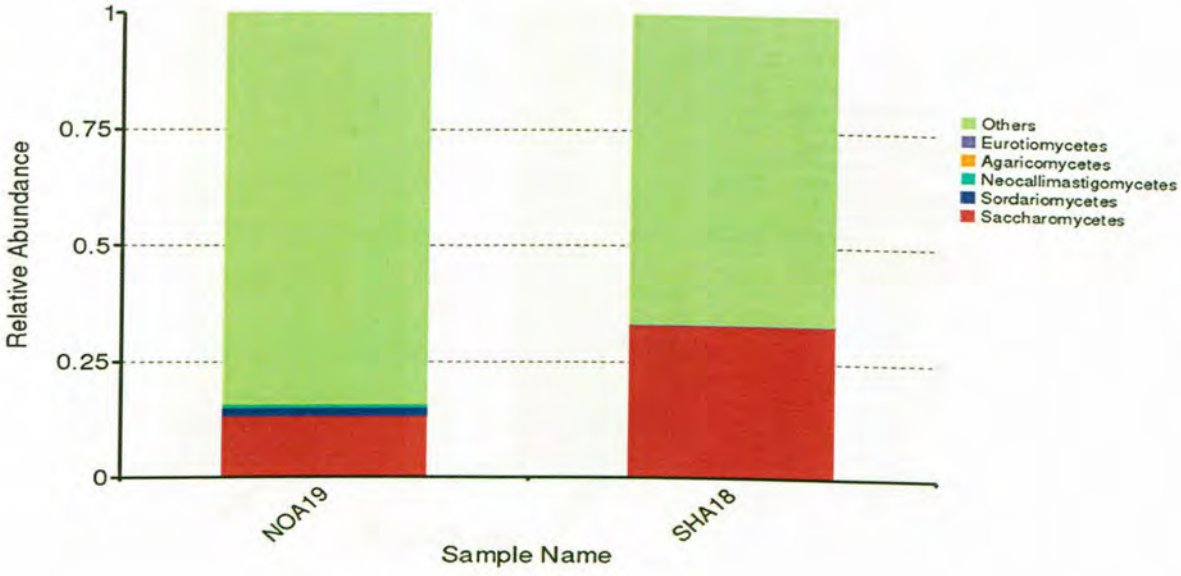

Figure 33. Class for 18S rRNA for Noab and Shalom Table Olives

The order for 18S rRNA (Figure 34) represents the OTUs of both sites using the 18S rRNA sequencing to determine the relative abundance of each sample.

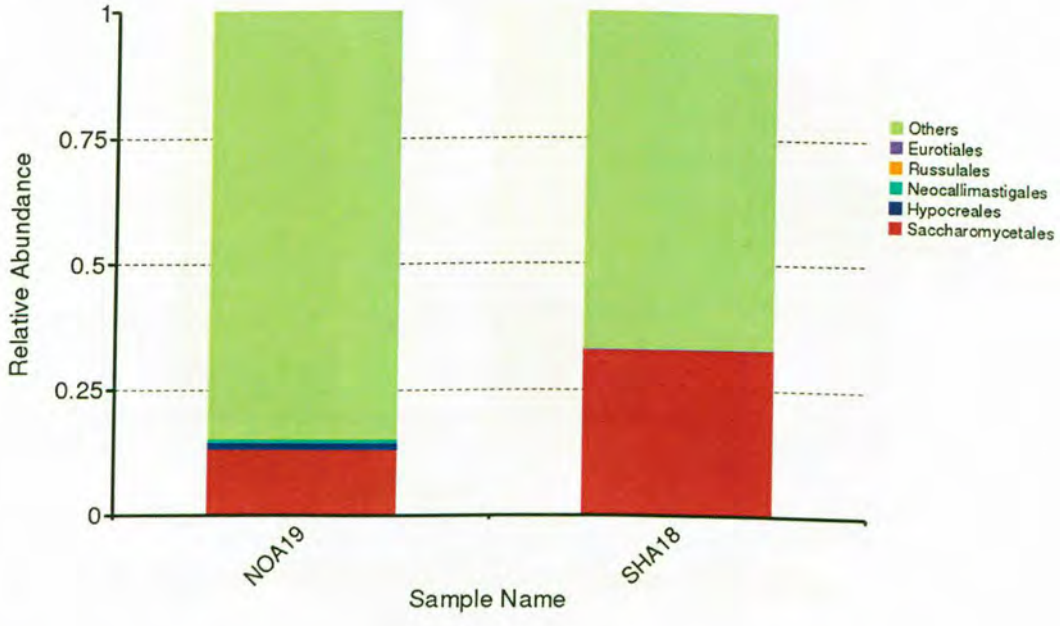

Figure 34. Order for 18S rRNA for Noab and Shalom Table Olives

The family for 18S rRNA (Figure 35) represents the OTU of both sites using the 18S rRNA sequencing to determine the relative abundance of each sample.

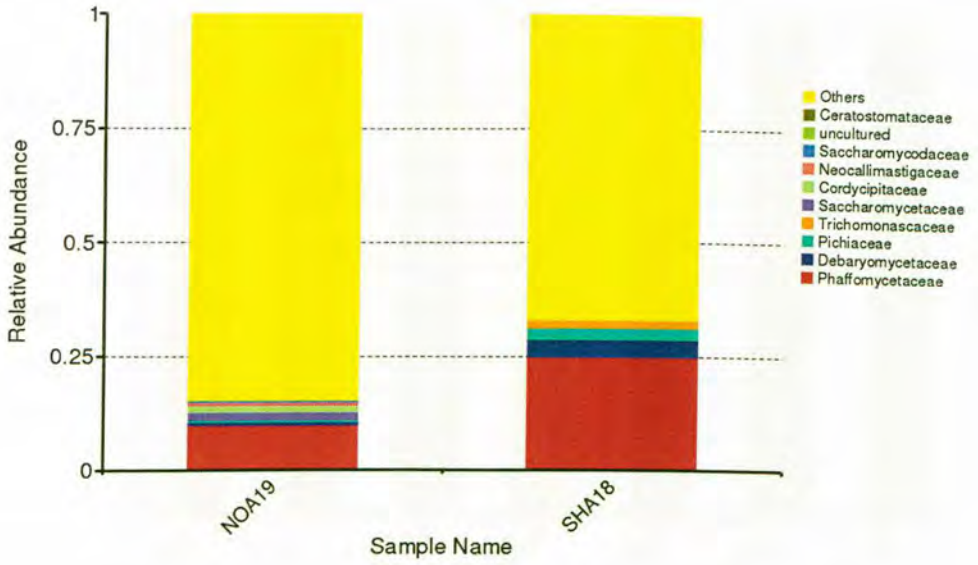

Figure 35. Family for 18S rRNA for Noab and Shalom Table Olives

The genus for 18S rRNA (Figure 36) represents the OTU of both sites using the 18S rRNA sequencing to determine the relative abundance of each sample.

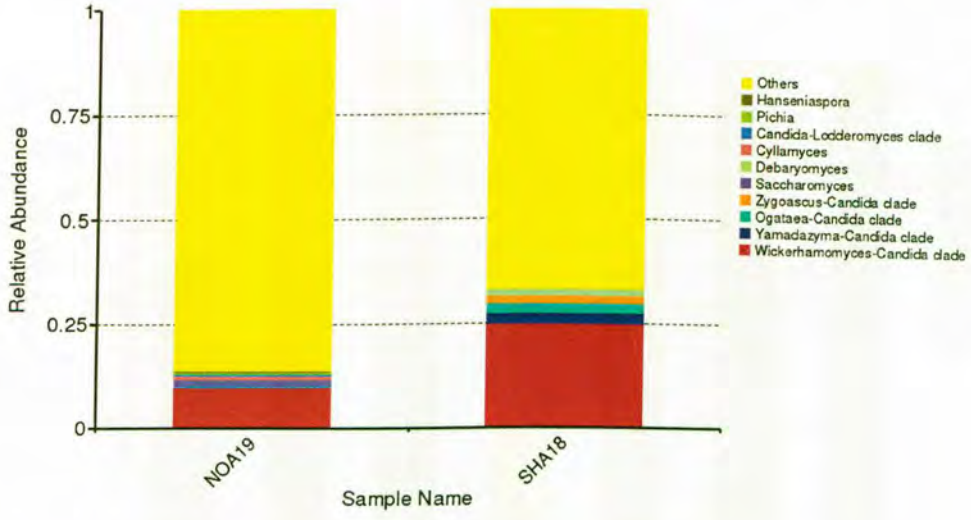

Figure 36. Genus for 18S rRNA for Noab and Shalom Table Olives

The phylum for ITS (Figure 37) represents the OTU of both sites using the ITS sequencing to determine the relative abundance of each sample.

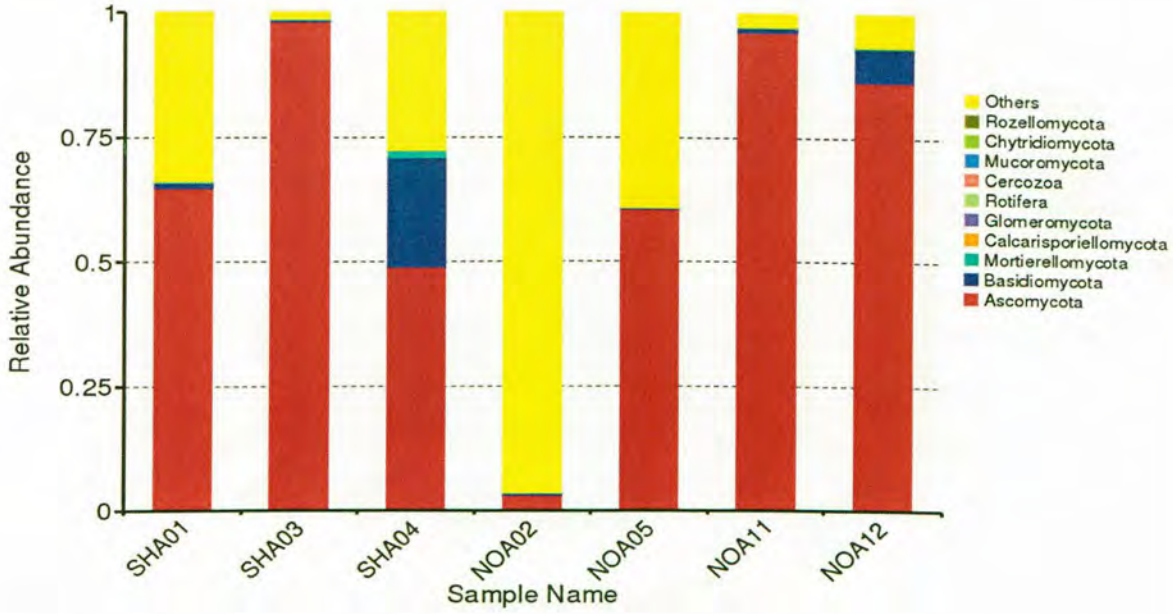

Figure 37. Phylum for ITS for Noab and Shalom Table Olives

The class for ITS (Figure 38) represents the OTU of both sites using the ITS sequencing to determine the relative abundance of each sample.

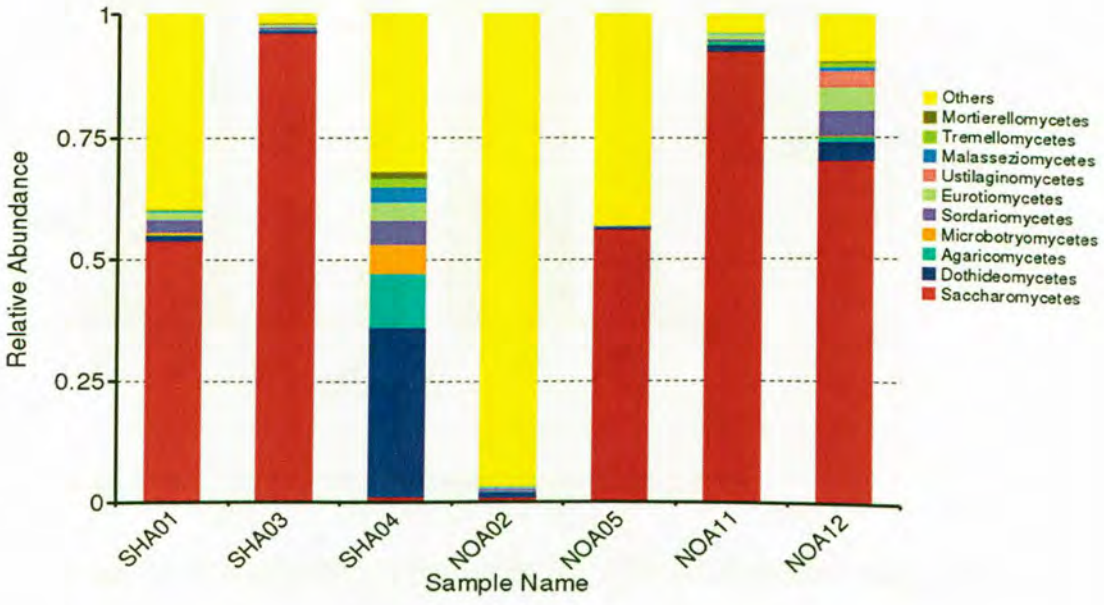

Figure 38. Class for ITS for Noab and Shalom Table Olives

The order for ITS (Figure 39) represents the OTU of both sites using the ITS sequencing to determine the relative abundance of each sample.

