ANTIMICROBIAL ACTIVITY OF FUNGAL SECONDARY METABOLITES ISOLATED FROM PEARL MILLET ON GUT MICROFLORA

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ABSTRACT

Pearl millet also known as mahangu in Namibia is a crop commonly found regions in the Northern part of Namibia such as Zambezi and Ohangwena region. Pearl millet is a common staple food of a certain percentage of Namibians. Traditional methods used for processing millet comprise of threshing, cleaning, washing, soaking, germination, wet and dry milling and fermentation. The fungal species gain access to grain matrices, depending on the type of environmental conditions certain secondary metabolites are generated this may occur during the pre-harvest, storage, transportation, processing, and marketing stages. Hazardous secondary metabolites with antimicrobial properties for instance polyketide, non-ribosomal peptides, alkaloids, and terpenes are produced by fungi and can cause food poisoning and possibly affect the microflora in the gut. It is important to identify and document whether fungi that produce antimicrobial secondary metabolites are present in mahangu that may affect the microflora. Even though sophisticated machinery exist, traditional methods of food storage and preparation are still being used in certain parts of the country. These traditional methods are seldom done in unhygienic conditions resulting in contamination of the food. The effect of food poisoning by fungi in Namibia is unknown, to the best of my knowledge there are fewer studies in Namibia that focused on the effect of antimicrobial fungi secondary metabolites on the microflora found in the gut. For this reason this study focused on isolating fungal species from pearl millet and testing their antimicrobial ability on four gut microflora *E. coli* ATCC 33849, *K. pneumoniae* ATCC BAA- 2146, *L. monocytogenes* and *L. plantarum* ATCC 8014. The pearl millet was bought from the local market and serial dilution was performed using distilled water. The solution of pearl millet with a dilution of 1:100 000 was plated on Sabouraud Dextrose Agar, it was then sub cultured until pure fungal isolates was obtained. Fungi were identified on the basis of colony characteristics and microscopic examinations. The following six types of fungi were identified *Aspergillus flavus, Aspergillus niger, Saccharomyces* sp., *Rhodotorula* sp., *Trichosporon* sp. *and Saccharomyces* sp. Ethyl acetate was used for extraction of the secondary metabolites and agar well diffusion was used to test for antimicrobial activity. Then Minimum Inhibitory Concentration was carried out only with fungi which produced zones of inhibition against the selected bacteria, lastly Minimum Bactericidal Concentration was carried out. The analytic test used showed that there was a significant difference in the antimicrobial activity among different fungal species p= 0.019. The fungus *A. niger* did not show any form of antimicrobial activity while *Saccharomyces* sp. showed the highest inhibitory effects with a mean value of 12.16 ± 0.21. The fungus *A. flavus* showed more inhibitory activity against the following bacterial strain *L. plantarum*, *L. monocytogenes* and *K. pneumoniae* at 7.5 mg/ml when MIC was carried out. The extract from *A. flavus* was the only extract exhibiting bactericidal effects and this was seen against *K. pneumoniae* and *L. plantarum.* It is evident that gut microflora can be affected by secondary metabolites of fungi from pearl millet which can lead to detrimental effects in humans. Further studies are required to detect secondary metabolites that could cause harm to the gut microflora also what dosage level is required to cause an effect in vivo on gut microflora.

Keywords: Pearl millet, Secondary metabolites, Antimicrobial activity, Gut Microflora,

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND/OR ACRONYMS

DON	: deoxynivalenol
PAT	patulin
GIT	gastrointestinal tract
GALT	gut associated lymphoid tissue
OTA	ochratoxin A
SCFA	short chain fatty acid
LAB	Lactobacillus
w/v	weight per volume
FUM	fumonisins
ZEA	zearalenone
AF	aflatoxin
AFB1	aflatoxin B1
mg/kg	milligram per kilogram
µg/kg	microgram per kilogram
DNA	deoxyribonucleic acid
*μ*L	microliter

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DEDICATION

This thesis is dedicated to :

My parents, Helena and Erich Nghaamwa who taught me perseverance, hard work and faith.

My siblings, Talenge and Dankie who are my motivation to always excel in what I do.

To my grandmother, Lea Uuwanga who taught me never to give up and to always have faith.

To my partner and friend Mweneni Mwafangeo who supported, guided and motivated me always.

DECLARATION

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

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Pamela MN Nghaamwa	…… …	…30/11/21..
Name of Student	Signature	Date

CHAPTER 1 INTRODUCTION

1.1Background

According to Shifiona, Dongyang and Zhiquan (2016) consumption of pearl millet represents one fifth of the national cereal consumption in Namibia. Millets are customarily cultivated under intense environmental conditions such as high temperature and inconsistent precipitation, as well as on acidic and infertile soils that have of poor water holding capacity (Osamwonyi and Wakil, 2012). Common nutrients found in millets are B vitamins, especially niacin, B17, B6 and folic acid, calcium, iron, potassium, magnesium and zinc including proteins and carbohydrates (Osamwonyi and Wakil, 2012). Traditional methods used for processing millet comprise of threshing, cleaning, washing, soaking, germination, wet and dry milling and fermentation (Osamwonyi and Wakil, 2012). Most traditional fermentation techniques in Africa utilise different natural microflora that are adapted to the varied environmental and non-sterile conditions produced by the different processes (Osamwonyi and Wakil, 2012). This eventually leads to challenges like food spoilage and food borne diseases. Many fungal species grow on food products, frequently contributing to spoilage of fruits, vegetables and other fresh or prepared products (Osamwonyi and Wakil, 2012).

According to Misihairabgwi et al. (2017) aflatoxins and fumonisins are reportedly prevalent in major dietary and export targeted crops in Southern Africa, with fewer reports of deoxynivalenol (DON) and patulin (PAT) contamination. Acute mycotoxicoses have been observed in Africa and persistent exposure to low amounts of numerous mycotoxins is a risk factor for human diseases including cancer and childhood stunting (Misihairabgwi et al., 2017). Secondary crops such as sorghum, millet, wheat and cassava are mainly used for home consumption, traditional beer brewing and for income generation through selling in the informal sector (Misihairabgwi et al., 2017). Low-income economies usually consist of high food insecurity, food scarcity and undiversified diets, predisposing the population to ingesting mycotoxin contaminated foods (Misihairabgwi et al., 2017).

The globalization of trade and travel has increased the emergence and reemergence of certain food-borne pathogens (Bhalla, Monika, Sheetal and Savitri, 2019). This could be a result of weak public health infrastructure, economic problems, changing health policies, poverty, uncontrolled population displacement and urbanization, ineffective disease control programs and resistant microbial strains (Bhalla, Monika, Sheetal and Savitri, 2019).

Fungi produce a wide variety of molecules referred to as secondary metabolites such as, polyketides, as well as non-ribosomal peptides and terpenes. Secondary metabolites are not involved in metabolic processes of growth and energy generation but exhibit a range of biological activities that contribute to the survival of the organism in an occupied ecological niche (Boruta, 2017). Exposure to mycotoxins is generally by ingestion of contaminated food. The staple foods of Namibians are millet, milk, maize and sorghum and it is believed that most fungi grow on these food products and in most cases produce mycotoxins. Impacts of mycotoxins on human wellbeing depends on the degree of exposure, which includes dosage, period, type of mycotoxins, physiological and dietary status and lastly synergistic impacts that may occur between different synthetic chemicals to which animals or people are exposed to (Liew, and Mohd-Redzwan 2018).

The gastrointestinal tract (GIT) is the original site for interaction of ingested mycotoxins. Certain mycotoxins will be more rapidly absorbed, whereas some will get further along the GIT (Broom, 2015). Their effect on the body depends on whether the absorbed mycotoxin enters the systemic circulation or not, the cells of the GIT will hypothetically be exposed to ingested mycotoxins and in the high concentrations (Broom, 2015). Furthermore, mycotoxins that advance along the GIT will interact with the microbial cells present in the intestine and affect them. The intestinal microbiota has a crucial part in determining the health and performance of a human being. A well-functioning microbiota inhibits colonisation of the intestinal epithelium by pathogens and infiltration of the gut barrier, controls the gut-associated lymphoid tissue (GALT) and systemic immunity, and impacts gastrointestinal development (Broom, 2015). The combined effects of the gut microflora improve digestive efficiency and utilisation of nutrients. Literature indicates a healthy gut microbiota in humans plays a significant role in the health and host immune system. Therefore it has been established that stable gut microbiota can eradicate mycotoxins from a host (Liew and Mohd-Redzwan 2018). The level of exposure to mycotoxins and possible health effects differ from individual to individual. Therefore, this study will focus on identifying possible fungal species isolated from locally produced pearl millet that are capable of producing secondary metabolites. This study was also designed to determine the effects of extracted secondary metabolites from these fungi on selected gut microflora.

1.2 Statement of the problem

Over the past few years, Namibia has been affected by climate change which has resulted in both long periods of severe drought and extreme flooding, the latter often favoring the growth of fungi and production of secondary metabolites which contaminate foods like pearl millet and eventually cause disease. Namibians especially in the Northern regions depend on maize, sorghum and pearl millet production as their staple food. The method used to harvest pearl millet and the way it is stored, makes the conditions suitable for fungal growth and the production of secondary metabolites. Food poisoning can often occur because pearl millet can be consumed raw in the form of traditional beverages and can also be cooked and referred to as porridge. Certain fungi and their secondary metabolites can withstand high temperatures during preparation such as cooking. As well as withstand acidic conditions found in the stomach, certain fungi can modulate environmental pH and survive in the stomach. Therefore if the fungal strains or antibacterial secondary metabolites are ingested and spread to the digestive tract this can cause an imbalance in the gut microflora. This disturbance of the gut microflora can lead to various health effects such as bloating, stool change, reduced ability of the body to absorb nutrients and lastly affect the function of the immune system. This can lead to the person becoming susceptible to opportunistic pathogens and additional illnesses. Therefore the aim of this project, was to identify fungal species found in pearl millet and determine whether the fungal species possess secondary metabolites with antibacterial properties on selected gut microflora.

1.3 Objectives

The specific objectives were to:

a) Isolate and culture fungal strains from pearl millet.

b) Identify the fungal species isolated from the pearl millet.

c) To extract intracellular and extracellular fungal antimicrobial secondary metabolites from isolated fungal strains.

d) To evaluate the antimicrobial activity of the crude fungal extract for against gut microflora.

1.4 Hypotheses of the Study

There are several antimicrobial secondary metabolites fungi found in pearl millet which are hazardous to the gut microflora. These secondary metabolites of the isolated fungi have the potential to affect the gut microflora negatively by inhibiting the growth of the microorganisms.

1.5 Significance of the Study

This study analysed potential toxic effects of secondary metabolites from contaminated food and beverages especially from pearl millet on gut microflora. The data will inform the Government on formulating guidelines on proper storage of pearl millet and also allow the government to organize training for local farmers on how to store the grain and preventative measures to inhibit the growth of fungi. Companies like Namib Mills that manufacture pearl millet can be educated on the potential toxic fungal strains that can be found in their products and take precautions. The study will determine whether gut microflora is affected by fungal metabolites which can result in severe illness.

1.6 Limitation of the Study

Not all secondary metabolites produced by fungi isolated from the pearl millet were extracted also not all fungal strains that are present in the pearl millet will develop on the selected growth media.

1.7 Delimitation of the Study

The study was conducted *in vitro* and the outside environment might not mimic the intestinal microflora environment.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

In Northern parts of Namibia where conditions are warm, moist, and most likely unhygienic during the traditional malting and milling processes, the growth of mycotoxigenic fungi can be activated (Nafuka et al., 2019). As stated by Nafuka et al. (2019), mycotoxigenic fungi can gain access to grain matrices, therefore generating mycotoxins during the pre-harvest, storage, transportation, processing, and marketing stages. Hazardous mycotoxins are aflatoxins, ochratoxins, fumonisins, patulin, and ergot alkaloids, produced by fungi such as *Aspergillus, Penicillium, Claviceps*, and *Fusarium* genera respectively (Nafuka et al., 2019). A study by Dunaiski and Denning (2019) specifies that an estimated 5% of the Namibian population is affected by fungal infections.

Even though mycotoxin contamination is a major concern in many countries in Southern Africa, relatively few studies have been conducted on dietary mycotoxins in Africa compared to other continents, primarily due to shortage of advanced laboratory equipment, inadequate research funds, capacity and expertise, and limited surveillance systems (Misihairabgwi et al. 2017). Inadequate information on dietary mycotoxins exists outside the borders of South Africa, while monitoring and implementation of regulatory standards are rare or non-existent (Misihairabgwi et al. 2017).

Secondary metabolites generated by fungi are known to cause mycotoxicoses in both human and animals. A study by Alassane-Kpembi, Pinton, and Oswald (2019) revealed changes in the gut microbiota of mice, as a result of long term feeding of aflatoxin B1. Secondary metabolites can be carcinogenic, mutagenic, teratogenic, estrogenic, neurotoxic, hepatotoxic, nephrotoxic and cytotoxic or may induce immunosuppression in humans (Misihairabgwi et al. 2017). Findings by Rafiq et al.(2018) state that fungi not only affect bacterial strains but also fungal species like *Candida* subspecies.