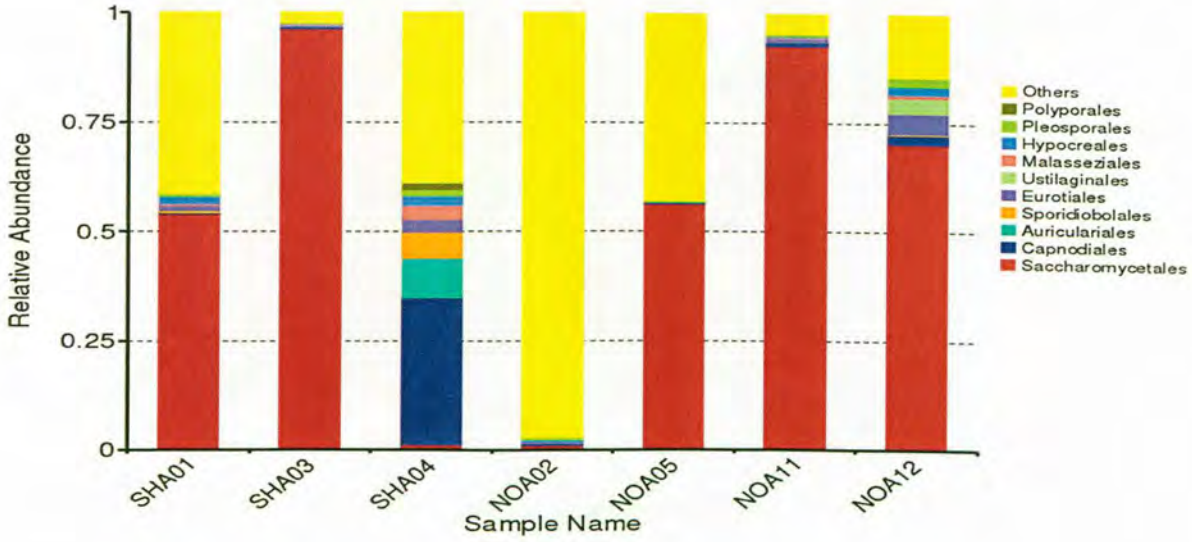

Figure 39. Order for ITS for Noab and Shalom Table Olives

The family for ITS (Figure 40) represents the OTU of both sites using the ITS sequencing to determine the relative abundance of each sample.

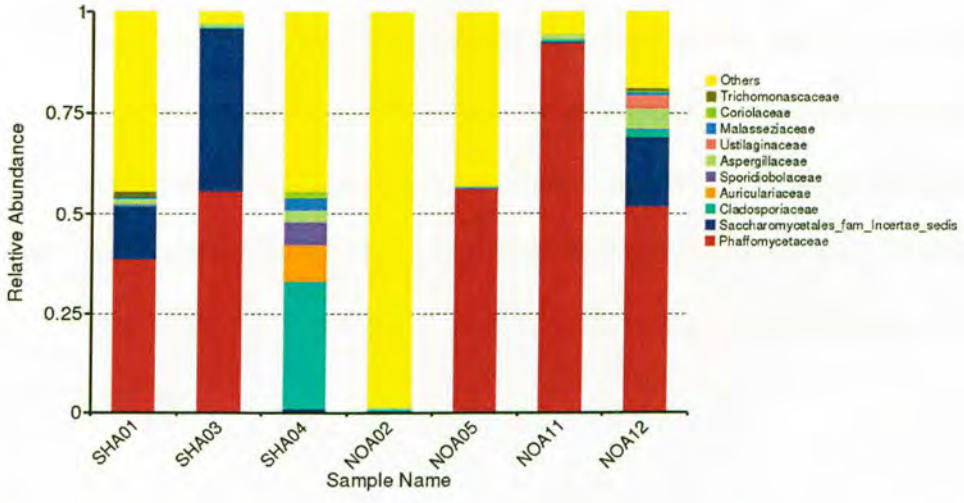

Figure 40. Family for ITS for Noab and Shalom Table Olives

The genus for ITS (Figure 41) represents the OTU of both sites using the ITS sequencing to determine the relative abundance of each sample.

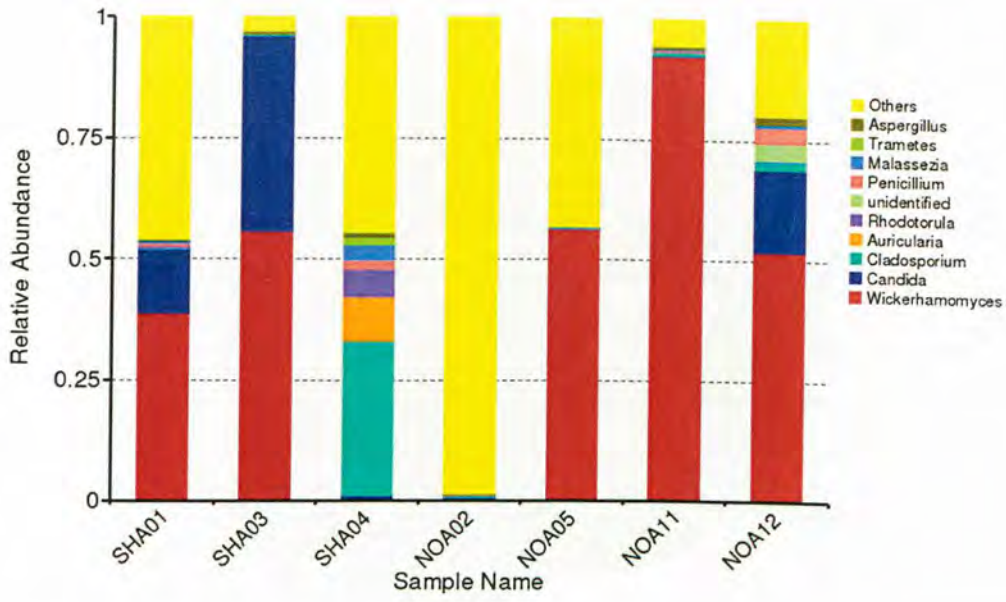

Figure 41. Genus for ITS for Noab and Shalom Table Olives

The Venn chart, the number of OTUs that commonly or uniquely owned between different samples (number 2 to 5) can be visually displayed (Chen & Boutros 2011). Combined with the species represented by OTU, the commonly owned microorganism can be defined in different environmental samples. A representative OTU-Venn graphs are represented by different colors. The number of overlapping numbers between different color patterns is the number of OTUs shared between the two samples, while the number in non-overlapping part is the number of unique OTU that owned by the related sample. The Venn diagrams for 16S rRNA, 18S rRNA and ITS for the group 1 and group 2 table olive samples are shown in figures 42, 43 and 44 respectively.

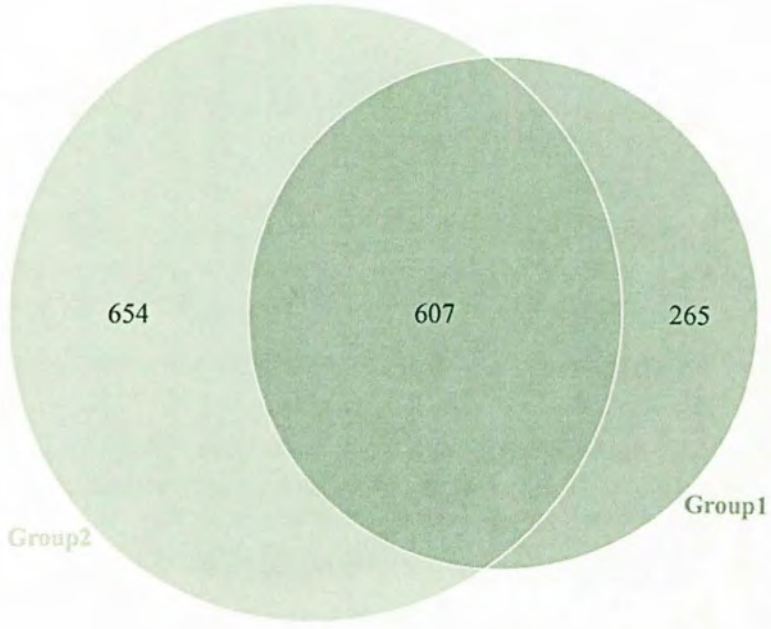

Figure 42. Venn Diagram for 16S rRNA for Noab (Group 2) and Shalom (Group 1) Table Olives

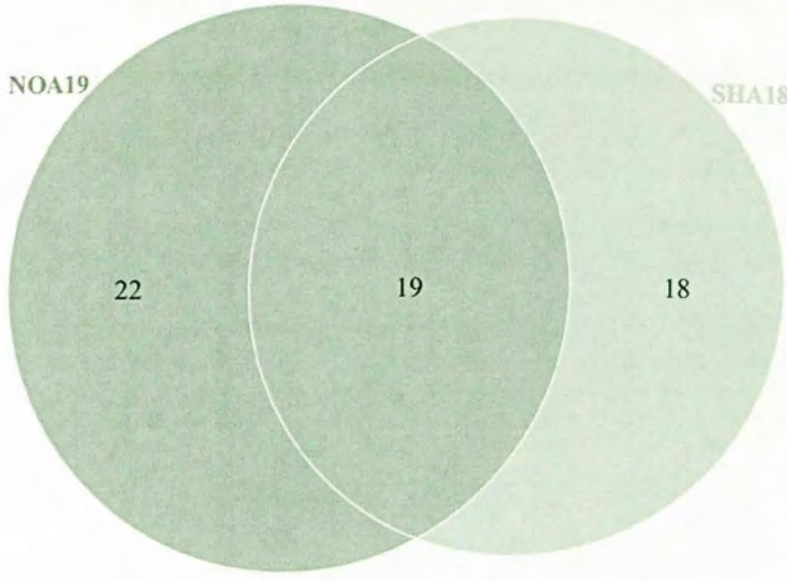

Figure 43. Venn Diagram for 18S rRNA for Noab (NOA19) and Shalom (NOA18) Table Olives

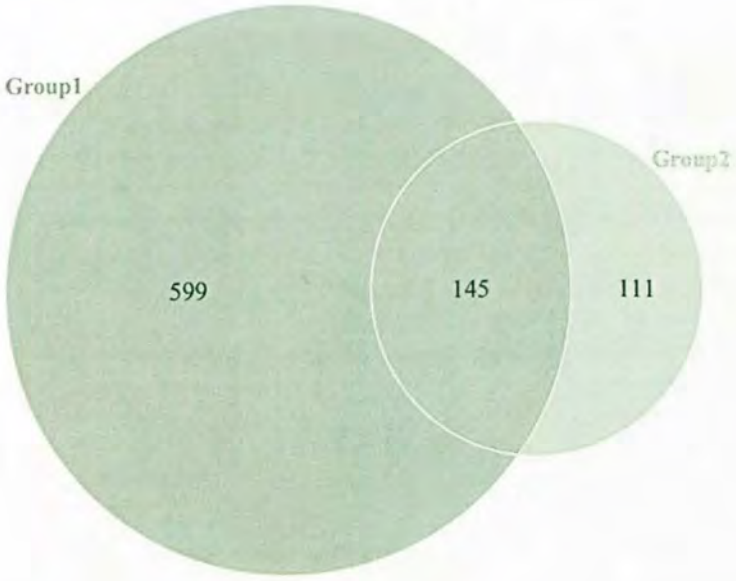

Figure 44. Venn Diagram for ITS for Noab (Group 2) and Shalom (Group 1) Table Olives

The Venn diagrams show that a total of 607 OTUs (66%) were shared among Noab and Shalom table olives for 16S rRNA analysis (Figure 42). For 18S rRNA analysis 19 OTUs (48%) were shared among Noab and Shalom table olives (Figure 43). While 145 OTUs (20%) were shared between Noab and Shalom table olives for ITS analysis (Figure 44).

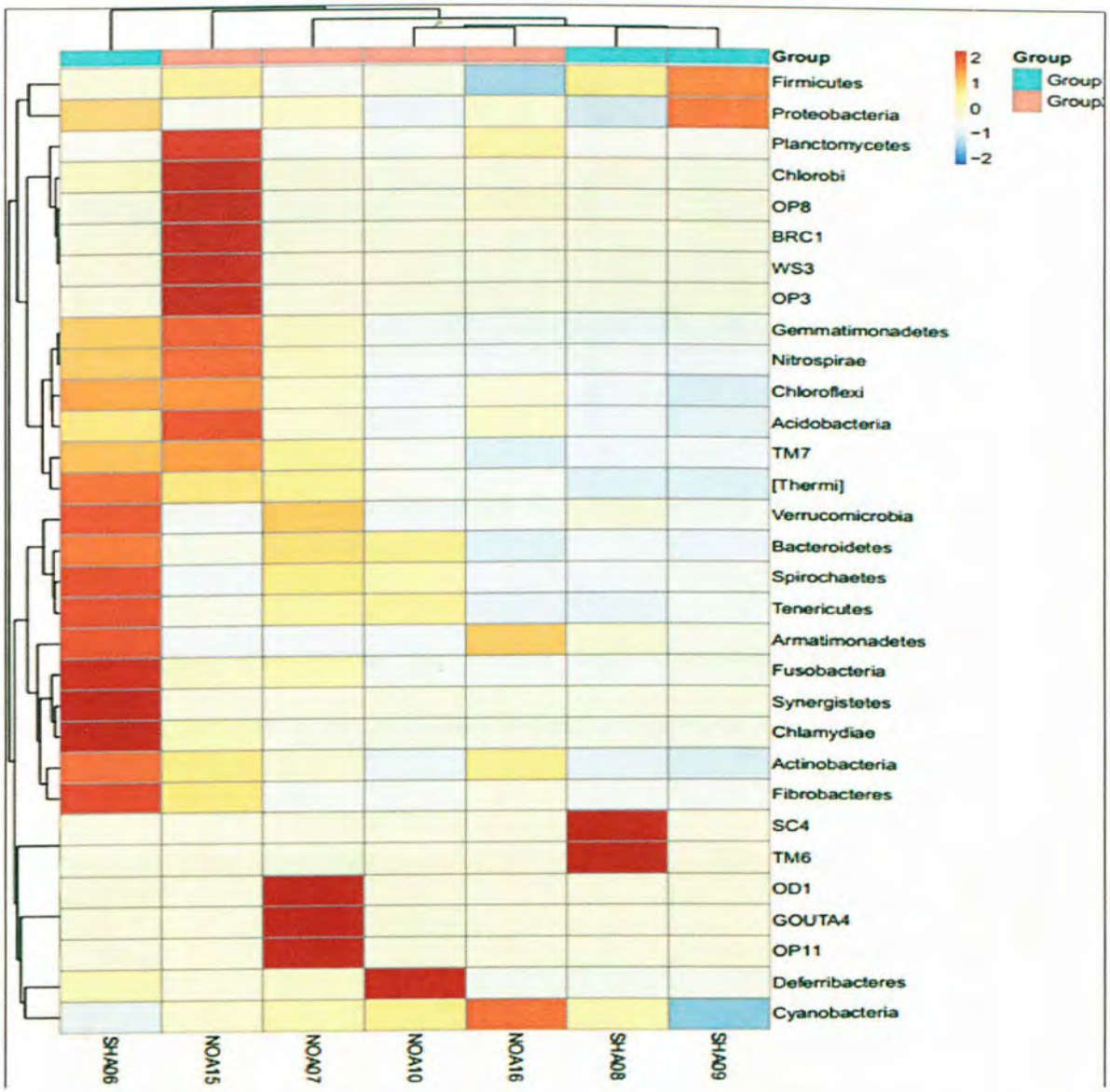

Figure 45. Species Abundance 16S rRNA Heat Map between Noab and Shalom Table Olives

For 16S rRNA, *Chlorobi, OP8, BRC1, WS3, OP3* for NOA15, *Fusobacteria, Synergistetes* and *Chlamydia* for SHA06, *SC4* and *TM6* for SHA08, *OD1, GOUTA4* and *OP11* for NOA07 and *Deferribacteres* for NOA07 had the highest abundance of species (Figure 45).

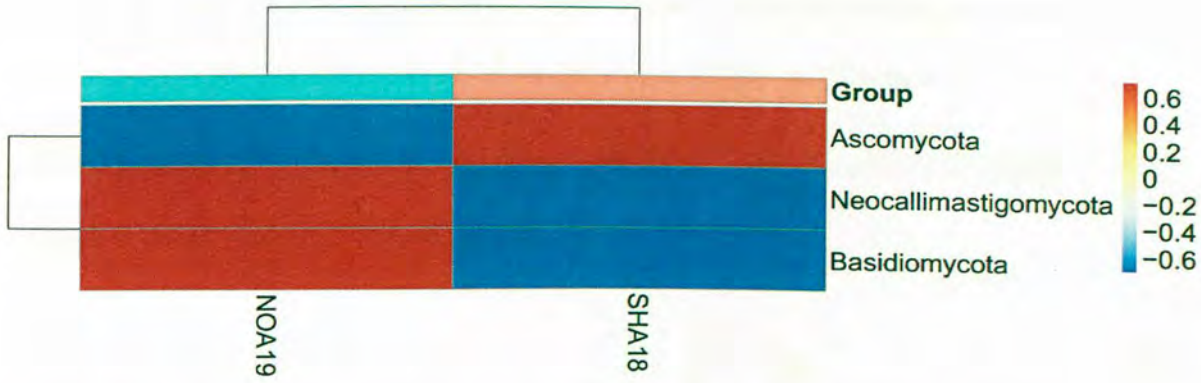

Figure 46. Species Abundance 18S rRNA Heat Map between Noab and Shalom Table Olives

For 18S rRNA, Ascomycota for SHA18 and *Neocallimastigomycota* and *Basidiomycota* for NOA19 had the highest abundance of species (Figure 46).

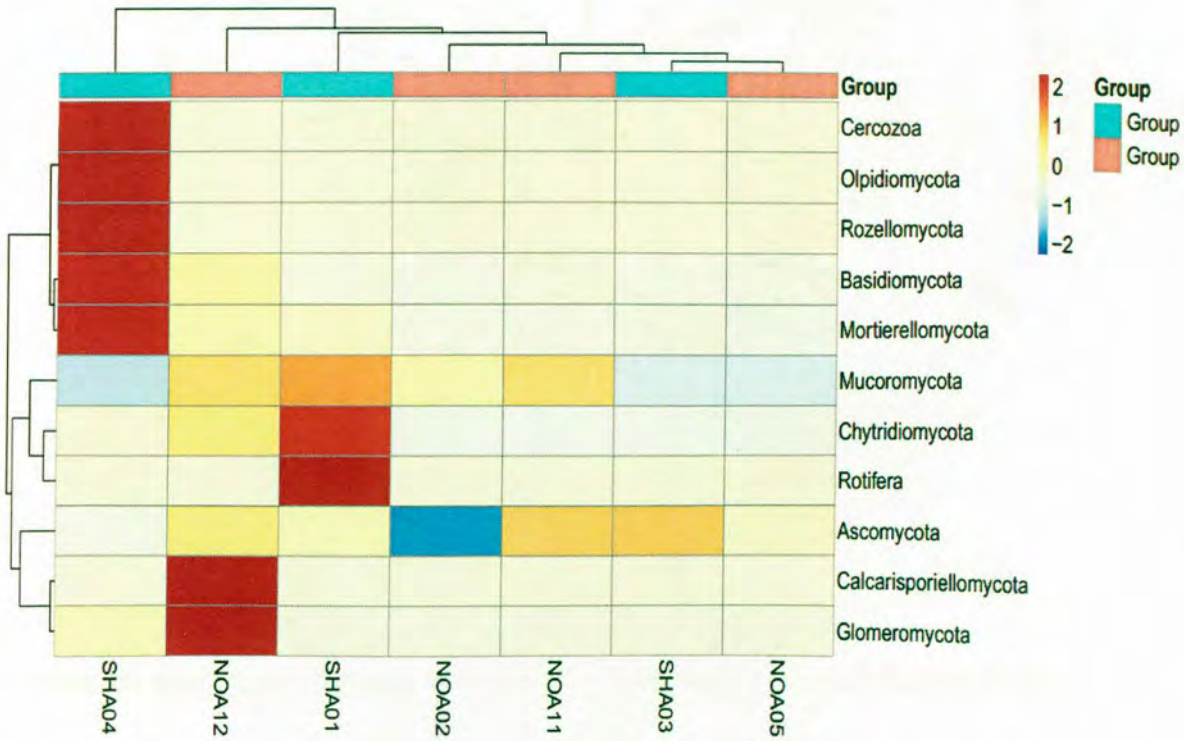

Figure 47. Species Abundance ITS Heat Map between Noab and Shalom Table Olives

For ITS, *Cercozoa, Olpidiomycota, Rozellomycota, Basidiomycota* and *Mortierellomycota* for SHA04, Rotifera for SHA01 and *Calcarisporiellomycota* and *Glomeromycota* for NOA12 had the highest abundance of species (Figure 47).

Figure 48 shows the phylogenetic tree of the OTU at the genus level for 16S rRNA analysis between Noab and Shalom table olives.

Figure 48. Species phylogenetic tree 16S rRNA between Noab and Shalom Table Olives

The classification tree can be divided into single sample taxonomy analysis and multiple sample taxonomy analysis. Single sample analysis can be used to understand the distribution of sequences in individual samples at various taxonomic levels. Multiple sample analysis can compare the sequence abundance difference of multiple samples at different taxonomic level.

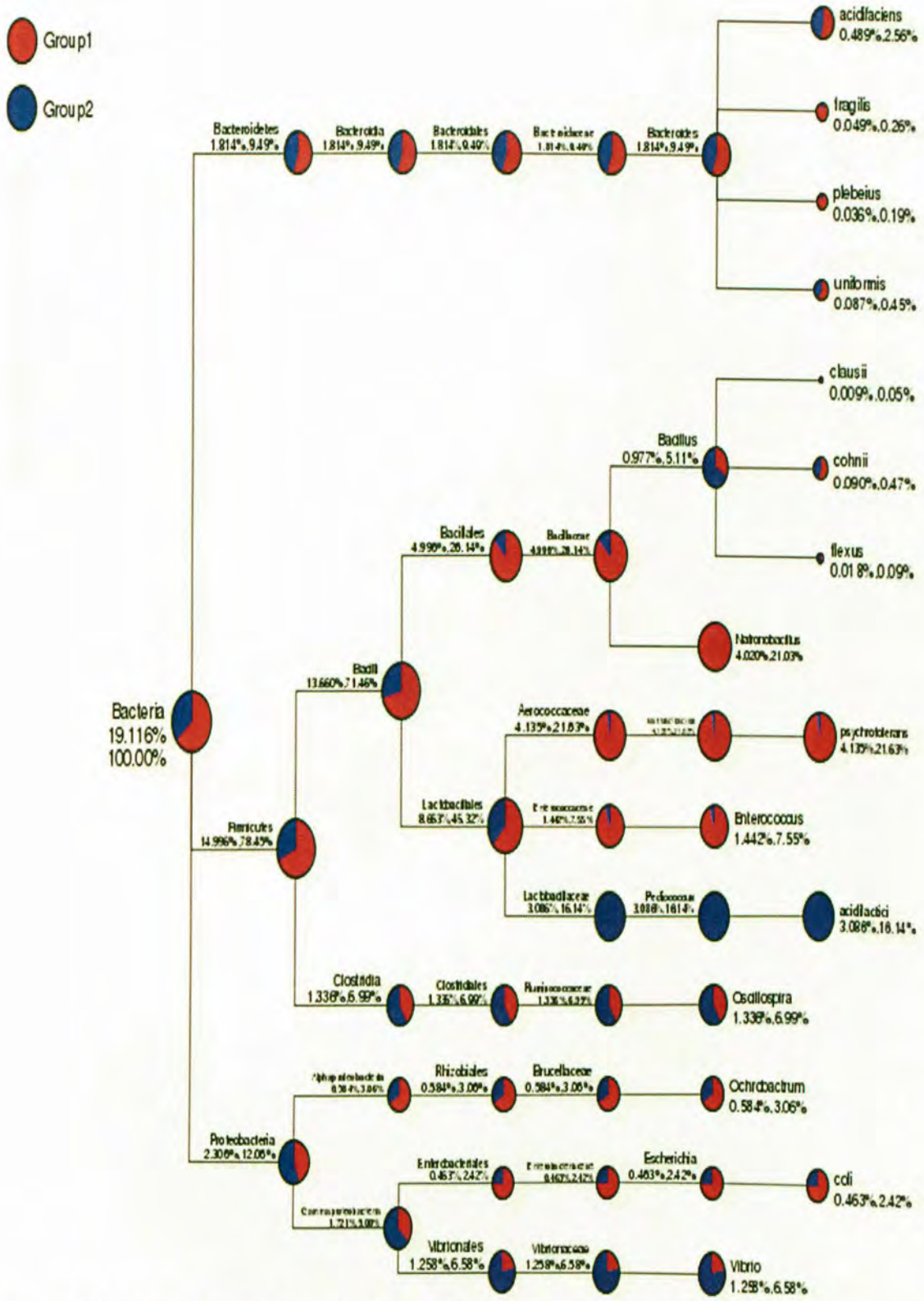

Figure 49. Multiple sample taxonomic tree for 16S rRNA between Noab and Shalom Table Olives