Recent studies by Broom (2015) demonstrated that ochratoxin A (OTA) considerably reduced acetic, butyric and total short chain fatty acid (SCFA) concentrations that was used in a dynamic simulation model of the descending human colon. This indicated that OTA has the potential to affect the composition and metabolism of the microbiota found in the colon. This work also revealed that OTA eliminated a strain of *Lactobacillus reuteri* from the descending colon microbiota, which was a permanent removal. The bacterium *L. reuteri*, is known to produce bacteriocin and reuterin therefore these can be a key resident of the GIT (Broom, 2015). These compounds produced have positive effects on intestinal disorders, infections and immune responses (Broom, 2015). In another study by Fei Zhang (2019) the pathogenesis of inflammatory bowel disease (IBD) this is caused by the loss of equilibrium among the gut microbiota, epithelial cells, and resident immune cells. The gut microbiota contains a large proportion of probiotic commensal Lactobacillus species; some natural microbiota and probiotics provide protection against IBD.

On the other hand not only can bacterial counts in the gut be reduced but also increased, pigs were exposed to low doses of DON which increased aerobic intestinal bacteria (Broom, 2015). Additionally, mycotoxins promote *Salmonella* invasion of the intestinal epithelial cells which affects the host susceptibility, bacterial metabolism including the expression of virulence factors (Broom, 2015).

2.2 Pearl Millet in Namibia

Pearl millet, *Pennisetum glaucum* as seen in figure 1, is an annual grass in the family Poaceae which is grown widely in Africa and India for its grain which can be used to make flour and other foodstuffs. Pearl millet is a very robust grass consisting of branches that can develop from the leaf axils of the main shoot during vegetative growth and grows by using its own adventitious roots . Its stem are slender and divided into distinctive nodes (Plantsvillage, 2020).

Figure 1. Harvested Pearl Millet

Pearl millet also known as mahangu as seen in figure 2a and figure 2b is a crop found in regions like Zambezi, Kavango East, Kavango West, Ohangwena, Omusati, Oshana, Oshikoto, and in parts of the Otjozondjupa region and in the Tsumkwe area. The crop is known to be highly adapted to reduced rainfall and the prevailing soil conditions in the North Central Regions and the Kavango (Namibian Agronomic Board, 2020).

Pearl millet is grown in Namibia under both irrigation and dry conditions. The production of grain in Namibia provides the foundation for the growth of agricultural income, rural employment, subsequently contributing to a reduction of import products and household food security (Shifiona, Dongyang and Zhiquan, 2016).

The Namibian Agronomic Board (2020) speculates that for many years, small-scale farmers have survived on the low yields obtained from mahangu. Mahangu farmers in Namibia are amongst the few people in Africa who have successfully developed an integrated food storage system where they can store their grain in storage baskets made of wood strips for almost five years (Namibian Agronomic Board, 2020). Storage in these baskets provides a suitable environment which allows fungi to grow. This growth usually involves the production of secondary metabolites secreted into the grains.

Hybrid assortments of pearl millet are propagated to advance disease resistance and increase yield productivity, a common hybrid variety that is used locally is Okashana 1. This variety was developed in India from a natural growing variety from Burkina Faso and it is known to produce twice the amount of yield. Okashana 1 grain is now also used in Chad, Mauritania and Benin as stated by Keulder (2019).

Figure 2A. Ground pearl millet **Figure 2B.** Cooked pearl millet

Namibia is known to have an erratic annual rainfall, which has an average of 350 mm–700 mm and the temperature can reach 40 °C in summer, and thus crop production activities are limited, mainly due to dry condition and poor soil fertility (Shifiona, Dongyang and Zhiquan, 2016). Izumi et al. (2018) described Namibia has experienced persistent periods of dry weather during the rainy season also known as the dry spell, which affects the country's main cropping areas of the densely inhabited northern region and have caused a significant decrease in the production of pearl millet in Namibia. According to Shifiona, Dongyang and Zhiquan (2016) the performance of agricultural production in Namibia fluctuates in response to climatic conditions, therefore food self-sufficiency varies from 35% to 75% of total demand. Commonly in north-central Namibia, more than 90% of the farmers cultivate pearl millet as a staple food crop (Izumi et al., 2018).

2.3 Contribution of Gut Microbiome to the Human Body

The microbiome makes vital contributions to energy homeostasis, metabolism, gut epithelial health, immunologic activity and neurodevelopment (Barko et al., 2017). The introduction of solid food during weaning transitions the gut whereby the gut microbiome evolves the ability to scavenge energy from complex carbohydrates, metabolize xenobiotics, and participate in vitamin biosynthesis for instance cobalamin, biotin and thiamine these are all features of a mature microbiome (Barko et al.,2017). Interestingly, bacterial communities involved with plant polysaccharide metabolism might be present before the introduction of solid food, preparing the infant gut for plant-derived nutrients (Barko et al., 2017). This finding emphasizes the impact of the gut microbiome on the development and wellbeing of humans. Recent studies in laboratory animals have shown that at a certain point the intestinal microbiome directs the development of the immune system, gut epithelium, and brain, among other body systems (Barko et al., 2017).

The static or cidal effect of the secondary metabolites, will affect the function on the gut microflora in the gastrointestinal (GI) tract. The authors Barko et al. (2017) further states that the ileum and colon are much more active immunologically than the proximal small intestine. The microbiome in small intestinal crypts, for instance regulates enterocyte proliferation by influencing DNA replication and gene expression, while the microbiome found at the tips of the villi regulate the expression of genes involved in metabolic and immune function (Barko et al., 2017). The enterocytes and paneth cells in turn regulate luminal bacteria through the production of mucus and antibacterial factors (Barko et al., 2017). The human eukaryotic microbiome includes pathogens, commensals and beneficial organisms.

The fungi harbor a wide diversity of organisms, with an overlap for the skin with the local environment (Thomas et al., 2017). The fungi include the *Ascomycota* for instance *Candida albicans*, *Basidiomycota* for instance *Cryptococcus neoformans*, *Microsporida* for instance *Encephalitozoon intestinalis*, *Zygomycota* for instance *Rhizopus microspores* (Thomas et al., 2017). As fungi are part of the environment and human alimentation, it may be difficult to differentiate between transient and commensal organisms, unless a disease or an opportunistic infection occurs (Thomas et al., 2017).

According to Thomas et al. (2017) as humans get older, the composition of the gut microbiome also changes, aging is accompanied by the onset of a myriad of clinical changes, including a basal pro-inflammatory state that directly interfaces with the microbiota of older adults and enhances their susceptibility to diseases that accompany aging. Studies in older adults demonstrated that the gut microbiota correlates with diet and abdominal inflammation, a variety of clinical problems in older adults have been made, for instance colitis, vulvovaginal atrophy, colorectal carcinoma and atherosclerotic disease (Thomas et al., 2017). With information like this knowing that Northern parts of Namibia consist of elderly people, these people survive on pearl millet as their daily source of nutrition. If the pearl millet becomes contaminated with fungal species and their metabolites. This pearl millet is then consumed by the elderly and can lead to serious medical conditions.

Certain foodborne molds and yeasts can also stimulate allergic reactions. Also fungus from the genus *Aspergillus* have the ability to produce large numbers of air-disseminated spores which can result in respiratory diseases in humans (Cai et al., 2020). It is further stated by Greene (2021) that these fungi are known to cause superficial, cutaneous, and deep-seated mucosa-associated infections in immunocompetent hosts and disseminated, life threatening infections in immunocompromised or postoperative patients. Most common species, especially from invasive infection is *Trichosporon inkin* (Kidd et al., 2016).

2.4 Fungal Growth

Fungi is known to have originated from a flagellated ancestor, but most current diversity encompasses non-flagellated cells that often grow in a form of simple multicellularity called the mycelium, a true cellular network that sometimes extends over large areas (Naranjo-Ortiz and Gabaldón, 2020). Next from this mycelial growth, many fungal organisms can switch to a unicellular growth form for instance yeast, but this often depends on the environmental conditions or on the stage of the life cycle of the fungi (Naranjo-Ortiz and Gabaldón, 2020). In several lineages of yeast and mold throughout the fungal tree of life, mycelial growth has been abandoned, often completely but sometimes only to be recovered later where some other lineages have taken multicellularity one step further, originating complex fruiting bodies (Naranjo-Ortiz and Gabaldón, 2020). It is further stated by Naranjo-Ortiz and Gabaldón (2020) that the physical size of fungal networks allows the instantaneous exploitation of a diverse range of nutrient sources, which is a huge benefit for a sessile microbe. Furthermore, fungi are capable of forming tissues using their filamentous cells, these are used as support structures for the distribution of propagative spores (Kües, Khonsuntia & Subba, 2018). The origin of this complex multicellularity presents many similarities with that of plants and animals.

The mycelia of fungi are able to sense a wide range of physical and chemical signals. According to Naranjo-Ortiz and Gabaldón (2020), fungal sensory systems are functionally very identical to those found in plants. For example, fungi are able to sense diverse wavelengths of light to adjust to their surroundings, similar to phototropin-mediated signaling found in plants (Naranjo-Ortiz and Gabaldón, 2020). Several fungi gene expressions can be affected by light, including those involved in important processes such as reproduction, morphogenesis, virulence and metabolism (Naranjo-Ortiz and Gabaldón, 2020).

The fruiting bodies of fungi are made from dikaryotic mycelium, producing a vegetative hyphal tissue which protects fertile hyphae and this is the site of meiosis (Naranjo-Ortiz and Gabaldón, 2020).

Fungal species play an important role in the environment but a certain percentage can be pathogenic towards humans and can grow in different morphologies which plays an important part in their virulence. Certain fungal pathogens grow as yeast which is about the size of a red blood cell and are therefore well suited for bloodstream dissemination (Min, Neiman and Konopka, 2020). On the other hand, molds which are multicellular chains of filamentous hyphal cells grow invasively and disseminate into tissues (Min, Neiman and Konopka, 2020). Additional morphological forms can be seen in some fungi, such as large spherules that consist of hundreds of endospores (Min, Neiman and Konopka, 2020).

2.5 Harmful Fungal Species in Food

Food is an essential component for the very existence and sustenance of life, whether it be of plant or animal origin. It contains nutrients like carbohydrates, protein, fat, vitamins, and minerals, which provide nutritional support for the growth and maintenance of an organism. On the other hand, it is also known to cause major health problems for people around the globe, as it acts as a common transmission route of diseases due to the presence of microorganisms and contaminants (Bhalla, Monika, Sheetal and Savitri, 2019). Food supports the growth of microorganisms, and sometimes it contains anti-nutritional components and toxins that a plant or animal produce for self-defense (Bhalla, Monika, Sheetal and Savitri, 2019).

Bhalla, Monika, Sheetal and Savitri (2019) further state that microorganisms causing food-borne illness are found in a wide range of foods with various virulence factors and it may illicit a diverse range of adverse responses that may be acute, chronic, or intermittent. Some microbial pathogens are invasive and may cause generalised infections while pathogens produce toxins that cause severe damage in susceptible tissues and organs (Bhalla, Monika, Sheetal and Savitri, 2019). A definition provided by Bhalla, Monika, Sheetal and Savitri (2019) states that food-borne diseases are defined as any disease that is infectious or toxic in nature and is caused by ingestion of contaminated food.

Several foodborne molds and possibly yeasts, may also be hazardous to human or animal health because of their ability to produce toxic secondary metabolites. Most secondary metabolites consist of properties that allow the metabolites to be stable compounds that are not destroyed during food processing or home cooking (Tournas and Stack, 2021). Even though the mold or yeast is destroyed during the process of food preparation, the secreted toxin may still be present (Tournas and Stack, 2021). Therefore this can be speculated for the secondary metabolites or spores of fungi. During the process of ingestion of contaminated pearl millet as known as mahangu, secondary metabolite or spores may endure the acidic conditions of the stomach. This might lead to the gut where the pH level ranges from 5-7 this is the ideal pH for fungal spores to germinate also for secondary metabolites to cause a negative impact on gut microflora. This can either cause bacteriocidal or bacteriostatic effect. Upon ingestion of contaminated food, the GIT is particularly affected by the harmful secondary metabolites because the intestinal membrane in the GIT mainly functions as a filter and it is in direct contact with the toxic secondary metabolites found in contaminated food. However, some mycotoxins have been found to exert their detrimental effects in the GIT. For instance, mycotoxins can alter the normal intestinal functions such as barrier function and nutrient absorption (Liew and Mohd-Redzwan, 2018).