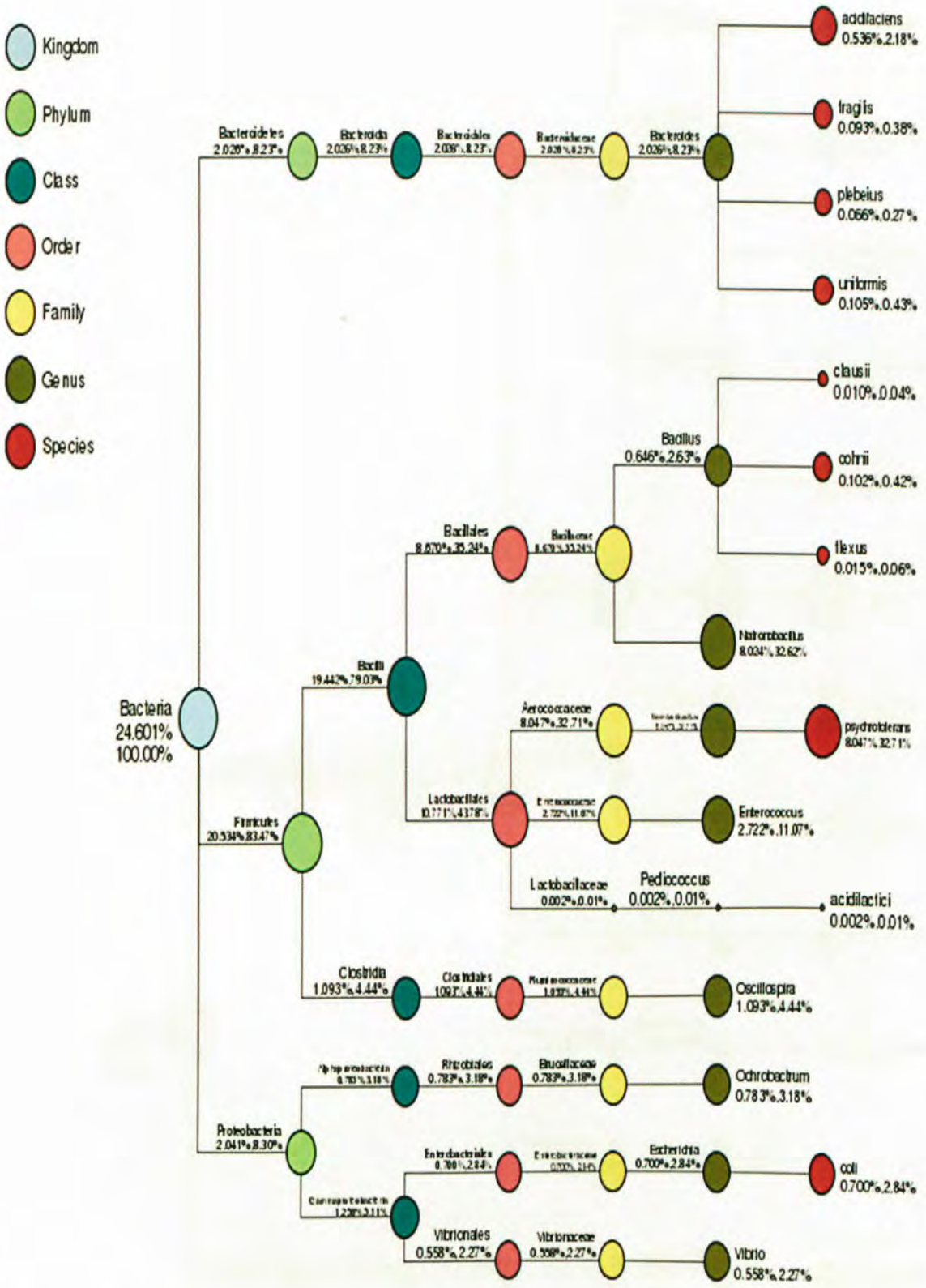

Figure 50. Single sample taxonomic tree for 16S rRNA between Noab and Shalom Table Olives

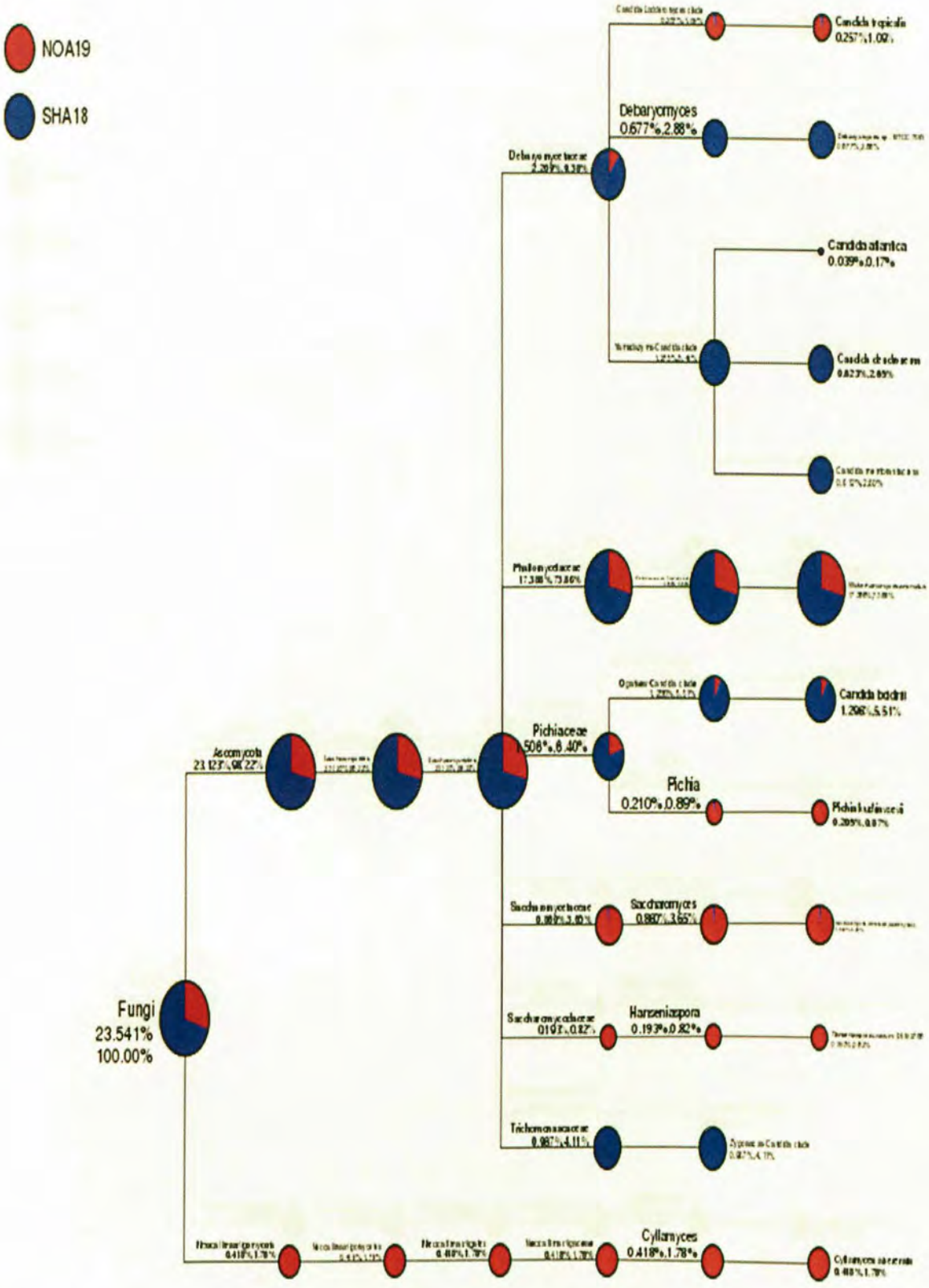

Figure 51. Multiple sample taxonomic tree for 18S rRNA between Noab and Shalom Table Olives

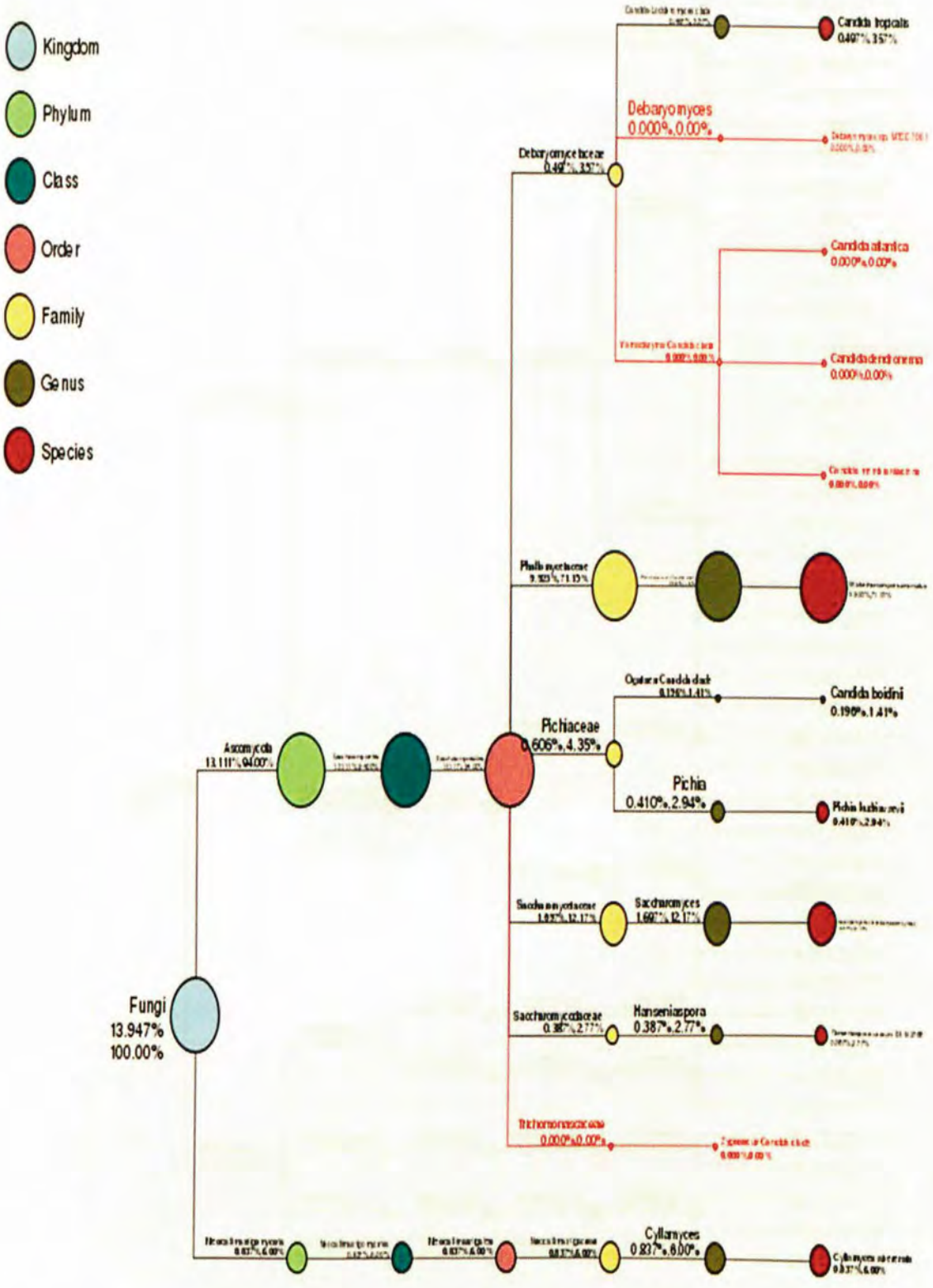

Figure 52. Single sample taxonomic tree for 18S rRNA between Noab and Shalom Table Olives