Foodborne diseases are the major contributors to the estimated 1.5 billion annual episodes of diarrhea in children under the age of 5 years, and up to 70% of such episodes are due to ingestion of contaminated foods. A study conducted in Namibia by Nawases et al. (2019) found that dried Cleome gynandra contained most of the fungal species which were *Pichia burtonii, P. macrostoma, Dothidealus*, and *A. parasiticus*. However, *Aspergillus* species were present in all the food samples and *A. parasiticus* was found in all food samples except the ground nuts. These could be an indication that the fungus can survive in low water activity.

The chronic sequelae also known as secondary complications following food-borne infections are also the cause of concern recognised together with the variability of human response, these secondary complications could be more serious and result in terrible chronic disorders or even death. A child that is from a homestead where there is malnutrition and infection, does not survive under these circumstance (Bhalla, Monika, Sheetal and Savitri, 2019).

Yeasts and molds are known to cause various deterioration and decomposition of foods. Yeast and mold can invade and grow on practically any type of food at any time; fungi invade crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage and also grow on processed foods and food mixtures (Tournas and Stack, 2021). The ability to detect fungi in or on foods depends on food type, organisms involved, and degree of invasion. The severity of contaminated food can range from being slightly blemished, severely blemished or completely decomposed (Tournas and Stack, 2021). The actual growth of fungi on food can be observed as rot spots which consist of various sizes and colors, unsightly scabs, slime, white cottony mycelium, or highly colored sporulating molds (Tournas and Stack, 2021). Abnormal flavors and odors can also be produced. When food is contaminated by yeasts and molds this can result in substantial economic losses to producer, processor, and consumer.

Obligate saprophytic fungi obtain nutrients from dead organic matter during their entire life, while many phytopathogenic and entomopathogenic fungi survive on saprophytic growth when they form propagules at the end of their life cycle. Mycoparasitic fungi like *Trichoderma, Gliocladium* and *Clonostachys,* with a wide range of hosts feed either on living fungal mycelia or on dead organic matter and may also colonize living plants. The interspecific competition is separated into exploitation competition, which arises when the use of a resource by one species reduces its availability for another species and into interference competition, which usually occurs when one species directly restrains the growth or spread of a competitor. In microbial communities, exploitation competition lowers the chances of the competitor to obtain the substrate by its depletion on the other hand interference competition constrains competitor's growth by antibiosis (Chatterjee et al., 2016).

The first requirement for fungi to survive and grow is accessibility of nitrogen and energy sources, when grain is intact this is a great strategy to reduce fungal exposure to energy and amino acids inside the grain kernel. Grain has a cellulose or polyester covering to protect the grain from exposure (Rodberg, 2021). As long as the protective barrier surrounds the grain, mold or yeast growth on grain transpires at a slower rate, if at all (Rodberg, 2021).

A study done on fermented drinks and yeast focused on the interactions between yeasts and *Lactobacillus* (LAB), it was recognized that the interaction can also be both synergistic and antagonistic, but are usually mutual benefit (Johansen et al., 2019). Mutualistic interactions between yeasts and LAB was observed in a traditional drink ogi. This is a drink which consist of none or very low alcohol and is a cereal-based beverage

. The yeast are known to benefit from a decline in pH by the acidification which is caused by the activity of the LAB, simultaneously a substantial higher growth of *Lactobacillus plantarum* has been shown when co-cultured with either *Saccharomyces cerevisiae* or *Pichia kudriavzevii*, this indicates that these yeast species provide growth factors for the LAB, most likely amino acids. Mutualistic interactions have equally been described amongst species of LAB and yeasts originating from indigenous sub-Saharan African fermented milk products (Johansen et al., 2019).

In cereal based foods, the presence of anti-nutritional factors such as the highly charged phytate produced by yeast limits the bioavailability of divalent ions through the process of chelation of cation minerals such as $Fe2^+$, $Zn2^+$, $Ca2^+$, and $Mg2^+$. The phytate complexes made by chelation are insoluble at bodily pH and therefore, the divalent ions cannot be absorbed in the human intestine (Johansen et al., 2019). Most of the yeast species are capable of surviving at conditions equivalent to the human gastrointestinal tract which is at low pH (2.5), in a mass concentration of 0.3% and bile salts at 37°C (Johansen et al., 2019).

According to Johansen et al. (2019), if yeast had to be compared to mold, food borne yeasts are generally not known to be antagonistic pathogens and are barely linked with outbreaks of foodborne gastroenteritis, intoxications or other infections.

Numerous species of the genus *Candida* are broadly disseminated in nature and have commonly been isolated from indigenous sub-Saharan African fermented products. Most of these species are harmless, but on the other hand some *Candida* spp. can take advantage of a locally or systemically impaired immune system to proliferate in the host and cause diseases generally termed candidiasis. These species *Candida albicans* is the most frequently isolated cause of candidiasis, these species cause about 41–47% of total yeast infections globally. However, other candidiasis infections are also widespread with worldwide incidence in invasive candidiasis as follows *Candida glabrata* 18–27%, *Candida parapsilosis* 16–18%, *Candida tropicalis* 9–11%, and *Candida krusei* 1–3% (Johansen et al., 2019).

During the production of mycotoxins usually on grains, this production is extremely dependent on pre-harvest and postharvest environmental factors for example temperature and moisture content (Smith, Madec, Coton and Hymery, 2016). These secondary metabolites are frequently subdivided firstly into field mycotoxins produced on cereal crops before or instantly after harvest primarily by *Fusarium spp*., and secondly storage mycotoxins, predominantly secreted by *Aspergillus spp.* and *Penicillium spp*. during commodity drying and storage (Smith et al., 2016). There are a few factors that can rapture the grain coating. When rapid drying of grain occurs, it causes the development of cracks or fissures which can reduce the protective barrier, consequently resulting in dry matter loss of stored grain (Rodberg, 2021).. Also insect damage can expose starch to mold and wild yeast (Rodberg, 2021). Finally, grinding the grain kernels removes the protective coating and exposes the starch, fat and nitrogen to microbes, therefore grinding grain should be delayed until the time of usage to minimize exposure to starch which leads to fungal degradation (Rodberg, 2021).

A valuable observation detected by Cai et al. (2020) was that the stover gained no spoilage at 31℃ after 120 days of exposure in the field. The meteorological conditions affecting the survival of yeast and mold clearly explain why the stover can be stored for such a long time during the dry season in West Africa (Cai et al., 2020). The temperature in West Africa is similar to the Northern parts of Namibia, as temperature can reach up to 37℃. Therefore locals are also able to use stored pearl millet after months not only because of temperature but also because of moisture content. But a statement made by Tournas and Stack (2021) concludes that the temperature can range from 10-35°C, with a few species capable of growth below or above this range. For moisture requirements of foodborne molds are relatively low; most species can grow at a water activity (aw) of 0.85 or less, although yeasts generally require a higher water activity (Tournas and Stack, 2021). But this statement is dependent on a number of factors, if conditions including nutrient level and oxygen level are in appropriate amounts. Also if microbial competitors are absent or less, then growth is possible at temperatures ranging above 30 °C. The northern parts of Namibia temperatures are high but pearl millet are dried therefore affecting the moisture content, which results in inhibiting the growth of mold and yeast. Only when environmental conditions are favorable especially an increase in moisture content and nutrient level will result in growth of fungi.

The effects of secondary metabolites are known to be liver or kidney deterioration, other mycotoxins are neurotoxins, while still others interferes with protein synthesis (Sweeney, 1998). Fungi contaminants of human food belong mainly to three genera: *Aspergillus*, *Fusarium* and *Penicillium* (Sweeney, 1998). While *Fusarium* species are destructive plant pathogens producing mycotoxins before, or immediately post-harvesting, *Penicillium* and *Aspergillus* species are more frequently found as contaminants of food during drying and storage (Sweeney, 1998).

In a study conducted by Jurjevic et al. (2015) the following fungi were isolated from pearl millet *Fusarium chlamydosporum* 19%, *Curvularia spp*. 14%, *F. semitectum* 16%, *Alternaria spp*. 9%, *Aspergillus flavus* 8%, "*Helminthosporium" spp*. 6%, and *F. moniliforme* 3%. The study also revealed isolation frequency was rarely affected by temperature, relative humidity or atmosphere treatments, but was affected by storage duration for some fungi (Jurjevic et al., 2015). According to the findings of Jurjevic et al. (2015) changes in isolation of toxigenic fungi occurred in high-moisture pearl millet.

It is stated by Europa (2021) that fungi can grow over a wide range of temperatures and, in general the rate of mold growth specifically will decrease with decreasing temperature and available water. In grains, molds are known to utilize inter-granular water vapor for metabolic processes and growth. When focusing on moisture content, different grains will consist of a different water holding capacity thus, these grains will support different rates and type of mold growth (Europa, 2021). In addition the metabolic activity of insects and mites can contribute and increase the moisture content and temperature of the infested grain (Europa, 2021). Arthropods can also act as carriers of mold spores and furthermore their fecal material can be utilized as a food source by mold.

When the body's gut microbiota is disturbed it leaves the body vulnerable since there is a reduced amount of gut microflora to counteract the secondary metabolites. Subsequently this leads to the uptake of mycotoxins which leads to tissue disruption. In addition, this affects the intestinal functions and impair local immune response. Therefore maintaining a balanced gut microflora is essential since only an unbalanced gut microflora is more susceptible to the effects of pathogenic fungi.

2.5 Mold and Yeast

Filamentous fungi are ubiquitous in nature and can be isolated from their environments under varying conditions in terms of temperature, pH and humidity (Nielsen, 2018). Various filamentous fungi are saprophytes, which means that they grow on dead organic matter that they degrade through the secretion of enzymes. They play important roles in the ecosystem as principal decomposers (Nielsen, 2018). Another aspect where fungi plays an important role in the ecosystem is the secretion of bioactive compounds, which are used as a means of communication with other organisms (Nielsen, 2018). Colonization of nutrient sources is achieved by growing in a network of branching tubes, hyphae, where transportation of nutrients between cells takes place through septate perforations (Nielsen, 2018).

Conditions favourable for the growth of yeast are normally cool temperatures, these conditions are unfavourable for bacteria species that are responsible for the fermentation and preservation of ensiled feeds (Dairy land Laboratories, 2021). Yeast have adapted a mechanism to establish a suitable environment for growth. Firstly high endemic yeast population is ensiled, during slow fermentations moderate growth of yeast occurs until oxygen is depleted in the silage (Dairy land Laboratories, 2021).

2.6 Secondary Metabolites

Secondary metabolites are derived from central metabolic pathways and primary metabolite pools, acyl-CoA is the critical initial building blocks that feed into the synthesis of polyketide for instance like aflatoxin and terpene for example, carotene with secondary metabolites and amino acids being used for the synthesis of non-ribosomal peptide secondary metabolites for instance penicillin (Keller, 2018). Secondary metabolites are crucial players in fungal development, protection against toxins and actively assist with the interactions of fungi with other organisms. The biosynthetic enzymes that produce secondary metabolites are encoded by genes which are physically clustered in the genome, and are clled biosynthetic gene clusters (Pfannenstiel and Keller, 2019).

According to Dairyland Laboratories (2021) certain molds produce mycotoxins under certain conditions, but the presence or absence of mold does not directly specify the presence or absence of mycotoxins. Phytochemical and chromatographic analysis indicated a variety of secondary metabolites in all of the fugal extracts with the highest antimicrobial activity observed in *A. flavus* from its external secondary metabolites (Alkhulaifi et al., 2019). For instance, *Fusarium* molds have been described to grow prolifically at 25-30°C without producing much mycotoxin, but when the temperature was decreased close to freezing temperatures *Fusarium* mold yielded large quantities of mycotoxins with minimal mold growth (Dairyland Laboratories, 2021). Molds can be detrimental to animal performance regardless of mycotoxin contamination.

In a research article by Freimoser, Rueda-Mejia, Tilocca and Migheli (2019) yeast secreted enzymes in nutrient poor conditions and serve the provision of nutrients like carbon sources and amino acids that are released from targeted cells, which may lead to the killing of these cells this is also known as mycoparasitism or fungivory. The type of enzymes secreted are chitinases, glucanases and proteases (Freimoser et al., 2019).

Also yeast are capable of producing volatile organic compounds (VOC) these are small and less than 300 Da molecules with low water solubility and high vapour pressure

(Freimoser et al., 2019). These VOC include an array of molecular classes, which consist of hydrocarbons, alcohols, thioalcohols, aldehydes, ketones, thioesters, cyclohexanes, heterocyclic compounds, phenols and benzene derivatives (Freimoser et al., 2019). The chemical composition of each blend of volatiles is not constant for that reason it may change depending on the producing yeast, the antagonized pathogen and the ecological niche where the cross-talking species are growing (Freimoser et al., 2019). One of the yeast *S. cerevisiae* is able to secrete a killer activity and hydrolytic enzymes as well as organic volatile compounds, specifically alcohols and esters (Freimoser et al., 2019).

Many of the fungi synthesize some secondary metabolites during their growth in different foods. It was stated by Keller (2018) that a certain concentrations of a metabolite could function as a signal rather than as a toxin. This possibility was illustrated by the gradient-dependent effects of Pseudomonas aeruginosa phenazine on Aspergillus, whereby a high concentration of phenazine is antifungal but a moderate concentration induces prolific sporulation in the fungus These metabolites are highly toxic and carcinogenic in nature and are called mycotoxins. Mycotoxins have caused many dreadful epidemics in the past, during 1942-1948 alimentary toxin aleukia became responsible for claiming 100,000 lives in Russia (Bhalla, Monika, Sheetal and Savitri, 2019).

Research indicates that mycotoxins appear to have no function in the normal metabolism involving growth of the fungus. These structures vary from single heterocyclic rings with molecular weights of scarcely 50 Da, to groups of irregularly arranged 6 or 8 membered rings with total molecular weights greater than 500 Da. Because of their small molecular size, these molecules do not induce any response in the human immune system. That is why secondary metabolites are a major potential danger in the human diet this because of our inability to detect them biologically (Pitt, 2000).

Deoxynivalenol (DON), fumonisins (FUM) and zearalenone (ZEA) are the most predominant mycotoxins in the world, with an incidence of 66%, 56%, and 53%, respectively, based on the 6844 analyzed agricultural commodity samples. Since mycotoxins commonly co-occur, other mycotoxins can also potentially occur with aflatoxin (AF) which consist of a worldwide prevalence of 22%. The occurrence of the pairs AF and FUM and also AF and ZEA binary combinations as well as the AFs, ochratoxin A (OTA) and ZEA ternary combination were observed to be 29%, 21%, and 29% respectively (Smith et al., 2016). Focusing on African samples, more than 26 combinations of mycotoxins were observed; this includes AF and OTA was found to be the main combination (Smith et al, 2016). The AF and FUM also AF and ZEA binary combinations as well as the AF, OTA and ZEA ternary combination were also observed (Smith et al., 2016).

The occurrence of deoxynivalenol is not affected by storage conditions, the study also revealed that low concentrations of nivalenol were detected in most of the pearl millet grain incubated at 100% relative humidity (Jurjevic et al., 2015).

2.7 Negative impact of Secondary Metabolites on the Gut Microflora and the Body

There is over 98% of the gut microbiota which is composed of four phyla of bacteria these are *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria* which all play an important role in the digestive system (Huang, Fan, Ying and Chen, 2019*)*. The most dominant are from either *Firmicutes* or *Bacteroidetes*. Research has revealed that *Bacillus* subgroup of *Firmicutes* and *Actinobacteria* are more prevalent in the small intestine, while *Bacteroidetes* and *Lachnospiraceae* are more prevalent in the colon. Interesting fact is that the human gut microbiome is more similar among family members than unrelated individuals; intra-personal differences are minor compared to inter-personal differences in gut microbiota, indicating short term environmental change does not play a major part in microbiota composition (Huang et al., 2019). The fermentation process by gut microflora produces monosaccharides and short chain fatty acids which include acetate, butyrate, and propionate that are rich energy sources for the host (Liew and Mohd-Redzwan, 2018). Equally the metabolism pathways for the production of proteins, bile, and phytochemicals, as well as vitamins involve the gut microbiota and is said to be important to the human body (Liew and Mohd-Redzwan, 2018).

According to Pitt, (2000) mycotoxins consist of four basic kinds of toxicity: acute, chronic, mutagenic and teratogenic. The most frequently described effect of acute mycotoxin poisoning is deterioration of liver or kidney function, which in extreme cases may result in death (Pitt, 2000). However, certain mycotoxins act primarily by interfering with protein synthesis, and yield effects ranging from skin sensitivity or necrosis to extreme immunodeficiency (Pitt, 2000). Mycotoxins with neurotoxic effects, may in low doses cause sustained trembling in animals; only considerable elevated doses cause brain damage or death (Pitt, 2000). Long-term effects of low levels of secondary metabolites ingested also vary. The major chronic effect of many mycotoxins involves producing cancerous cells, especially of the liver (Pitt, 2000). Some toxins affect DNA replication, and hence can produce mutagenic or teratogenic effects (Pitt, 2000).

Secondary metabolites can disrupt the following functions of the gastrointestinal tract microbiome; fermentation of dietary fibers into short chain fatty acid which counts up to 10% of the human energy source, degradation of complex polysaccharides, and synthesis of indispensable vitamins and amino acids (Huang, et al., 2019). The gut microflora also produce multiple metabolites that function in protecting epithelial lining integrity, stimulating intestinal angiogenesis, and regulating immune response (Huang, et al., 2019). These reflect that gut microflora are not just commensal with human hosts, but there is a mutualistic relationship with the host.

Cell morphology similarly affects how fungi interact with the immune system. Yeast cells can be phagocytosed by leukocytes but their long hyphal filaments are too large to be attacked in this manner (Erwig and Gow, 2016). Remarkably, some yeasts have developed strategies for surviving in the phagosome, and therefore use this trojan horse mechanism to their advantage to disseminate within the host (Erwig and Gow, 2016).According to Min, Neiman and Konopka (2020) spores which enter the body are small enough to be phagocytosed by macrophages, therefore the beneficial part of transforming to filamentous growth for fungi is that the emerging hyphae can penetrate through the macrophages membrane this is because they are too large to be phagocytosed. Remarkably, in vitro studies conducted by Ellett et al. (2017) have revealed that interaction with neutrophils can encourage hyphal branching as an evasive.

Typically, when hyphae are too big to be phagocytosed, neutrophil extracellular traps (NETs) are developed where the neutrophil DNA is utilized to entrap the hyphal filaments and offer a platform in which antimicrobial peptides and other toxic compounds are focused on the fungus (Urban and Nett, 2019).

CHAPTER 3. RESEARCH METHOD

3.1 Research Design

This study focused on qualitative data by identifying the type of fungi found in the pearl millet and on quantitative data which was the antimicrobial activity of the fungi against selected bacteria.

3.2 Isolation and culturing of fungal strains from the contaminated pearl millet

The stored pearl millet can be a source of nutrients for various microorganisms such as fungi. Therefore in this study culturing and identifying the fungal species from pearl millet was essential procedure in the methodology.

Following a protocol by Mishra and Daradhiyar (1991) with a few modifications, the pearl millet was purchased from an open market at Single Quarters in Windhoek. The pearl millet was then stored at room temperature 27 °C in a closed container for at least 3 months. Serial dilution was performed, this was done on the collected pearl millet sample by mixing 1g of the pearl millet with 100ml of distilled water, and this was the stock solution. Then 5 tubes containing 9 ml of distilled water was obtained and labelled from 1-5. Test tube 1 obtained 1 ml of the stock solution, then 1 ml of the mixture in test tube 1 was transferred to test tube 2, this process was carried out for test tubes 3, 4 and 5. Test tube 5 with a dilution of 1:100 000 was selected and 1ml of the mixture was plated on 10 Sabouraud Dextrose Agar (SDA) plates. These plates were kept in an incubator for 7-14 days at a temperature of 27 °C. After growth of fungi was observed, pure cultures were obtained by cutting a small piece of agar consisting of the fungal culture and placed on new SDA agar medium.

3.3 Identification of fungal species isolated from the pearl millet

Fungal species exist in different shapes, forms and colors. For this reason identifying the isolated fungi was important because this specified which type of fungi are inhabitants of pearl millet.

The fungal colonies growing on the SDA plates were isolated and examined. Examination of fungi was based on the colony morphology on the SDA plates. Each SDA plate was examined focusing on the form, elevation and margin of the colonies. Furthermore the colonies were examined microscopically by using a compound microscope. Forceps were used to collect samples of the fungal isolates, which were placed on a slide. Then 2 to 3 drops of lactophenol blue was added to the slide consisting of the fungal sample, the slides were viewed under the microscope. Fungi were identified based on of colony characteristics and microscopic examinations using a compendium of soil fungi (Dunn, Domsch, Gams and Anderson, 1982).

3.4 Extracting intracellular and extracellular fungal secondary metabolites from isolated fungal strains

The production of intracellular and extracellular secondary metabolites by fungal species is common this can be caused by different factors. Fungi produce intracellular metabolites enclosed in the cell membrane therefore it is important to extract these metabolites. Extracellular metabolites mainly produced as by-products of metabolic processes are secreted into the environment. Both intracellular and extracellular secondary metabolites have the potential to affect the growth of bacteria. Hence the secondary metabolites were extracted for antibacterial purposes.

3.4.1 Extraction of Extracellular metabolite

After a pure culture of fungal strains were obtained, fungi cultures were transferred by using a razor blade and cutting small sample pieces of fungi growing on SDA and inoculating the fungal species into a 100ml of broth. The broth was made of 40g glucose, 10g peptone and 1L of distilled water in a 1L container. After the broth cooled down antibiotics namely nalidixic acid with a concentration of 10mg/ml was added to the broth.

Extracellular metabolites were extracted from the fungi, following the procedure by Osamwonyi and Wakil (2012) with a few modifications. After the incubation period in the broth for 7 days the fungus was obtained by the filtering method. Using Whatman filter paper the mycelial biomass and filtrate was obtained. The fermented broth was collected in 50ml falcon tubes and equal amounts of ethyl acetate was added to the broth to homogenize it. The mixture of ethyl acetate and fermented broth in the falcon tubes was then centrifuged at 4000 revs/min without the mycelial biomass for 20min.

After centrifuging 2 layers formed, the top layer was removed and placed into beakers while the bottom layer was discarded. The beakers consisting of the top layer liquid were placed under a fume hood until the liquid evaporated. Once the liquid evaporated 10 ml of ethyl acetate was added into the beakers containing the extract. The liquid consisting of the extract were stored in glass vile in a dark cool place.

3.4.2 Extraction of Intracellular Metabolites

The mycelial biomass was firstly washed with double distilled water. The mycelial biomass was collected by filtration using Whatman filter paper, this was weighed after collecting the mycelial mass. The mycelial was then dried in an oven and was also weighed. The dried fungal mass was crushed with a mortar and pestle, 10 ml of ethyl acetate was added to a beaker and 2 g dried mycelial were also added. The beakers containing the mixture was placed on a shaker for 24hr. The liquid was removed and placed in a separate container and the solids were discarded.

The intracellular extracts and the extracellular extracts were both mixed into one container and stored in a glass vile in a refrigerator (Osamwonyi and Wakil, 2012).

3.5 Evaluation of crude fungal extract for antibacterial activity against gut microflora

Certain secondary metabolites are known to possess antibiotic properties. For this reason this study focused on the antibacterial activity of the secondary metabolites from the isolated fungi on selected bacteria strains: *Escherichia coli* ATCC 33849, *Klebsiella pneumoniae* ATCC BAA- 2146, *Listeria monocytogenes* and *Lactobacillus plantarum* ATCC 8014.

3.5.1 Preparation and culturing of test bacteria

Nutrient broth medium was prepared by dissolving 3.2g per 200ml of distilled water for the growth of the bacterial strains where the pH was adjusted to 7.0. About 9ml of the broth was then transferred into screw capped test tubes and then autoclaved. The nutrient broth was inoculated with the bacterial strains in screw-capped tubes. The broth was incubated at 37 °C for 24 hours. The broth cultures were removed to avoid over growth and compared with the McFarland turbidity standard utilizing the spectrophotometer at a wavelength of 1.5 absorbance unit. The McFarland 0.5 barium sulfate turbidity standard was prepared according to the protocol provided by Raham et al (2014). Shortly 0.5 ml of 0.048 M barium chloride was added to 99.5 ml of 0.36 M sulfuric acid. The barium sulfate turbidity standard was prepared in advance and stored in screw-capped test tubes. If the inoculum was not the same as the turbidity standard, the strains were mixed with normal saline solution to obtain the same turbidity as the 0.5 McFarland turbidity standard 1.5 x 10^8 colony forming unit (CFU) ml^{-}1. These broth cultures were then stored at 4 °C.

3.5.2 Antimicrobial activity of extracted secondary metabolites

Antibacterial activity of secondary metabolites extracted from the isolated fungi was screened against 100 μL of *Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes* and *Lactobacillus plantarum* . The medium used was Muller Hinton agar (MHA) and the Agar well diffusion method was used to screen for antibacterial activity. Six equidistant wells of 6 mm in both diameter and depth were made on the plates using sterile cork borers. Approximately, 50 μL of prepared secondary metabolite extract was then dispensed into the wells, allowed to equilibrate at room temperature for 30 minutes, and then incubated overnight at 37 °C (Gasu, Ahor and Borquaye, 2018). Zones of growth inhibition measured in mm were measured as the diameter of the clear zone around each well. The assay was performed in independent triplicates and the averages of the three experiments taken. Nalidixic acid was used as a positive control for bacteria strains while ethyl acetate was used as a negative control.