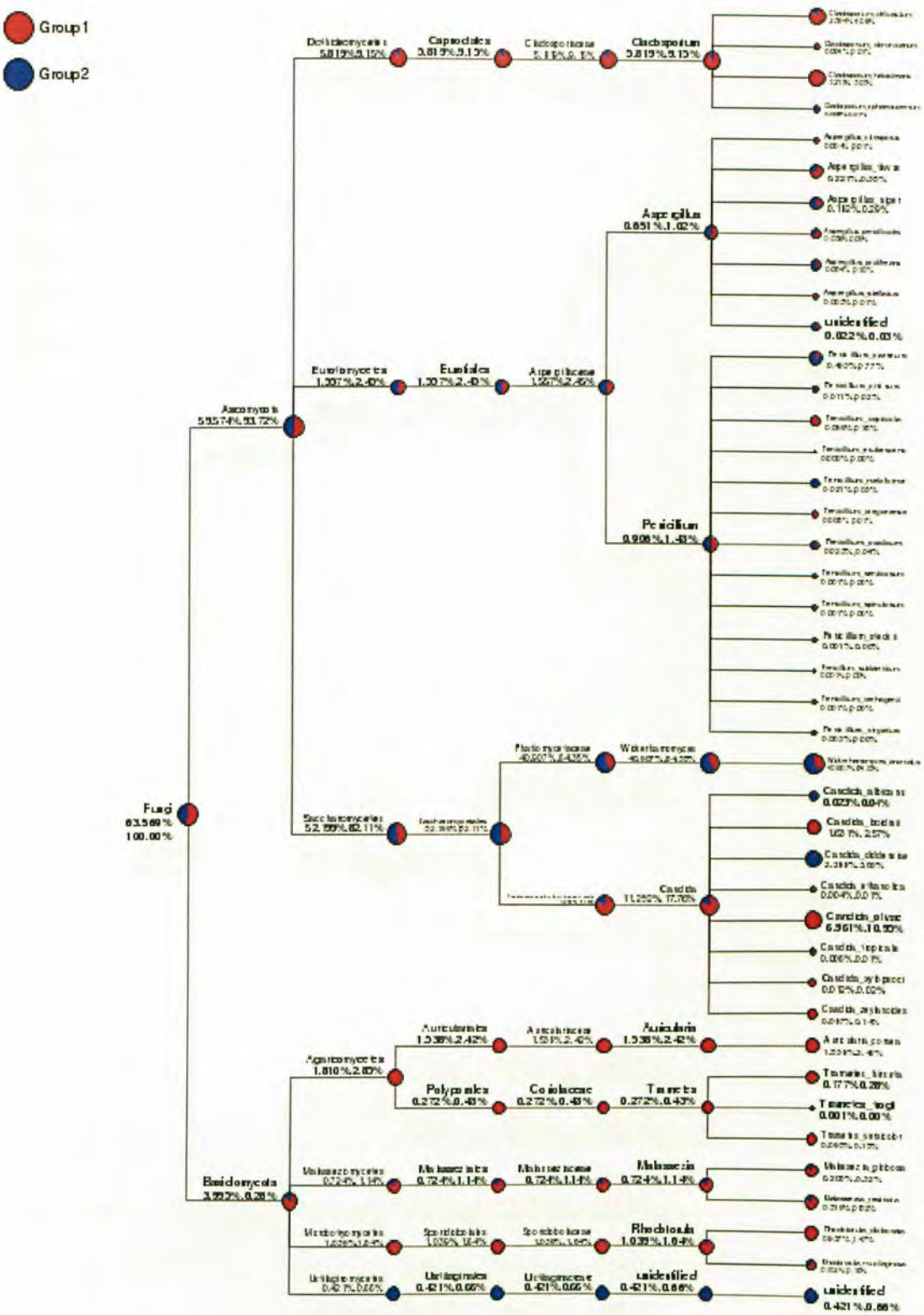

Figure 53. Multiple sample taxonomic tree for ITS between Noab and Shalom Table Olives

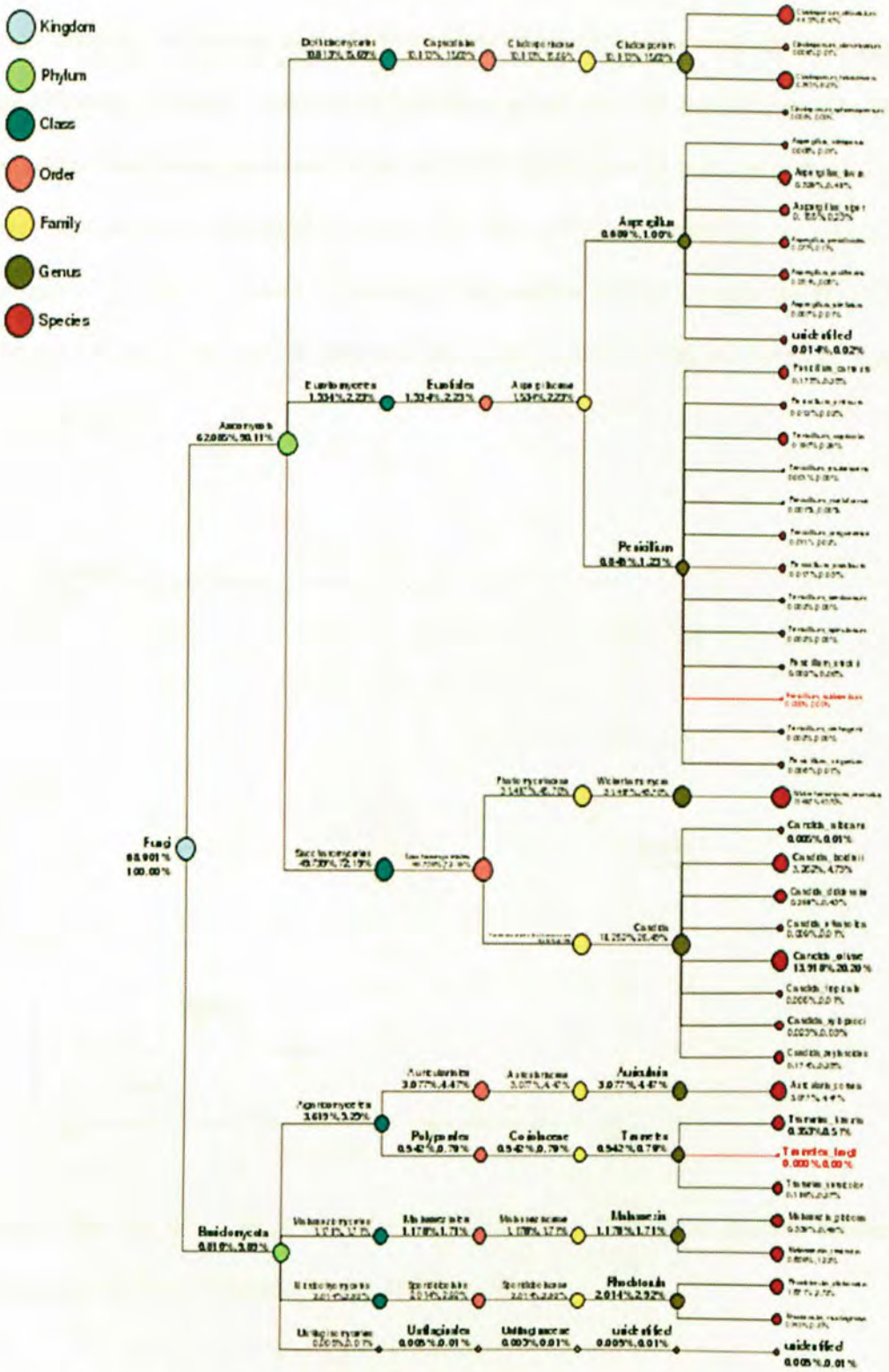

Figure 54. Single sample taxonomic tree for ITS between Noab and Shalom Table Olives.

Principal component analysis is a statistical procedure, using an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. The first principal component accounts for the variability in the data as much as possible, and each succeeding component accounts for the remaining variability as much as possible. For the microbial community composition of the samples (OTU, 97% similarity), the more similar they are, the closer sample points in the PCA figure could be get.

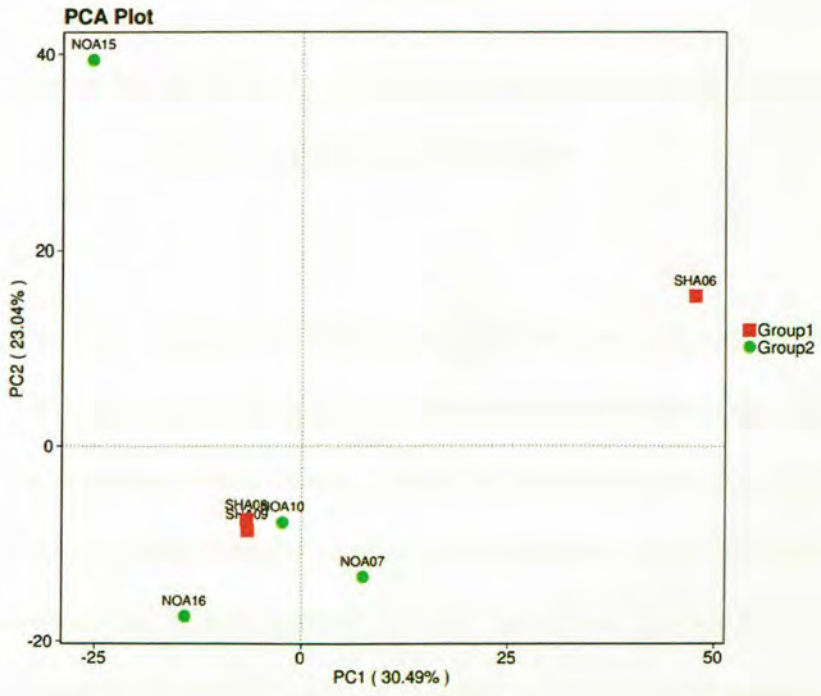

Figure 55. 16S rRNA PCA on the relative abundance of bacterial phylum between Noab (Group 2) and Shalom (Group 1) Table Olive

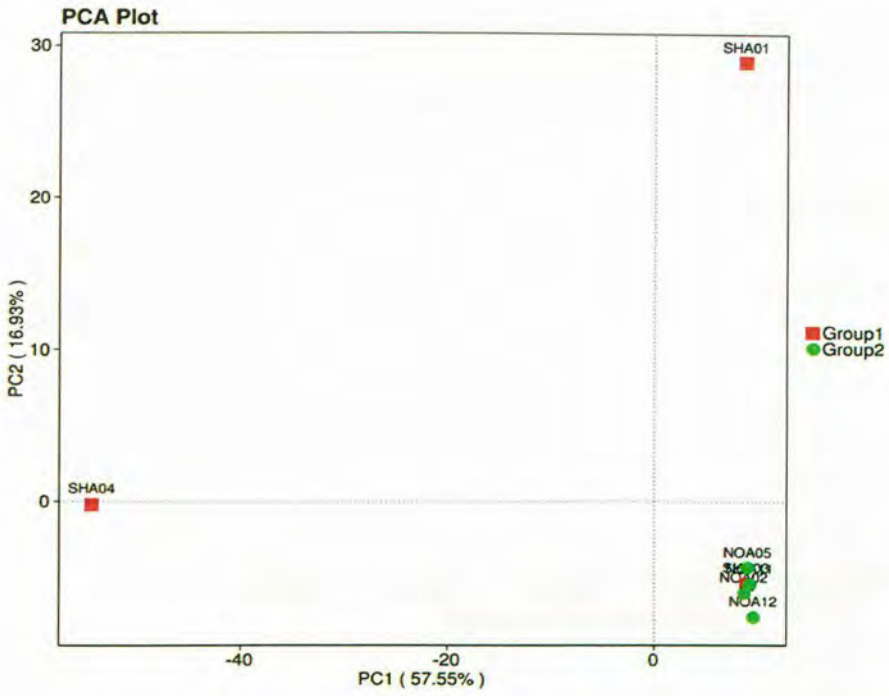

Figure 56. ITS PCA on the relative abundance of bacterial phylum between Noab (Group 2) and Shalom (Group 1) Table Olives

The level of alpha diversity was calculated and displayed with Mothur (Version 1.30). In order to compare the diversity indices between the samples, standardized the sequence number in each sample in the analysis process. Furthermore, the Alpha diversity, Beta diversity analysis, and significant difference analysis can be used to explore the distinct between samples. Microbial diversity can be assessed within a community (alpha diversity) or between the collections of samples (beta diversity).