3.5.3 Determination of minimum inhibitory concentration (MIC)

The extracted secondary metabolites which inhibited the bacterial growth were further subjected to Minimum Inhibitory Concentration (MIC) to determine the lowest concentration of inhibiting bacterial growth. This method was carried out by using Gasu, Ahor and Borquaye (2018) procedures with a few modifications. Approximately 500 ml of nutrient broth was made and sterilized using an autoclave. Minimum inhibitory concentration (MIC) was carried out using 1 ml 96 well plates. Only the fungal strains which showed zones of inhibition were selected for MIC. The positive control was nalidixic acid and the negative control was the broth consisting of only the bacterial culture. To each of the wells except for the positive and negative control wells, 100 µl of the fungal crude extract and bacterial culture were added respectively. The concentrations were 30 mg/ml, 15 mg/ml, 7.5 mg/ml and 3.7 mg/ml. The 96 well plates were covered and were incubated at 37 °C for 24 h and examined for growth. The lowest dilution well in which growth was inhibited was the MIC of the culture.

3.5.4 Determining Minimum Bactericidal Concentration (MBC)

The different concentrations from the MIC were used for Minimum Bactericidal Concentration (MBC) to determine the lowest level of secondary metabolites required to cause bacterial death. Following the MBC procedure of Gasu, Ahor and Borquaye (2018) with a few modifications. MBC of the fungal crude extract was determined by MIC aliquots after the 24-hour incubation period, 50 μL aliquots from wells with the fungal extract concentrations greater than the MIC were plated on sterile MHA agar plates. The agar plates were incubated at 37∘C for 24 hours. The MBC was recorded as the lowest extract concentration killing 99.9% of the bacterial, which is the least fungal extract concentration that showed no visible growth of the microorganisms on the surface of the agar plates. Each experiment was repeated three times.

3.6 Data Analysis

The qualitative results from the zones of inhibition were subjected to SPSS for statistical analysis. Shapiro- Wilk test was used to test data for normality, the p-value > 0.05 showed that the data were normally distributed. The zones of inhibition were analyzed by One Way ANOVA. The MIC results were recorded by using Microsoft Excel 2007 to formulate a graph and analyze the data. The qualitative data which were the characterization of the fungi isolated from pearl millet were tabulated with morphological descriptions.

3.7 Research Ethics

Ethical clearance was obtained from the University Research and Ethics Committee (UREG) and Centre for Postgraduate Studies (CPGS). The selected bacterial strains *Escherichia coli ATCC 33849, Klebsiella pneumoniae ATCC BAA- 2146, Listeria monocytogenes* and *Lactobacillus plantarum ATCC 8014* were purchased. Standard laboratory practices and sterile techniques were conducted, also the waste was disposed in their respective containers to avoid any contamination of the environment or health hazards. No human trials and samples were involved in this study.

Chapter 4: RESULTS

4.1 Isolation and culturing of fungal strains from the contaminated pearl millet

For Objective 1 of this study, we wanted to isolate, characterize and identify fungal species that may contaminate the pearl millet during growth and storage. After obtaining pure cultures of the fungal isolates, the fungal species were identified by observing the colonies morphology on the agar plates using microscopy. Growth and colony characteristics included the color, shape, margin and the elevation of the colonies. As shown in Table 1 *Aspergillus niger, Rhodotorula* spp., *Trichosporon* spp., *Saccharomyces* spp., *Aspergillus flavus* and *Saccharomyces* spp were identified. Morphological features of the methylene blue stained isolates are shown in Fig. 3. The presence or absence of conidiophores, hyphae, spores and branching properties were features that assisted with the identification of the fungi. Identification was done using a compendium of soil fungi (Dunn, Domsch, Gams and Anderson, 1982).

As indicated in Table 1, *Trichosporon* sp. had septated hyphe and its colonies appeared wrinkled, rugose, verrucose and white. *Aspergillus flavus* on the other hand showed filamentous hyphe with conidiospores and colonies appeared velvety and irregular; umbonate, green and yellow in colour. *Saccharomyces* sp. was wrinkled, rugose and white and showed budding structures. On the other hand the other *Saccharomyces* sp. was irregular, flat, undulate but also showed budding features with a creamy colour. Meanwhile *Aspergillus niger* had a wooly texture with filamentous hyphae and colourless conidiophores and dark conidiospores. However, *Rhodotorula* showed budding structures with a waxy colony texture, circular, raised, entire and pink in color.

Table 1. Characterization of Fungi isolated from Pearl Millet based on morphology observed on media and under a Light microscope, focusing on the presence or absence of conidiophores, hyphae, spores, branching properties, color, shape, margin and the elevation of the colonies at a magnification of 400x using immersion oil.

Number	Colony Appearance On SDA plate	Appearance of Slide culture	Fungal Name
1	Wrinkled, rugose, verrucose and white	Septated hyphae	*Trichosporon* sp.
2	Velvety colony texture irregular, umbonate and green and yellow.	Filamentous hyphae, conidiospore	*A. flavus*
3	Wrinkled, rugose, raised and white.	Budding yeast cell	*Saccharomyces* sp.
4	Wooly, filamentous, filiform with a black and white.	Smooth and colourless conidiophore and dark conidiospores.	*A. niger*
5	Irregular, flat, undulate with a creamy colour.	Budding yeast cell	*Saccharomyces sp.*
6	Waxy colony texture circular, raised, entire and pink	Budding yeast cell	*Rhodotorula sp.*

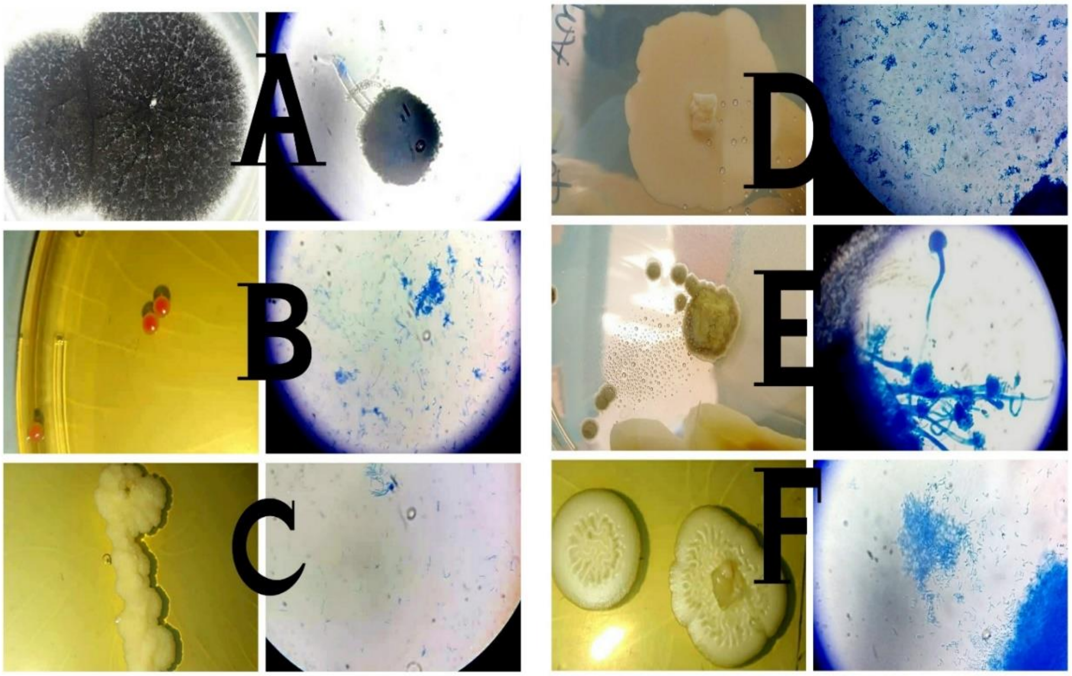


Figure 3. Fungal morphology of isolated *A. niger, Rhodotorula* spp.*, Trichosporon* spp.

Saccharomyces spp., *A. flavus* and *Saccharomyces* spp. On the left are fungi on SDA agar and on the right fungi stained with methylene blue viewed under the Light microscope at magnification 400x using immersion oil.

A- *A. niger* , B- *Rhodotorula* spp., C- *Trichosporon* spp.

D- *Saccharomyces* spp., E- *A. flavus* , F- *Saccharomyces* spp

4.2 Evaluation of crude fungal extract for antibacterial activity against gut microflora using the Agar well diffusion method

The secondary metabolites produced by the fungi were extracted and tested against bacteria strains normally found within the gastrointestinal tract as described in Section 3.4 and 3.5 of the previous chapter. This was done to establish which of the isolated fungal strains would affect the growth of the bacterial strains. Each of the test bacteria: *Escherichia coli* ATCC 33849, *Klebsiella pneumoniae* ATCC BAA- 2146, *Listeria monocytogenes* and *Lactobacillus plantarum* ATCC 8014 were tested against the extracts of all six isolated fungi. Agar well diffusion plates were done in triplicate for each test bacteria and the zones of inhibition were measured in millimeter and averaged.

The results below in Table 2, indicates the overall effect of the fungal extracts on all the bacteria these were, *Escherichia coli* ATCC 33849, *Klebsiella pneumoniae* ATCC BAA- 2146, *Listeria monocytogenes* and *Lactobacillus plantarum* ATCC 8014. This was done to identify which of the isolated fungi *A. niger*, *Rhodotorula* spp., *Trichosporon* spp.,*Saccharomyces* spp., *A.flavus* and *Saccharomyces* spp. has the greatest antibacterial effect on the gut microflora.

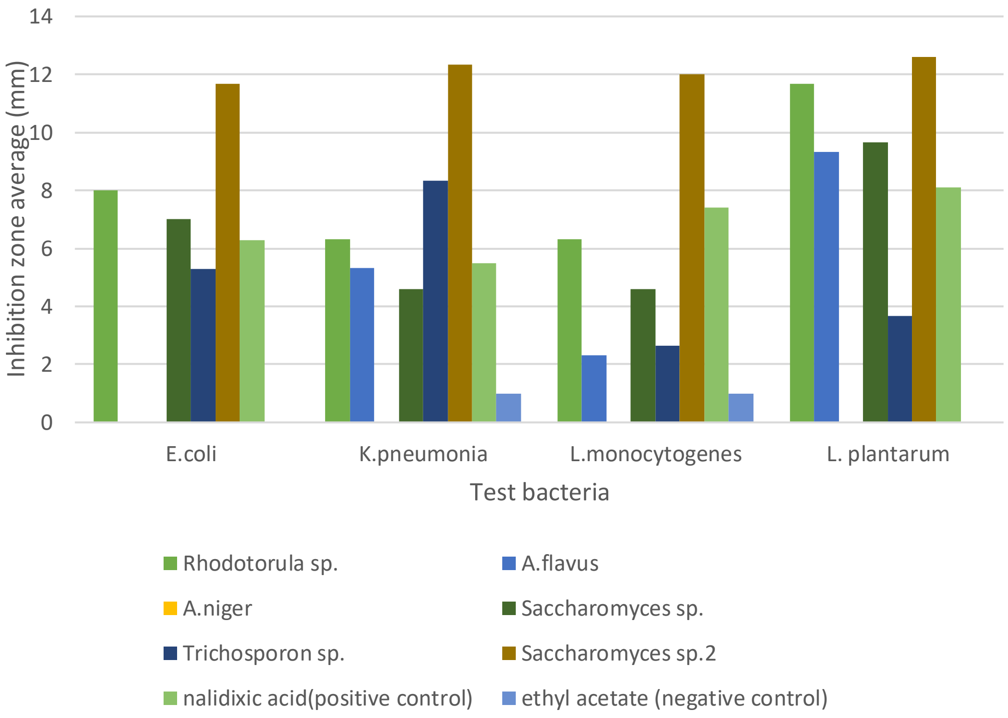
The results observed in Figure 4, specifies which fungal isolate had an antibacterial effect on the gut microflora namely *Escherichia coli* ATCC 33849, *Klebsiella pneumoniae* ATCC BAA- 2146, *Listeria monocytogenes* and *Lactobacillus plantarum* ATCC 8014. This was done to determine how the individual bacterial strains react to the secondary metabolites produced by the fungal isolates by the presence of a zone of inhibition.

Table 2. Zones of Inhibition from Fungal extracts by Agar Well Diffusion method (mm)

Fungal Species	Mean ± Standard Error of the zones on inhibition (mm in diameter)
Trichosporon sp.	4.98 ± 1.23
A. flavus	4.24 ± 2.01
Saccharomyces sp.	6.49 ± 1.19
A. niger	No zone of inhibition
Saccharomyces sp.	12.16 ± 0.21
Rhodotorula sp.	8.08 ±1.25

In Table 2, these observations were reported as mean ± standard deviation values from triplicated experiments. The Levene Statistics value was p= 0.019 this indicates that the population variance of the fungi are not equal. The mean difference level was p=0.05. The fungi *A. niger* did not show any form of antimicrobial activity and an inhibition zone value of 0.0 mm was observed. Also *Saccharomyces* sp. and *Rhodotorula* sp. showed the highest inhibitory effects with a mean value for the zone of ihibition which was 12.16 ± 0.21 mm and 8.08 ±1.25 mm respectively (Table.2). The zones of inhibition had a sig. value p = 0.019 which was less then statistical value of p= 0.05 .

Figure 4. Determining Zones of Inhibitions of *A. niger* , *Rhodotorula* spp.,*Trichosporon* spp.,*Saccharomyces* spp., *A.flavus and Saccharomyces* spp. *extracts on E. coli, K. pneumoniae, L. monocytogenes* and *L. plantarum* using Agar Well Diffusion method (mm) this was repeated 3 times (Gasu, Ahor and Borquaye, 2018).



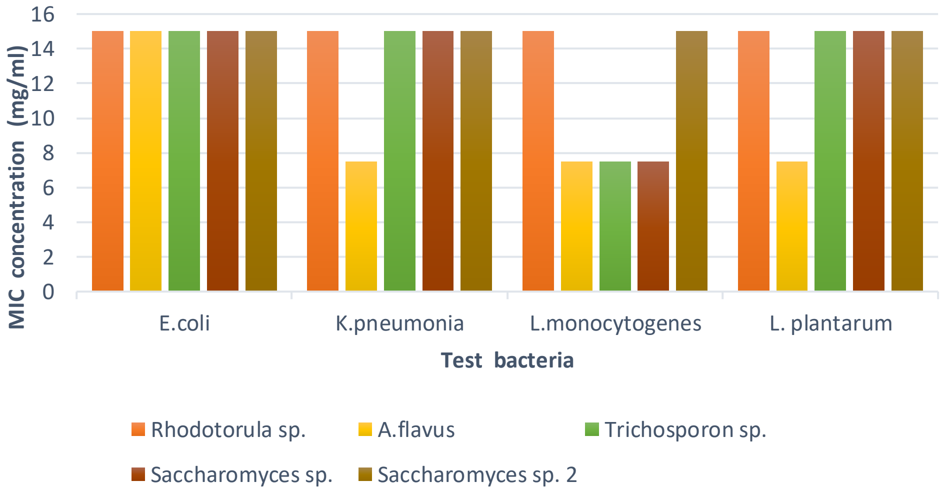
The above graph in Figure 4. shows the antibacterial effect of fungal extract at a concentration of 0.2 g/ml on *E. coli, K. pneumoniae, L. monocytogenes* and *L. plantarum*. The positive control was nalidixic acid and the negative control was ethyl acetate as shown in Figure 5 where the negative control showed no zones of inhibition. The positive control nalidixic acid showed zones of inhibition against the test bacterial strains. No inhibitory effect was shown by *A. niger* against the selected bacterial strains. The genus *Aspergillus* had no inhibitory effect on *E. coli* but *A. flavus* was the only fungus from the genus *Aspergillus* with inhibitory effect on *K. pneumoniae, L.*

monocytogenes and *L. plantarum*. The fungal crude extracts from *Rhodotorula* spp., *Trichosporon* spp., *Saccharomyces* spp. and Saccharomyces spp. showed antibacterial properties. The fungi *Saccharomyces* sp. showed inhibitory effect against all the bacterial strains.

4.3 Determination of minimum inhibitory concentration (MIC)

The fungal strains that showed antibacterial activity were selected to determine the smallest amount of the extract that would inhibit the growth of the test bacteria, which is the Minimum Inhibitory Concentration (MIC). The determination of MIC for the fungal crude extract was carried out using the 96 well plate method as described in the previous section 3.5.3. The following concentrations of the fungal crude extract were used for MIC 30mg/ml, 7.5 mg/ml and 3.7 mg/ml. A minimum inhibitory concentration was observed at 7.5 mg/ml for *A. flavus* against *K. pneumoniae*, *L. monocytogenes* and *L. plantarum*. In Figure 5 the minimum inhibitory concentration of the different fungal extracts on the selected bacteria is shown. Secondary metabolites extracted from *A. flavus* showed more sensitivity against the following bacterial strain *L. plantarum, L. monocytogenes* and *K. pneumoniae* at 7.5 mg/ml. The least sensitivity was detected in *E. coli* at 15 mg/ ml.

Figure 5. Determining the Minimum Inhibitory Concentration of *A. niger* , *Rhodotorula* spp., *Trichosporon* spp.,*Saccharomyces* spp., *A. flavus* and *Saccharomyces* spp. extracts on *E. coli*, *K. pneumoniae*, *L. monocytogenes* and *L. plantarum* using the 96 well plate method (Gasu, Ahor and Borquaye, 2018).



4.4 Determining Minimum Bactericidal Concentration (MBC) from MIC aliquots

Although inhibition of growth was observed in the test bacteria, this doesn’t indicate whether the effect of the fungal extract caused a permanent or temporary effect on the growth of the bacteria. Therefore Minimum Bactericidal Concentration (MBC) was performed to determine whether the fungal extracts contain secondary metabolites that have a cidal or static effect on bacteria. The MBC was determined by the presence or absence of colonies on the agar, the formation of colonies indicated that the fungi metabolites consisted of static properties and the absence of bacterial colonies indicated that the fungi metabolites consisted of cidal properties. As seen in section 3.5.6 aliquots from MIC 96 well plates were used for MBC. In Table 3 the secondary metabolites extracted from *A. flavus* was the only extract exhibiting cidal effects on the bacteria *K. pneumoniae* and *L. plantarum* at a concentration of 30 mg/ml. Secondary metabolites extracted from the other fungal species all resulted in static effects. Static effect was observed for all the fungi namely *Rhodotorula* spp., *Trichosporon* spp., *Saccharomyces* spp., *A. flavus* and *Saccharomyces* spp at a concentration of 7.5 mg/ml against *L. monocytogenes*. The images in Figure 8 as seen in the appendix show that at 30 mg/ml *A. flavus* secondary metabolite extract caused a cidal effect on *K. pneumoniae* and *L. plantarum.* While in Figure 9 in the appendix growth was observed in *E.coli* and *L.monocytogenes* with fungal extracts from *Saccharomyces sp.* and *Aspergillus flavus* respectively.

Table 3.Determining the Minimum Bactericidal Concentration (MBC) of *A. niger* , *Rhodotorula* spp.,*Trichosporon* spp.,*Saccharomyces* spp., *A. flavus and Saccharomyces* spp. extracts on *E. coli, K. pneumoniae, L. monocytogenes* and *L. plantarum* from MIC aliquots (mg/ml). The presence of bacteria colonies on agar plates indicate a static effect and the absence of bacteria colonies on agar plates indicate a cidal effect (Gasu, Ahor and Borquaye, 2018).

Fungal Species	*E. coli*		*K. pneumoniae*		*L. monocytogenes*		*L. plantarum*	
	(mg/ml)	Effect	(mg/ml)	Effect	(mg/ml)	Effect	(mg/ml)	Effect
Trichosporon sp.	15	Static	15	Static	7.5	static	15	Static
A. flavus	15	Static	30	Cidal	7.5	static	30	Cidal
Saccharomyes sp.	15	Static	15	Static	7.5	static	15	Static
Saccharomyes sp.	15	Static	15	Static	7.5	static	15	Static
Rhodotorula sp.	15	Static	15	Static	7.5	static	15	Static

CHAPTER 5 DISCUSSION

5.1 Microscopic and Macroscopic Description of Isolated Fungi

The isolated fungi listed in Table 1 consisted of different macroscopic characteristics as seen on the agar plates as well as microscopic morphological characteristics as observed under the microscope. Both yeasts and molds were observed and isolated. More yeasts were obtained compared to molds. One of the yeast isolates was *Rhodotorula* spp., the colonies were coral red to salmon-coloured, smooth, highly glossy to semi-glossy. These yeast cells had a mucoid to pasty texture. When it was observed under microscope it had a globose or more elongated budding yeast-like cells or blastoconidia Fig. 3 B. Visible colonies of *Rhodotorula* spp. appeared between 4-6 days and had no morphological variations during the incubation period, there was only an increase in the number of colonies.

The colonies of *Trichosporon* spp. are white, forms a finely cerebriform shape without a margin Fig. 3 C. The colonies were also raised and had a waxy appearance, it also developed radial furrows and irregular folds. Microscopically little dots were observed these were conidia produced in clusters at the septa. Branching hyphae was also viewed under the microscope. The appearance of *Trichosporon* spp colonies occurred at the same time as *Rhodotorula* spp., but there was a morphological change as the colonies increased in size with an increase in folds and colonies linked to each other.

Two of the yeast colonies were *Saccharomyces* spp. these colonies were white to cream, smooth and glabrous. The yeast colonies had an irregular, flat and undulate shape Fig. 3 D. Small yeast cells were observed under the microscope budding yeast cells was also observed, this was observed for both *Saccharomyces* spp.

The colonies of *Saccharomyces* spp. in Fig. 3 F has a wrinkled, rugose, raised morphology. The growth of the other *Saccharomyces* spp. in Fig. 3 F was faster as it was observed on day 4. Compared to Fig. 3 D where growth was observed on day 6.

The two isolated mold strains were both *Aspergillus* genus. But the isolated mold consisted of different morphology both microscopically and macroscopically. Conidial heads in shades of yellow-green was observed macroscopically in *A.flavus* (Fig. 3 E) also the texture was woolly to cottony. The colonies were raised forming a hill shape form, with the top exhibiting a brighter yellow color, while the lower part had a yellow green color. With the bottom of the colony surrounded by brown color hyphae known as a sclerotia. Microscopically, conidial heads were typically radiated and some biseriate with some presenting features of heads with phialides attached directly to the vesicle meaning that it was uniseriate. Furthermore conidiophore stipes were hyaline and coarsely roughened. Colonies were observed within 3 days of incubation with bright yellow conidia observed and as the colonies aged a green yellow color appeared.

For *A.niger*, colonies consist of white conidiophore on the outer circle and towards the inside a dense layer of black conidial heads Fig. 3 A. Microscopically, *A.niger* Fig. 4 A exhibited smooth walled stipes and black or near black-coloured conidia, the conidial heads had a circular shape with a black color. The conidiophore stipes were smooth-walled, some conidiophore stipes was hyaline or turning dark towards the vesicle. The conidial heads were biseriate with the phialides borne on metulae. Conidia appeared globose and black with a rough-wall. The growth of *A. niger* was faster compared to all the other isolated yeast and fungi, with colonies appearing within 1 day of incubation.

5.2 Yeast and Mold found on Pearl Millet

Fungal species known as *A. flavus* was isolated from the pearl millet and this can be supported by Navi (1999) whose study observed *A. flavus* on grain seeds and colonies on seed were dispersing and very light yellow-green, deep yellow-green, olive brown. The conidiophores were swollen apically and borne numerous conidia-bearing cells with conidia in long, dry chains. This resembles what was observed in the current study Fig 3. E.

When *A. niger* is found on grain the colonies on seed grows slowly also consisting of a compact to fairly loose white to faintly yellow basal mycelium, which eventually bears a large amount of erect and usually crowded conidial structures, typically with a carbon black but every so often deep brown-black color (Navi, 1999). This is usually covering the entire colony except for a narrow growing margin. A similar morphology was observed during the current study as seen in Fig. 3 A. Conidial heads were typically large and black it seemed to be compacted at first and also spherical, commonly reach 700-800 µm in diameter (Navi, 1999). The fungi *Aspergillus niger* is found worldwide and occurs on a variety of surfaces including grains, spoiled fruits and vegetables, exposed cotton textiles and fabrics, leather, dairy products, and other protein-rich substrata. The genus Aspergillus is common to favor heat and drought stress during pre-harvest and also heat and humidity after harvesting.

According to Kidd, Halliday, Alexiou and Ellis (2016), *Rhodotorula* Fig.3 B species are common environmental basidiomycetous yeasts, which can be found in soil, ocean and lake water, fruit juice and milk, and even on shower curtains. The genus contains 46 species of which three have been described as rare human pathogens these are *R. rubra*, *R. glutinis* and *R. minuta*. Colonies are budding yeast-like cells or blastoconidia, 2.3-5.0 x 4.0-10 µm (Kidd et al., 2016).

Saccharomyces sp., Fig 3 D and F can grow at water activities as low as 0.62, which normally range in water activities from 0.50 to 0.94, depending on the water content of forages (Hernández et al.,2018). According to Hernández et al. (2018) numerous mechanisms contribute to spoilage by *Saccharomyces* spp, such as the production of lytic enzymes these are lipases, proteases, and cellulase also gas, utilization of organic acids, discoloration, and off-flavors.

Trichosporon sp., are yeast like fungi that exist in nature as soil saprophytes and these fungi belong to the family Cryptococcaceae (Greene, 2021). On culture plates, they form hyaline yeast like cells, mycelia, and characteristic arthroconidia (Greene, 2021). The description provided by Greene (2021) can be seen in Fig. 3 C. *Trichosporon* sp. species are fungal species that can be part of the normal flora of the human skin and gastrointestinal tract (Guo et al., 2018). This taxonomy of the genus *Trichosporon* sp.

recently has undergone extensive changes, creating a new taxonomy with no less than 50 species being proposed to be maintained within the genus and 17 of which are medically relevant (Guo et al., 2018).