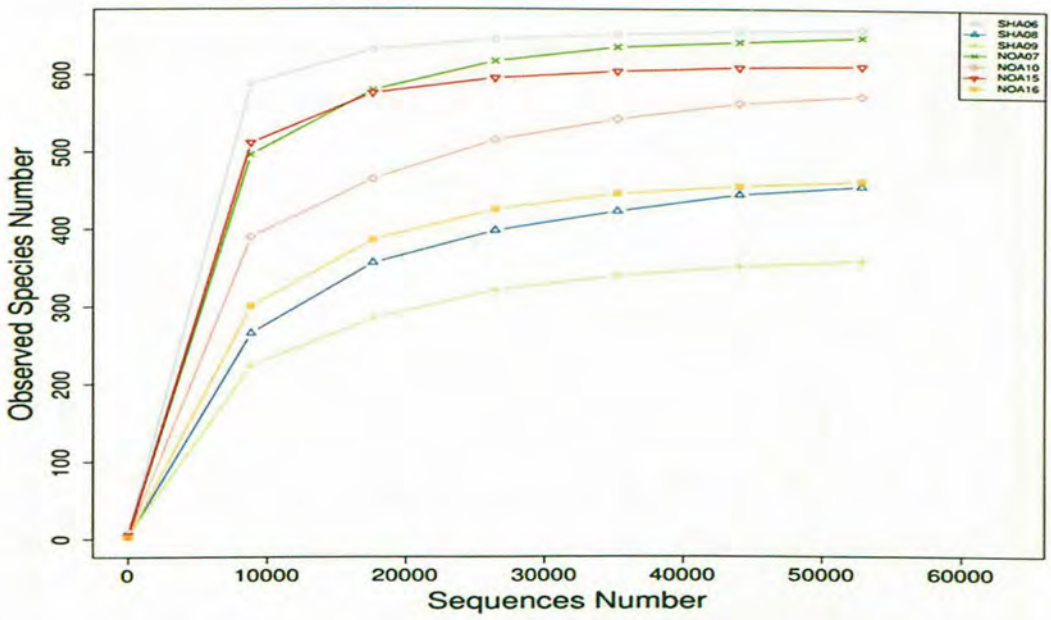

Figure 57. Rarefaction curve of the 16S rRNA sequences for Noab and Shalom Table Olives

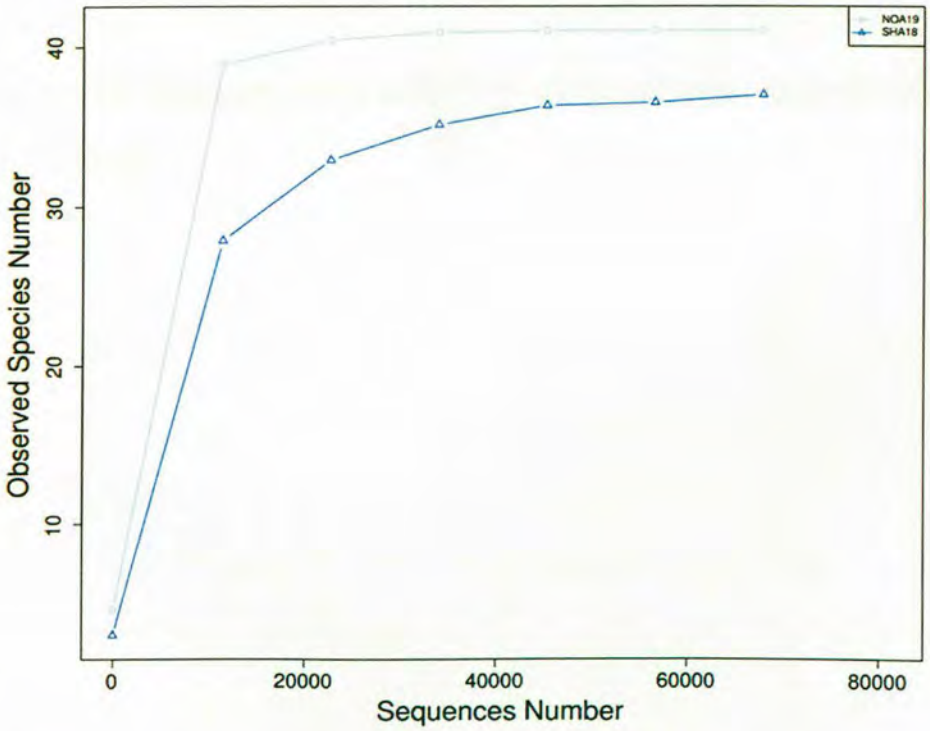

Figure 58. Rarefaction curve of the 18S rRNA sequences for Noab and Shalom Table Olives

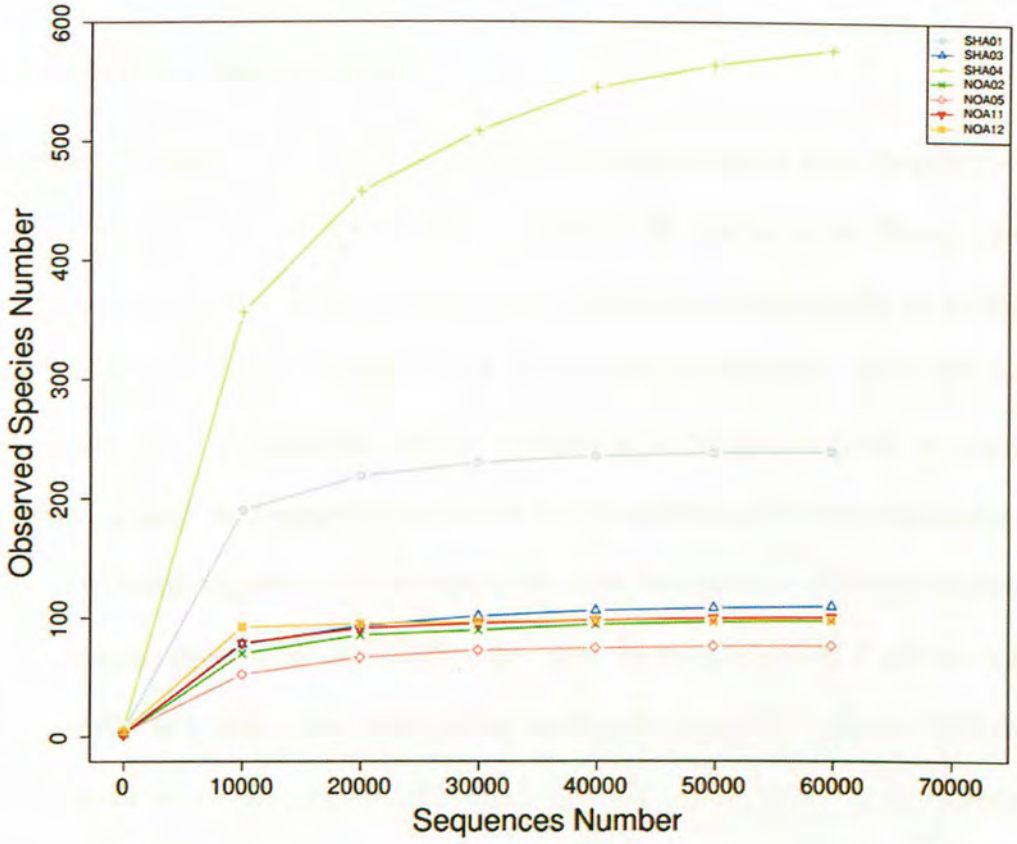

Figure 59. Rarefaction curve of the 16S rRNA sequences for Noab and Shalom Table Olives

CHAPTER 5: DISCUSSION

5.1 Table Olive Processing Steps

The study focused on two sites with two different approaches in olive fermentation. Noab farm used mixed fermentation and a double lid method while Shalom farm used the cloth method. Noab site used mixed fermentation and a double lid method while Shalom site used the cloth method. In terms of olive fruit size, Noab farm had the largest olive (Manzanilla) and the smallest olive (Frantoio) grown at higher altitude (around 1600 meters above the sea level) and Shalom farm used the medium size fruit (Mission) grown at lower altitude for olive fermentation. Although Frantoio is not commonly used as a table olive but more for oil production, it can be used successfully as a table olive. Shalom farm table olive preparation method is more well known by submerging the olives with a sealed plastic bag or heavy plate or cloth (Yada 2007). Noab farm table olives uses the same approach, but is modified by using the first lid as weight and the second lid with a small hole for CO^2 to escape and this prevents the lid from popping when it is opened.

5.2 Physicochemical and Kinetic Factors

Noab and Shalom table olives had no statistically significant difference between their pH values during the whole fermentation process from 0-150 days. Therefore, a similar pH during the fermentation process should have occurred between both processes in these two olive farms. The alkaline pH (8.9 for Noab olive samples and 7.9 for Shalom olive samples) dropped to around 4.5 and continued throughout the fermentation process (Figure 9), this can be an indication of the presence of lactic acid fermentation which reduces the growth of Gram-negative bacteria counts (Hamdi 2008). The LAB and yeast population consume the sugars of the table olive

fruit, causing it drop and stabilize to a pH of around 4-5 until the fermentation process ends. The pH plays an important role, as a constant pH of 4-5 allows successful fermentation and safety of the final product (Medina-Pradas & Arroyo-López 2015). In some cases, lactic acid is not produced in the amounts needed for the adequate fermentation of olives, and spoilage occurs through subsequent contamination by other microorganisms (Poiana & Romeo 2006). As expected, the pH values of the two sites decreased during the fermentation, ranging from 4 to 5 from day 30 till the end of the fermentation process. According to Kailis and Harris (2007), at high salt concentrations (8-10%), the pH of the brine that should be between 4.0-4.5, but if the pH increases the commercial value and safety of the olive product is jeopardised. The pH condition value of our experimental conditions were similar to those observed by other studies such as by Lanciotti *et al.* (1999), with effective fermentations of table olives achieving brine pH values of 4-5 or less.

There was no significant difference with respect to the combined acidity of the table olives from the two sites. Noab and Shalom olive samples had similar combined acidity, this can be an indication that the different varieties of table olives (Mission, Manzanilla and Frantoio) needed the same amount of acid conversion to reach the pH of 4.5 for all the cultivars were the same (Panagou, Tassou & Skandamis 2006). The combined acidity of both table olives did increase over 150 days of fermentation in relation to the beginning (Fig. 10). This is largely due to the presence of organic acids such as acetic, malic and lactic acids (Pereira *et al.* 2015). Even though the study had a minimum of 0.008 NaOH/L and a maximum of 0.128 NaOH/L, it still corresponds to the range reported by Leal-Sanchez *et al.* (2003). The combined acidity was similar to Lucena-Padrós *et al.* (2014) of 0.14 (±0.04) NaOH/L and 0.16 (±0.01) NaOH/L and closely resembles the combined acidity of this study. Combined

acidity of table olives plays a vital role in stabilizing and maintaining the final pH (Lopez-Lopez *et al.* 2004). The buffering capacity of brine contains multiple acids such as organic acid salts, mainly sodium lactate and sodium acetate present in the table olive brine. The low pH values may be attributed to the production of lactic acid by the LAB. As combined acidity increased, the concentration required of an organic acid to reach a specific pH value also increase. When combined acidity is low, a low pH can easily be reached and this helps the olive stability, because it permits most of the organic acids to be in their un-dissociated (antimicrobial) form (Lopez-Lopez *et al.* 2004).

The titratable acidity did increase over 150 days of fermentation in relation to the beginning (Fig. 12). This is largely due to the presence of organic acids such as acetic, malic and lactic acids (Pereira *et al.* 2015). Noab and Shalom olive samples had mean titratable acidity level values of 1.38 ± 0.17 g/L and 1.69 ± 0.22 g/L, respectively. In Lucena-Padrós *et al.* (2014) the titratable acidity was 1.14g/L (±0.04) g/L and 0.78 (±0.08) g/L and similar to the titratable acidity in this study. In the present study, pH showed a correlation with titratable acidity. As the pH decreases, the titratable acidity increases significantly. Titratable acidity was also not affected by the different variety of table olives used in the fermentation process.

NaCl is commonly used in brine to reduce the bitterness of table olives and make them more edible. The average values of NaCl content in the table olive in brine studied were 9.57 ± 0.11 % NaCl and 9.24 ± 0.15 % NaCl for Noab and Shalom, respectively. Figure 14 indicates that there was a slight decrease in NaCl as the fermentation period increased. According to data analysis, there was no significant difference between the average NaCl content in table olives between Noab and Shalom. There are no uniform specifications for the salt concentration in table olives,

this makes comparisons difficult (Lopez-Lopez *et al.* 2004). Traditional spontaneous fermentation system, which consists of brining in an 11% NaCl solution (Leal-Sánchez *et al.* 2003)

Both table olives had total phenol content that decreased over time. There was a significant difference between the olive samples from Noab and Shalom sites in terms of their total phenolic content as shown in Figure 16 and Table 10. The type of olive variety did not affect the total phenol content in this study. Total phenol content consists mainly of oleuropein, which should get reduced to make olives edible, therefore a decrease in total phenol content is good for olive processing methods. The total phenol count was similar to that reported by Kamtekar, Keer & Patil (2014) of 3.5725 ± 0.2336 GAE / mL.