5.3 Antimicrobial inhibitory activity of isolated Mold and Yeast

The effectiveness of the antimicrobial activity of the isolated fungi was determined by the test of homogeneity of variance, with a sig value p= 0.019 as seen in Table 2 in the appendix. This indicates that the extracted secondary metabolites do significantly affect the growth of the gut microflora negatively. For this reason the research hypothesis is not rejected which implies that there was antimicrobial secondary metabolite producing fungi found in pearl millet which are hazardous to the gut microflora. Also that these secondary metabolites have the potential to affect the gut microflora negatively by inhibiting the growth of the microorganisms or affecting the cellular structure of the microflora.

There was only one isolated fungi *A. niger* that didn't show any zones of inhibition against any of the selected gut microflora as seen in the appendix Fig 6. On the other hand an isolated fungi from the genus *Saccharomyces* spp. exhibited the highest mean and standard error value which was 12.16 ± 0.21 mm. This genus was followed by *Rhodotorula* spp. 8.08 ±1.25 mm, *Saccharomyces* sp. 6.49 ± 1.19 mm, *Trichosporon* sp 4.98 ± 1.23 mm and *A. flavus* 4.24 ± 2.01 mm in that order Table 2. This shows that *Saccharomyces* sp has a greater potential to exert an inhibitory effect on the gut microflora when compared to the other isolated fungi. Although *A. niger* did not present any inhibitory effect, *A. flavus* displayed inhibitory effects. The genus *Aspergillus* produces secondary metabolites for instance mycotoxins which is commonly secreted into their surroundings for instance aflatoxin. But it appeared that *A. niger* produced a reduced amount or did not produce any secondary metabolites that could cause an inhibitory effect on either of the following selected bacteria strains *E. coli, L. plantarum, L.monocytogenes* and *K. pneumoniae*. The fungi *Trichosporon* spp. are not considered to be primary pathogens because they are dispersed in the environment and found worldwide and form a minor percentage of normal cutaneous and mucosal flora of people and animals (Greene, 2021). This explains why *Trichosporon* spp. had one of the lowest mean and standard error value 4.98 ± 1.23 mm for its antimicrobial activity against the bacterial strains.

The homogenous subsets table as seen in the appendix for the antimicrobial activity of the mold and yeast extracted created 3 subsets in which the fungi were grouped according to homogenity. The first subset groups *A. flavus* and *Trichosporon* spp. and displays that these two fungi antimicrobial activity is similar in inhibiting the growth of gut microflora. But in subset 2 *A. flavus* and *Trichosporon* spp. are also grouped with *Saccharomyces* spp, and *Rhodotorula* spp. this indicates that although *A. flavus* and *Trichosporon* spp. are grouped separately in subset 1 their inhibitory effect on the selected gut microflora is not really dissimilar from *Saccharomyces* spp. and *Rhodotorula* spp. when their antimicrobial activity is compared. Yet again in subset 3, *Saccharomyces* spp. is paired *Rhodotorula* spp. although *Rhodotorula* spp. is already in subset 2. This just indicates that *Rhodotorula* spp. antimicrobial activity also resembles that of *Saccharomyces* spp. in subset 3.

5.4 The Minimum Inhibitory Concentration (MIC) of the Fungal Isolates

The minimum inhibitory effect varied amongst the isolated fungi against the test gut microflora. There was no MIC at 30 mg/ml for any of the bacteria, but most of the bacteria had a MIC at 15 mg/ml for instance *L. monocytogenes*, *K. pneumoniae*, *and E. coli* and *L. plantarum* Fig. 5. Hence only 15 mg/ml is required to inhibit the growth of the gut microflora. The lowest MIC on the bacterial strains was on *L. monocytogenes* with a MIC of 7.5 mg/ml, from extracts of *Saccharomyces* spp., *Trichosporon* spp. and *A. flavus*. The only bacteria which did not have a 7.5 mg/ml value was *E.coli*, this bacterium lowest value was only 15 mg/ml. The fungal isolates which were only able to exert 15 mg/ml on the bacteria was *Rhodotorula* spp. and *Saccharomyces* spp.

The fungi *A. flavus* had the lowest MIC with a value of 7.5 mg/ml for the following bacteria *L. monocytogenes*, *K. pneumoniae* and *L. plantarum*, except for *E. coli*. Fungal species which inhibited the growth of bacterial species at 7.5 mg/ml indicates that only a minimal amount of secondary metabolites are required to inhibit the growth of the gut microflora, this also shows the fungal isolates level of pathogenicity.

Microbes that reside in the gut aid host in the mycotoxin removal process through metabolizing or binding to the mycotoxins. Although some microbes possess the mycotoxin removal ability, it is possible that bacteria from the same genus, however are unable to remove mycotoxin (Liew and Mohd-Redzwan, 2018).

Fungi, are one of the most prolific sources of bioactive secondary metabolites, and is known as eukaryotic, heterotrophic microorganisms that have spread throughout the world and live symbiotically in diverse environmental conditions (Abdel-Razek, El-

Ghonemy and Shaaban, 2020). A valid point made by Keller (2018) states that a physiologically relevant concentrations of a secondary metabolite secreted by a fungal species could function as a signal rather than as a toxin. This possibility was illustrated by the gradient-dependent effects of *Pseudomonas aeruginosa* secondary metabolite phenazine on *Aspergillus*, whereby a high concentration of phenazine exhibited antifungal properties but a moderate concentration induced prolific sporulation in the fungus (Keller, 2018). Genetic, ecological and mechanistic studies now made available unquestionable evidence that fungal chemicals compose the core element of microbial ‘language’ that is fungi and microorganisms; fungi and plants; and fungi and insects interaction (Keller, 2018).

According to Chatterjee et al. (2016) that there is an ecological metabolite hypothesis postulates that articulates secreted secondary metabolites modulate interactions of their producers with other organisms. The current view of fungal ecological chemistry is that most, if not all, secreted fungal secondary metabolites are ecological metabolites (Chatterjee et al., 2016). The suggested function of ecological secondary metabolites are antibiotic activity that inhibits competing microorganisms by inhibiting the activity or synthesis of hydrolytic enzymes and the synthesis of other secondary metabolites (Chatterjee et al.,2016). In response to antibiotic metabolites produced by a competitor, a fungus may detoxify the metabolite or may synthesize and secrete its own toxic metabolites (Chatterjee et al., 2016).

5.5 The cidal or static effect of Isolated Fungi on Gut Microflora

The minimum bactericidal concentration (MBC) was observed at a concentration of 30 mg/ml Table 3 for both *K. pneumoniae* and *L. plantarum* this effect was a result of the secondary metabolites of *A. flavus* as seen in the appendix Fig. 7. The extracted secondary metabolites from the other isolated fungal species only caused a static effect as seen in the appendix Fig 8.

5.5.1 Cidal effect of *Aspergillus flavus*

A cidal effect was observed by *A. flavus K. pneumoniae* and *L. plantarum*. Members of the *Aspergillus* genus are quite well known to produce various bioactive secondary metabolites, most of which have been developed into human health therapeutic leads and will produce aflatoxin if the temperature is above 15°C and the water activity above 0.90 (Ward and Leduc, 2021). Certain species of the genus *Aspergillus* develop on plants for instance pearl millet and are later transferred to humans through the consumption of plant material (Ward and Leduc, 2021).

The fungi *A. flavus* is widely distributed in the environment and is a most reported food-borne fungus therefore this supports why this particular fungi is found on pearl millet. Naturally produced aflatoxins are of four types, B1, B2, G1, and G2. B and G refer to blue and green light emission by these compounds under ultraviolet light (Bhalla et al., 2019). The cidal effect observed by *A. flavus* is explainable because it is the second most common species to be identified in human infection for instance, contaminated maize was responsible for the death of hundreds of people in Kenya in recent years according to (Ward and Leduc, 2021). According to Alshannaq and Yu (2017) animal species showed LD50 values which ranges between 0.5–10 mg/kg body weights. Acute aflatoxicosis in humans causes certain symptoms these are vomiting, abdominal pain, pulmonary and cerebral edema, coma, convulsions, and also death (Alshannaq and Yu, 2017).

5.5.2 Importance of the genus *Lactobacillus* in the gut

With the cidal effect of *A. flavus* on *L. plantarum*, this can cause major consequences to the human body this is because it plays a vital role in neutralizing fungi in the gastrointestinal tract. A study conducted by Deepthi et al. (2016) has revealed that *L. plantarum* caused alteration of hyphal structures, swollen tips and disrupted the conidia also conidia germination inhibition assay restrained germination and showed deformed hyphae. The bacterium has a bio protective feature which was evident as the isolate inhibited fungal development in maize-kernel that was treated with the cell free supernatant of *L. plantarum* MYS6 (Deepthi et al., 2016).

Furthermore both the isolate and its extracellular metabolites lowered fumonisin content in feed model up to 0.505 mg/kg of feed and 0.3125 mg/kg of feed respectively when compared to the level of 0.870 mg/kg of feed in control (Deepthi et al., 2016). The study confirmed that the major antifungal compounds produced by the isolate were 10-Octadecenoic acid, methyl ester; palmitic acid, methyl ester; heptadecanoic acid, 16-methyl ester; stearic acid and lauric acid (Deepthi et al., 2016). The isolate *L. plantarum* MYS6 was able to reduce 61.7% of fumonisin by means of a binding mechanism (Deepthi et al., 2016). The binding process of *Lactobacillus* (LAB) is achieved by the peptidoglycan of *Lactobacillus* binging to the tricarballylic acid chains of fumonisin molecules (Deepthi et al., 2016). Both viable and non-viable LAB could bind fumonisin in vitro based on pH, genus, bacterial density and fumonisin analogue (Deepthi et al., 2016). Studies have shown that other major mycotoxins such as aflatoxin B1, zearalenone and some trichothecenes can also be neutralized by some probiotic LAB in vitro (Deepthi et al., 2016).

CHAPTER 6 CONCLUSION

Generally, this research has satisfied a comprehensive experimental study on the isolation, identification and antimicrobial activities of fungal species isolated from pearl millet. The study isolated the following six types of fungi were identified *Aspergillus flavus, Aspergillus niger, Saccharomyces sp., Rhodotorula sp., Trichosporon sp. and Saccharomyces sp*. The current study also revealed that there was a significant difference in the antimicrobial activity amongst the different fungal species. The fungus *A. niger* did not show any form of antimicrobial activity while *Saccharomyces* sp. showed the highest inhibitory during Agar Well Diffusion. The fungus *A. flavus* showed more inhibitory activity against the following bacterial strains *L. plantarum, L. monocytogenes* and *K. pneumoniae* at 7.5 mg/ml when MIC was carried out. Furthermore the crude extract from *A. flavus* was the only extract exhibiting bactericidal effects and this was seen against *K. pneumoniae* and *L. plantarum*.

On the other hand, it was interesting to observe that *L. plantarum* was the least susceptible to the crude extracts, indicating that the bacterium contains features which assist with neutralizing the secondary metabolites from fungi. This has also revealed that each species of bacteria reacts differently towards the secondary metabolites.

CHAPTER 7 RECOMMENDATION

Improvements in the production, storage, utilization, and consumption of pearl millet crops will greatly contribute to the reduction in contamination, increase food security and nutrition. Probiotics which generally helps restore the gut microbiota should be introduced to people in the northern parts of Namibia this will reduce the effects of antimicrobial secondary metabolites and could improve the health of the people. More studies are needed to elucidate the interaction between the gut microbiota and a combination of secondary metabolites and the consequences of such interactions. Also in depth research is required to find out the effects of temperature, water activity and pH on the production of secondary metabolites by fungi. Isolation, identification and dosage level of compounds found in the secondary metabolites are required and their toxicology impact on gut microflora. It is necessary to know if Namibians are suffering from any health issues caused by fungi therefore investigations are required. More studies on the bacterium *L. plantarum* is encouraged in order to determine the mechanism used and the metabolites secreted to avoid the negative effects of most pathogenic fungi.