5.3 Enumeration of LAB, Yeast and Moulds of Table Olives

The natural fermentation of olives relies upon indigenous microflora from raw table olive. The total plate for Noab and Shalom olive samples had a mean value between 3.83 ± 0.20 $\log_{10}$ CFU/ mL and of 3.67 ± 0.18 $\log_{10}$ CFU/ mL, respectively. As compared to other studies, the highest mean total plate value obtained in this study was lower than those obtained by Aponte *et al.* (2010) which ranged from 6.3 $\log_{10}$ CFU/ mL to 5.2 $\log_{10}$ CFU/ mL. The total plate count alone only gives an indication of all the countable microbial growth in brine of table olives over the fermentation timeline.

The mean LAB counts observed for Noab and Shalom table olives in brine, were 3.83 ± 0.20 $\log_{10}$ CFU/ mL and 3.67 ± 0.18 $\log_{10}$ CFU/ mL, respectively. LAB counts were not significantly different from each other ($P > 0.05$). The main factors that could have limited the adaptation of LAB to the brine environment are the

ambient temperature, the initial salt concentration, the nutrient availability and the presence of natural inhibitory compounds, since the fruits were not subjected to lye treatment (Fleming, Walter & Etchells 1973; Ruiz-Barba, Garrido-Fernandez & Jimenez-Diaz 1991, Ruiz-Barba *et al.* 1993).

The mean values for yeast and mould counts for Noab and Shalom table olives, were 4.14 ± 0.21 $\log_{10}$ CFU/ mL and 3.96 ± 0.27 $\log_{10}$ CFU/ mL, respectively. As fermentation began, changes on yeast population were noticed (Fig. 22), an increase pattern was generally observed throughout the fermentation process, being the highest at the end of the fermentation process. The high presence of yeast and mould in brine may be important in the contribution to aroma and flavour of the final product. Yeast tends to predominate on the skin surfaces and in the stomatal openings of olives, whereas bacteria predominated in the intercellular spaces of the sub-stomatal cells (Nychas *et al.* 2002).

Total coliform count is an indicator plate count used that can be used to measure and monitor the safety of the product. The mean of Noab olive samples (2.23 ± 0.13 $\log_{10}$ CFU/ mL) and Shalom olive samples (1.55 ± 0.17 $\log_{10}$ CFU/ mL) which indicated good microbial quality of table olive fermentation in brine. The low total coliform count indicates good hygiene practices employed and proper storage conditions. There are no reports of food borne outbreaks caused by these microorganisms in table olive so no standard has been developed yet (Medina-Pradas & Arroyo-López 2015).

5.4 Microbiological Results

The *Bacillus* genus is associated with a lot of different fermentation process and is mostly involved in the degradation of the product being fermented, allowing other

beneficial microorganisms to use the degraded parts as food. The *Bacillus* genera can be used in fermentation processes to break down tissue of the products and allow new microbial communities to become dominant in the fermentation process, such as LAB. Therefore, *Bacillus* spp. play a crucial role in the fermentation process to allow the fermentation to continue and diffusion to commence.

Even though, all the table olive samples had different climatic conditions and regions, homogeneity of genera isolated shows that the fermentation conditions for both sites were similar. The genus *Bacillus* originally included all rod-shaped bacteria, but now comprises only large, spore forming, Gram-positive bacilli that form chains and usually grow aerobically or anaerobically. They are common environmental organisms, frequently isolated in laboratories as contaminants of media or specimens. The *Bacillus* genus encompasses 70 species (Thwaite & Atkins 2012).

The strain *Bacillus licheniformis* had a 99% identification with isolate 1. In Soybean fermentation, *Bacillus licheniformis* was isolated and shown to have activity to increase insulin sensitizing (Yang *et al.* 2013). Sharma & Sharma (2017) reported that *Bacillus licheniformis* to have probiotic potential by being able to tolerate the gastric juices and having antagonism ability to reduce the level of pathogens. Table olive fermentation containing *B. licheniformis* or *B. paralicheniformis* can indicate the potential of having the same activity during fermentation.

B. cereus, *B. nitratireducens*, *B. thuringiensis* and *B. mycoides* had a 99% identification with isolate 2. *B. cereus* and *B. mycoides* have both been known to facilitate in the process of disintegrating cassava tissue during fermentation by cellulase activity (Amoa-Awua & Jakobsen 1995; Peng *et al.* 2003). The role of *B.*

cereus during fermentation could also be to facilitate in the breakdown of table olive tissue during fermentation. *B. nitratireducens* has also been isolated from fermented food and beverages, but it's exact role during fermentation is still to be understood (Liu *et al.* 2017). *B. thuringiensis* has been isolated from yogurt, but no study on the table olive fermentation has been done on *B. thuringiensis* (Amoa-Awua & Jakobsen 1995).

In addition, *Bacillus* cereus is one of the food-borne disease causing bacteria. Species of *Bacillus* and related genera have long been troublesome to food producers on account of their resistant endospores. Their spores may be present on various types of raw and cooked foods, and their ability to survive high cooking temperatures requires that cooked foods be served hot or cooled rapidly to prevent the growth of this bacteria (Tewari & Abdullah 2015) .

B. subtilis, *B. amyloliquefaciens* and *B. velezensis* had a 99% identification with isolate 3. Within the *Bacillus subtilis* group, *B. velezensis* and *B. subtilis* subsp. *subtilis* have received significant attention as biological resources for biotechnology-associated industries (Cho *et al.* 2018). *B. subtilis* and *B. amyloliquefaciens* are both commonly found in traditional Chinese soybean fermentation food (Peng *et al.* 2003). *B. amyloliquefaciens* produces a fibrolytic enzyme that can degrade the soybean tissue (Peng *et al.* 2003). As far as table olives are known, the enzymes commonly encountered are lipase and esterase that contribute to the formation of the aroma of the products (Bonatsou *et al.* 2017). Further studies need to be conducted to see if the enzyme from *B. amyloliquefaciens* can degrade the tissue of table olives.

B. cereus, *B. thuriensis* and *B. mycoides* had a 99% identification with isolate 4. The strain *B. cereus*, *B. velezensis* and *B. thuringiensis* had a 99% identification with

sequence 5. Due to spontaneous fermentation, the dominant strains at the end of fermentation cannot be controlled compared to the situation when starter cultures are being used

In this study, the phylogenetic characterization of all samples showed big differences between the two sites which covered the phyla including *Rozellomycota, Chytridiomycota, Mucoromycota, Cercozoa, Rotifera, Glomeromycota, Calcarisporiellomycota, Mortierellomycota, Basidiomycota, Ascomycota, Neocallimastigomycota, Fusabacteria, Acidobacteria, Chloroflexi, Deferribacteres, Verrucomicrobia, Actinobacteria, Bacteriodetes, Proteobacteria, Firmicutes* and *Cyanobacteria* (Figures 27,32 and 37) for 16S sRNA, 18S rRNA and ITS respectively. However, *Saccharomyces* and *Bacilli* classes were present in similar proportions for site 1 and site 2 samples (Figures 28, 33 and 38).

In terms of the orders distribution, *Lactobacillales, Streptophyta, Saccharomycetales* are present in both sites, while SHA09 had the highest relative abundance of *Enterobacteriales* and *Bacillales,* SHA08 had the highest relative abundance of *Lactobacillales,* SHA04 had the highest relative abundance *Capnodiales,* SHA03 had the highest relative abundance *Saccharomycetales* and NOA16 had the highest relative abundance of *Streptophyta* (Fig. 29, 34 and 39). The presence of both bacterial taxa in the fermentation of table olives and other vegetables is habitual, with a well-known negative role during fermentation for *Enterobacteriaceae*, and positive for *Lactobacillaceae* (Fernández, Adams & Fernández-Díez 1997). In general, the presence of food-borne pathogen in table olives is scarce, as it was also confirmed by pyrosequencing analysis by Cocolin *et al.* (2013) , who only found a low abundance of the genera *Escherichia, Staphylococcus, Clostridium* and *Listeria* during fermentation process of diverse Italian olive varieties. The presence

Pseudomonas genus was not observed during this study, however the *Pseudomonas* genus during fermentation was observed in Aloreña de Málaga table olives (Medina *et al.* 2016). *Pseudomonas* has proteolytic activity in table olives fermentation followed by decarboxylation and deamination of the resulting amino acids by heterofermentative lactobacilli that could cause an unusual type of spoilage characterized by a decrease in the acidity of brines and swelling (Harmon, Kautter & Mckee , 1987), and could also lead to biogenic amine formation. Globally, for fermented fruits the genera that accounted for >80% of the sequences were: *Celerinatantimonas* (53.5%), *Pseudomonas* (9.7%), unknown *Acetobacteraceae* (6.8%), *Modestobacter* (5.1%), *Propionibacterium* (5.0%), and an unknown *Lactobacillaceae* (3.0%) (Medina *et al.* 2016).

For 16S rRNA, the PCA for Shalom table olives had the highest relative abundance and Noab table olives which had the lowest relative abundance of phyla (Figure 55). For ITS, Shalom table olives had the highest relative abundance and Noab had the lowest relative abundance of phylum (Figure 37-41, 53 and 54).

The bacterial community was also analysed using a richness estimator or Chao1 Index. The Chao1 index varied from 37.5 to 688.11 (Tables 26,27 and 28). This kind of olives are characterized by a greater microbial diversity compared to treated processed olives because the natural process does not include the lye treatment (Hurtado *et al.* 2012). Overall, despite the diversity of sequencing depth between samples, a good coverage was obtained. There was satisfactory coverage of bacterial diversity for all samples with good coverage values above 0.90.

For 16S rRNA, the rarefaction curve showed that SHA06 had the highest sequence number and SHA09 the lowest sequence number (Figure 57). For 18S rRNA, the

rarefaction curve showed that NOA19 had the highest sequence number and SHA18 had the lowest sequence number (Figure 58). For ITS, the microbial communities is more diverse than 16S rRNA and 18S rRNA. the rarefaction curve showed that SHA04 had the highest sequence number and also it continued to spike and not level off and NOA05 had the lowest sequence number (Figure 59).

CHAPTER 6: CONCLUSION

Table olives is one of the fermented foods in Namibia and naturally associated with LAB and yeast. The olive tree is one of the oldest known cultivated trees and olive processing methods have been practiced for centuries. Noab and Shalom are the two table olive sites used in this study. The TPC (largely due to the olive variety used) and total coliform count (due mainly to handling or storage conditions) had significant differences between the two sites' table olive samples. The pH remained at around 4.5 which is the desired pH and should render the table olives safe for human consumption. The high NaCl content throughout the fermentation process should control the growth of spoilage microbes. The table olive samples are rich in phenols, which can be largely due to the different table olive cultivars used. The total plate count indicated that there was a high number of microorganisms growing throughout the fermentation process. The fermentation processes had similar rates of growth for both sites. The LAB count indicated high number of potential probiotic microorganisms during the fermentation of table olives. The total yeast can be due to the brine conditions that are favourable to the growth of yeast. The yeast of both sites could contribute significantly to the flavour of the final product. PCA shows that day 0, day 15 and day 30 are distinct from the other days in the number of microorganism's present. Days 60, 90 and 150 had the highest number of microorganisms and there is no difference between the LAB and yeast and mould count. Culture dependent method showed that *Bacillus* spp. was present in all olive samples. The present study documented both processing methods of table olive, microbial communities associated with olive fermentation in Namibian table olives and the physicochemical properties. The knowledge of the microbial community

involved in table olive fermentation is crucial for the safety of the final product and to define a procedure to control the fermentation process.

CHAPTER 7: RECOMMENDATIONS

The processing methods of table olives are well known worldwide, but they have not been optimized to full potential yet in Namibia. It is recommended that, further studies are needed to be done on the specific LAB and yeast strains during table olive fermentation that can ensure that organoleptic properties, safety and quality product remain constant. This will allow that table olive of the two sites to be sold not only to local markets, but be exported to international markets. In addition, the use of a starter culture can provide shorter fermentation periods and faster products on the markets. A detailed study is needed on the physicochemical and microbial properties of all Namibian sites involved in table olive production. This will provide a much better validation of the relationship between olive varieties (which works best in Namibia), microbial communities during olive fermentation and the effect of physicochemical properties on the final table olive product. Further studies are also necessary to determine the influence of these new microbial species on the quality and safety of table olives. Metagenomics gave an in depth insights into the microbial communities on the table olives in addition to giving an indication that with a suitable starter cultures better fermentation process can achieved.