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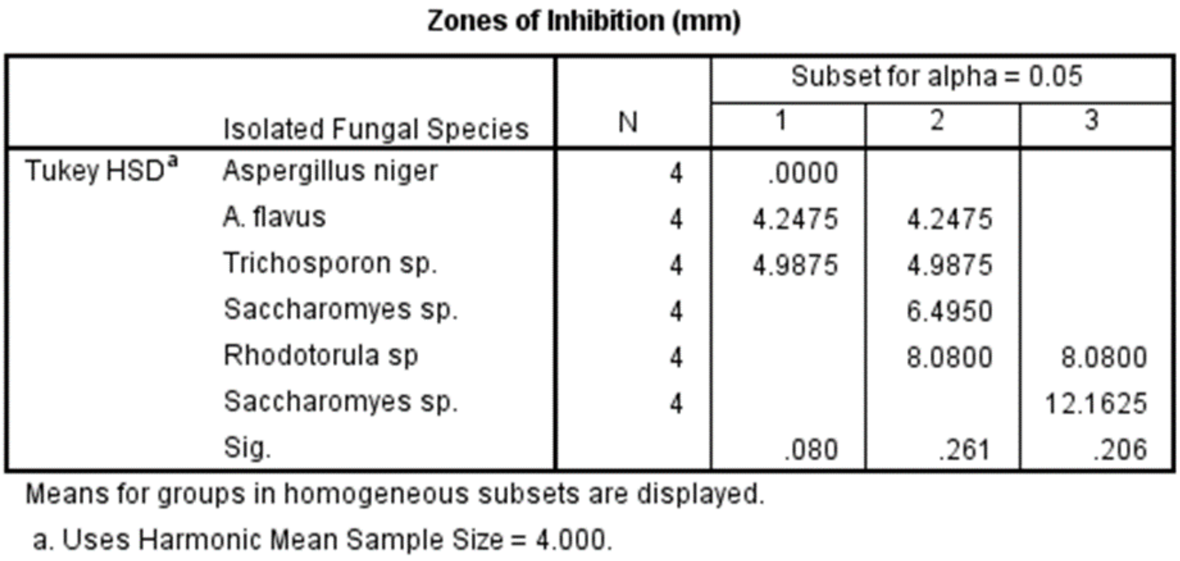
APPENDICES

1. Homogenous Subsets of Antimicrobial activity of Fungal extracts



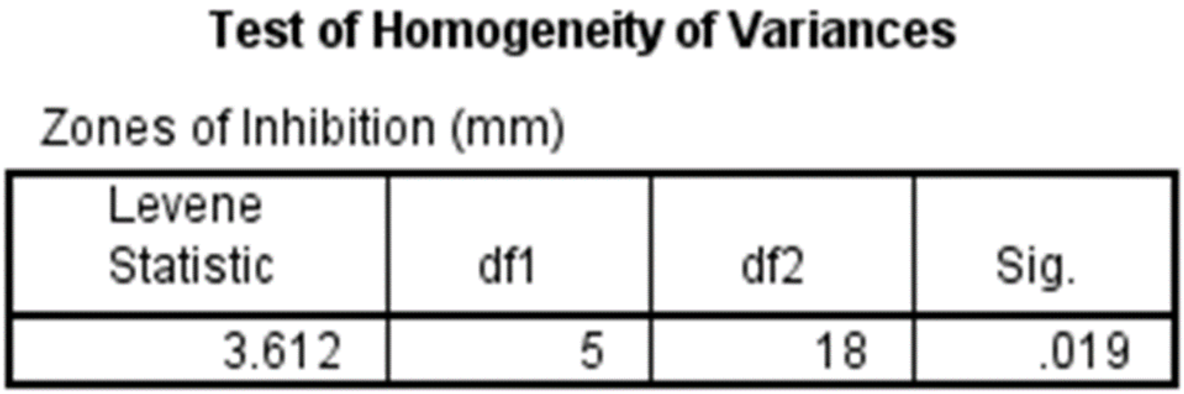
Zones of Inhibition (mm)

	Isolated Fungal Species	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Aspergillus niger	4	.0000		
	A. flavus	4	4.2475	4.2475	
	Trichosporon sp.	4	4.9875	4.9875	
	Saccharomyes sp.	4		6.4950	
	Rhodotorula sp	4		8.0800	8.0800
	Saccharomyes sp.	4			12.1625
	Sig.		.080	.261	.206

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

2. Test of Homogeneity Table for Zone of Inhibitions



Test of Homogeneity of Variances

Zones of Inhibition (mm)

Levene Statistic	df1	df2	Sig.
3.612	5	18	.019

3. Zones of inhibition (mm) agar plates of E. coli, L. plantarum, L.monocytogenes and K. pneumoniae

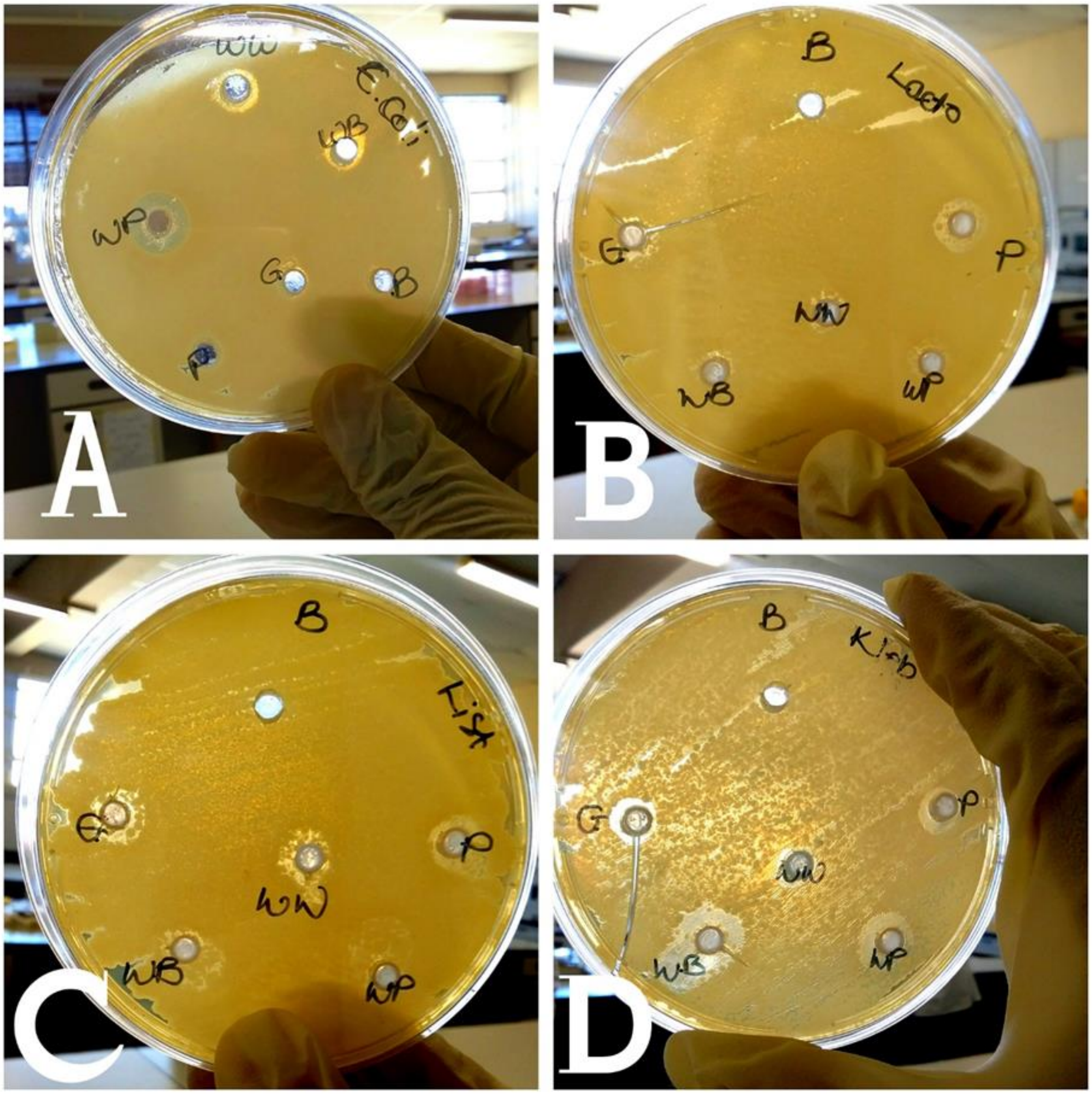


Figure 6. Zones of inhibition (mm) agar plates of the four test baterial strains are as follows: A- *E. coli*, B- *L. plantarum,* C- *L.monocytogenes,* D- *K. pneumoniae*

4. Minimum Inhibitory Concentration of *A. flavus* and *Saccharomyces sp.*

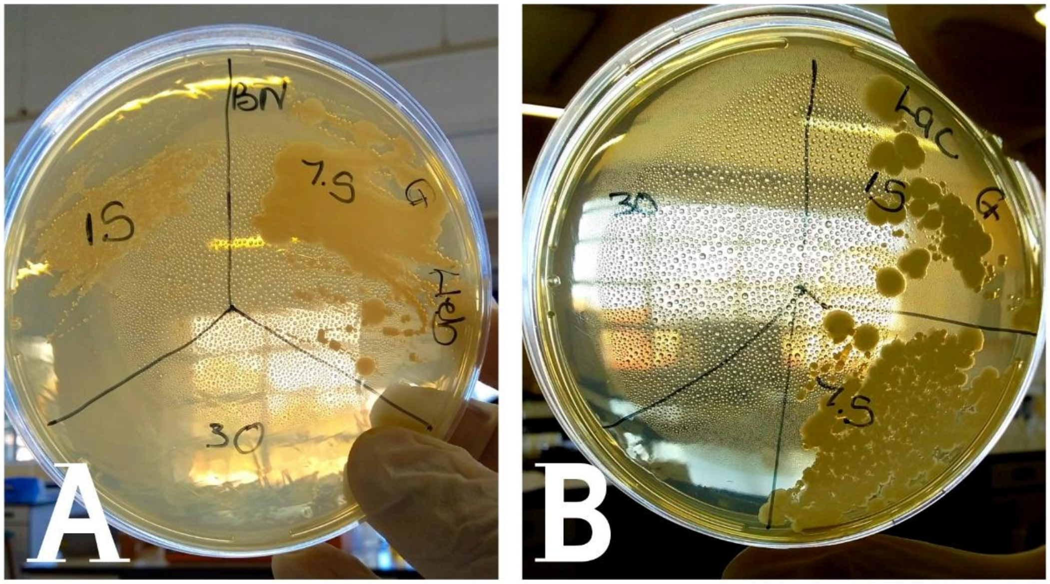


Figure 7. MBC plates, at 30 mg/ml of *A. flavus* secondary metabolite extract resulted to cidal effect: A- *A. flavus* extract caused a cidal effect towards *K. pneumoniae*, B- *A. flavus* extract caused a cidal effect towards *L. plantarum*.

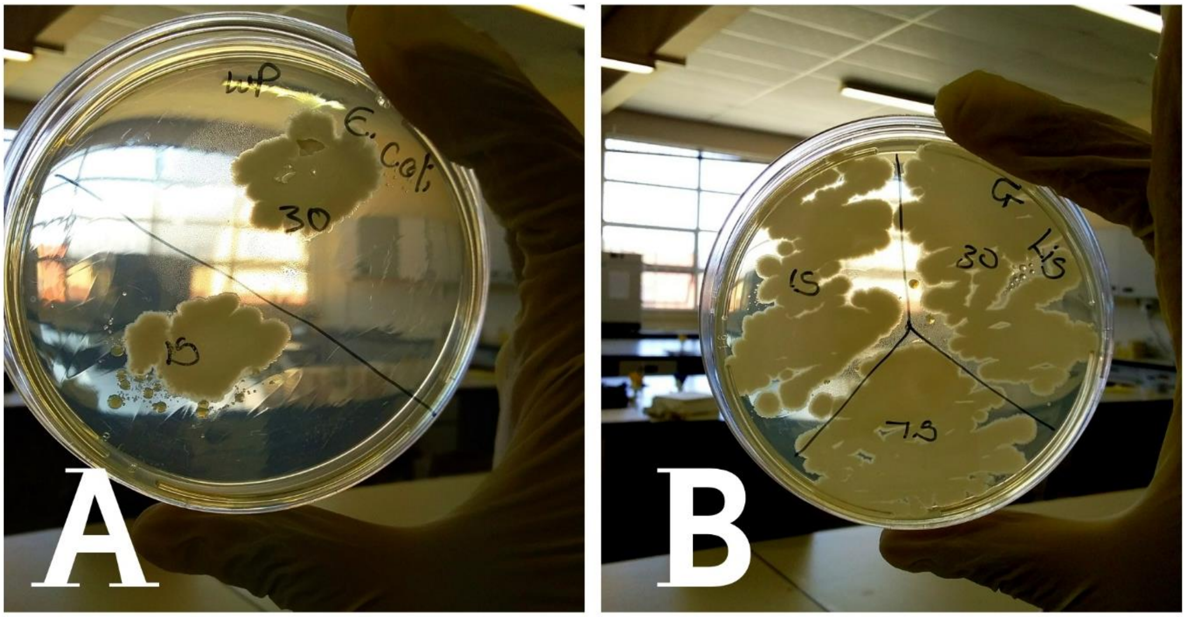


Figure 8. MBC plates, static effect observed: A- *E. coli* growth observed under concentrations of 15 mg/ml and 30 mg/ml *Saccharomyces sp*. secondary metabolite extract. B- *L. monocytogenes* growth observed under concentrations of 7.5 mg/ml, 15 mg/ml and 30 mg/ml *Aspergillus flavus* secondary metabolite extract.