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Appendix 1

Table 3. Independent Samples Test of the pH between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	1.781	0.186	-1.113	70	0.911	-0.3917	0.34773	-0.73270	0.65436
Equal variances not assumed			-1.113	64.182	0.911	-0.3917	0.34773	-0.73380	0.65547

Table 4. Independent Samples Test of Combined Acidity between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	Df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	0.373	0.545	0.485	34	0.631	0.006222	0.012831	-0.019853	0.032297
Equal variances not assumed			0.485	33.112	0.631	0.006222	0.012831	-0.019878	0.032323

Table 5. Pearson Correlation between pH and Combined Acidity of Noab and Shalom Table Olives

		Combined Acidity	pH
Combined Acidity	Pearson Correlation	1	-0.744**
	Sig. (2-tailed)		0.003
	N	12	12
pH	Pearson Correlation	-0.744**	1
	Sig. (2-tailed)	0.003	
	N	12	12

** Correlation is significant at the 0.01 level (2-tailed)

Table 6. Independent Samples Test of Titratable Acidity between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	Df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	3.965	0.055	-1.062	34	0.296	-0.30636	0.28856	-0.89278	0.28007
Equal variances not assumed			-1.062	32.330	0.296	-0.30636	0.28856	-0.89390	0.28119

Table 7. Pearson Correlation of Titratable Acidity between Noab and Shalom Table Olives

		Combined Acidity	pH
Combined Acidity	Pearson Correlation	1	-0.744**
	Sig. (2-tailed)		0.003
	N	12	12
pH	Pearson Correlation	-0.744**	1
	Sig. (2-tailed)	0.003	
	N	12	12

** Correlation is significant at the 0.01 level (2-tailed)

Table 8. Independent Sample Test NaCl of Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	Df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	2.154	0.151	1.732	34	0.092	0.33444	0.19306	-0.05790	0.72679
Equal variances not assumed			1.732	31.803	0.093	0.33444	0.19306	-0.05890	0.72779

Table 9. Pearson Correlation of NaCl and pH between Noab and Shalom Table Olives

		Combined Acidity	pH
pH	Pearson Correlation	1	0.616*
	Sig. (2-tailed)		0.033
	N	12	12
NaCl	Pearson Correlation	-0.616*	1
	Sig. (2-tailed)	0.033	
	N	12	12

** Correlation is significant at the 0.01 level (2-tailed)

Table 10. Independent Samples Test of Total Phenol Content between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	0.223	0.639	-5.325	34	0.000	-2.50517658	0.4704968863	-3.46134130	-1.54901187
Equal variances not assumed			-5.325	33.406	0.000	-2.50517658	0.4704968863	-3.46196745	-1.54838571

Table 11. Pearson Correlation of pH and Total Phenol Content between Noab and Shalom Table Olive

		Combined Acidity	pH
Combined Acidity	Pearson Correlation	1	0.502
	Sig. (2-tailed)		0.096
	N	12	12
pH	Pearson Correlation	-0.502	1
	Sig. (2-tailed)	0.096	
	N	12	12

Table 12. Independent Samples Test of Total Plate Count between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference Lower	Upper
Equal variances	3.175	0.079	0.353	70	0.725	0.10239	0.28988	-0.47576	0.68053
Equal variances not assumed			0.353	62.190	0.725	0.10239	0.28988	-0.47704	0.68181

Table 13. Independent Samples Test of Nutrient Agar and Plate Count Agar Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	0.430	0.514	0.836	70	0.406	0.24146	0.28870	-0.33433	0.81725
Equal variances not assumed			0.836	69.866	0.406	0.24146	0.28870	-0.33435	0.81727

Table 14. Independent Samples Test of LAB between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	0.334	0.565	0.590	106	0.556	0.16420	0.27825	-0.38745	0.71585
Equal variances not assumed			0.590	105.518	0.556	0.16420	0.27825	-0.38748	0.71588

Table 15. Levene's Test for LAB between Noab and Shalom Table Olives

Test of Homogeneity of Variances

Log CFU

Levene's Statistic	**df**	**df**	**Sig.**
0.315	2		0.730

Table 16. ANOVA for LAB Agars (MRS, M17 and Rogosa) between Noab and Shalom Table Olives

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.188	2	3.094	1.503	0.227
Within Groups	216.118	105	2.058		
Total	22.306	107			

Table 17. Independent Samples Test for Yeast and Mould between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	5.753	0.019	-0.510	70	0.612	-0.17576	0.34482	-0.86347	0.51195
Equal variances not assumed			-0.510	66.058	0.612	0.17676	0.34482	-0.86420	0.51267

Table 18. Independent Samples Test for SDA and YEGC between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	0.195	0.660	0.331	70	0.742	0.11417	0.34519	-0.57428	0.80262
Equal variances not assumed			0.331	69.656	0.742	0.11417	0.34519	-0.57437	0.80268

Table 19. Independent Samples Test for Total Coliform between Noab and Shalom Table Olives

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differences	Std. Error Differences	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances	0.037	0.849	-3.035	34	0.005	-0.67999	0.22402	-1.13525	-0.22474
Equal variances not assumed			-3.035	32.326	0.005	-0.67999	0.22402	-1.13612	-0.22387

Appendix 2

Table 21. Data statistics of quality control for 16S rRNA of Noab and Shalom Table Olives

Sample	PE	Raw	Clean	AvgLen(bp)	GC(%)	Q20(%)	Q30(%)	Clean(%)
SHA06	79634	71230	58230	417	53.76	96.51	93.18	73.12
NOA07	79939	70781	58639	413	54.1	96.32	92.82	73.35
SHA08	79934	71816	59833	418	53.15	96.15	92.57	74.85
SHA09	80011	72521	58524	428	53.38	96.37	93.06	73.14
NOA10	80098	71131	59739	412	54.4	96.15	92.5	74.58
NOA15	79575	71677	59400	417	54.15	96.34	92.88	74.65
NOA16	79751	71454	57633	411	55.62	96.27	92.74	72.27

PE Reads: the PE reads obtained from the sequence platform. Raw Tags: the merged tags. Clean Tags: the tags after QC. Effective Tags: the tags without the chimeric sequences and can be used by the subsequent analysis. AvgLen: the average length of the Effective Tags. GC (%): the percentage of G and C. Q20% & Q30%: the percentage of bases with a quality score equal to or higher than 20 (error rate <1%) and 30 (error rate <0.1%). Clean (%): the number of CleanTags take up of the number of Raw PE.

Table 25. Data statistics of quality control for ITS of Noab and Shalom Table Olives

Sample	PE	Raw	Clean	AvgLen(bp)	GC(%)	Q20(%)	Q30(%)	Clean(%)
SHA01	79999	73005	65623	287	49.7	98.33	95.92	82.03
NOA02	80048	68193	60961	398	46.95	95.05	91.39	76.16
SHA03	79926	74309	67002	267	37.2	98.78	97.07	83.83
SHA04	80273	72970	63975	248	47.63	98.97	97.43	79.7
NOA05	79793	72115	64787	284	51.44	98.33	95.8	81.19
NOA11	79938	74529	67125	261	38.99	98.94	97.39	83.97
NOA12	79691	73137	63656	264	41.82	98.8	97.07	79.88

PE Reads: the PE reads obtained from the sequence platform. Raw Tags: the merged tags. Clean Tags: the tags after QC. Effective Tags: the tags without the chimeric sequences and can be used by the subsequent analysis. AvgLen: the average length of the Effective Tags. GC (%): the percentage of G and C. Q20%&Q30%: the percentage of bases with a quality score equal to or higher than 20(error rate <1%) and 30 (error rate <0.1%). Clean (%): the number of CleanTags take up of the number of Raw PE.

Appendix 3

Table 27. Statistics of Alpha diversity indices for 16S rRNA for Noab and Shalom Table Olives

Sample	Observed	ACE	Chao1	Simpson	Shannon	Coverage
SHA06	658	661.7139	668.1111	0.953387	7.073486	0.999735
SHA08	455	478.4524	472	0.617354	2.351833	0.999035
SHA09	359	378.3624	381.0244	0.80801	3.18172	0.999186
NOA07	648	655.9392	662.5	0.775075	4.925757	0.999432
NOA10	572	603.4746	597.2857	0.694321	4.247066	0.998864
NOA15	611	612.8316	612.8	0.797127	4.496777	0.99983
NOA16	462	470.2758	470.2174	0.372301	1.984822	0.99947

Table 28. Statistics of Alpha diversity indices for 18S rRNA for Noab and Shalom Table Olives

Sample	Observed	ACE	Chao1	Simpson	Shannon	Coverage
NOA19	41	41	41	0.736	2.656	1
SHA18	37	37.673	37.5	0.505	1.5	1

Table 29. Statistics of Alpha diversity indices for ITS for Noab and Shalom Table Olives

Sample	Observed	ACE	Chao1	Simpson	Shannon	Coverage
SHA01	239	241.593	243.091	0.735	2.902	1
SHA03	109	111.223	111.1	0.545	1.502	1
SHA04	577	603.985	598.071	0.878	4.404	0.999
NOA02	97	101.458	115.333	0.129	0.643	1
NOA05	76	77.244	79.333	0.608	1.755	1
NOA11	100	101.645	103.75	0.208	0.948	1
NOA12	98	98.879	98.5	0.705	3.116	1

Appendix 4

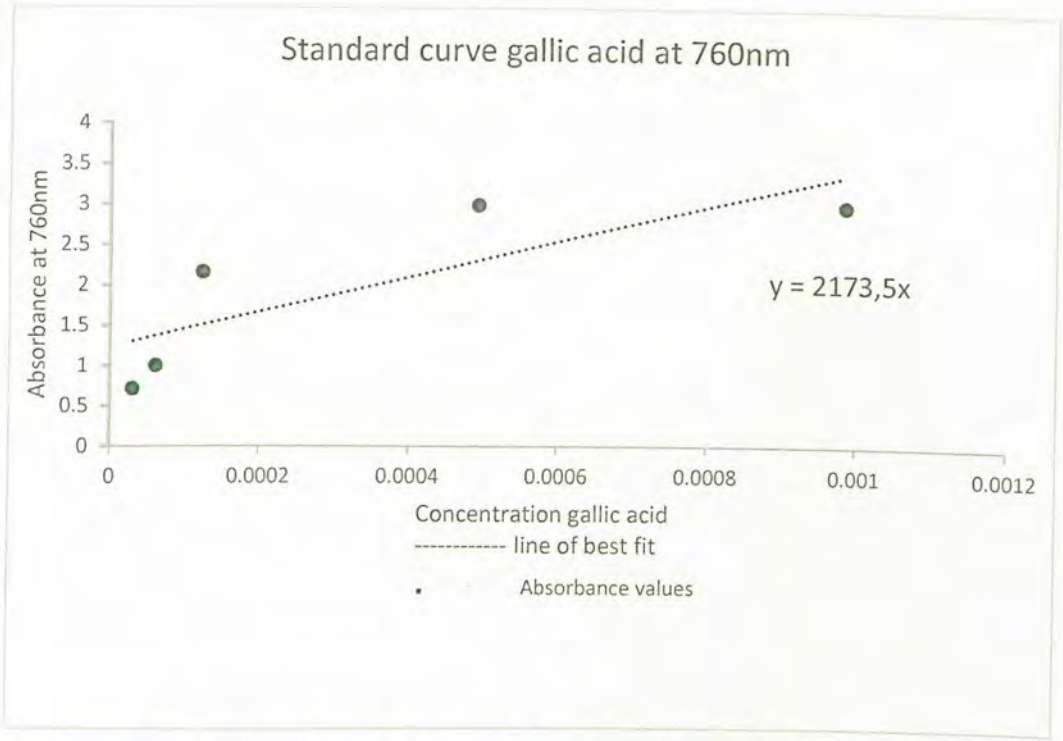

Figure 60. Gallic Acid Standard Curve of Noab and Shalom Table Olives

Table 30. Absorbance of the diluted stock solution (gallic acid solution) at 760 nm for Noab and Shalom Table Olives

Dilution factor	Concentration of stock solution (g/ml)	Volume of stock solution (ml)	Volume of solution (ml)	Absorbance in triplicate at 760nm			Average Absorbance at 760 nm
0	9.86×10^{-4}	3.000	0.000	3.00	3.00	3.00	3.00
2	4.93×10^{-4}	1.500	1.500	3.00	3.00	3.00	3.00
8	1.233×10^{-4}	0.3800	2.620	2.167	2.169	2.172	2.169
16	6.1625×10^{-4}	0.1875	2.8125	1.003	1.004	1.005	1.004
32	3.0813×10^{-4}	0.09375	2.90625	0.717	0.718	0.717	0.717