Colegio San Agustin
Senior High School Department
Southwoods Interchange, Brgy. San Francisco, 4024 Biñan City, Laguna, Philippines
Tel. No. (+63 2) 478-0167 local 273 ▪ Fax No. (+63 2) 478-0180

Gut microbiome characterization of *Holothuria nigra*

By

Marvin Ivan C. Mangubat

Ryan Oliver Almenanza

Princess Sofia Anne A. Rivera

Kristian Carl N. Tingson

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ENDORSEMENT

This is to certify that this capstone research project entitled **Gut microbiome characterization of *Holothuria nigra*** prepared and submitted by Marvin Ivan C. Mangubat, Ryan Oliver Almenanza, Kristian Carl N. Tingson, and Princess Sofia Anne A. Rivera in partial fulfillment of the requirements for Senior High School – Science, Technology, Engineering, and Mathematics Strand, is hereby accepted.

MR. JON RAY M. MAGLONZO, MSc
Research Mentor

MR. ROGELIO D. ALMENARIO
Panel Chairperson

MS. RONA J. SUANCO
Panel Member

DR. DARYLLE C. HILAPO, PhD
Panel Member

The senior high school department endorses the acceptance of this capstone research project as partial fulfillment of the requirements.

MRS. MARIA FE R. CHUA
Academic Coordinator
Senior High School Department

MRS. ANA LIZA BARABICHO-NAVAL, MAEd
Principal
Basic Education Department

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ABSTRACT

This study aims to isolate and characterize the bacteria in the microbiome of the digestive system of *Holothuria nigra*. It will serve as a foundation for future studies regarding the microbiome of *H. nigra*. The study aims to be a preliminary assessment of *H. nigra* microbiome to be used in future studies, especially in the field of antibacterial research. Antibiotic resistance is a debilitating problem that has plagued the world for years, and the lack of novel antibiotics makes this crisis grow stronger day by day. This urgent need for novel antibiotics led the researchers to delve into marine organisms, as most common antibiotics come from terrestrial bacteria. Marine invertebrate microbiomes are a potential source of antibacterial compounds as they contain a wide variety of bacteria. Specifically, *holothuriidae* harbors diverse microbiomes due to their consumption of bacteria-rich marine sediments. The researchers sought to isolate and find the morphological and biochemical characterization of the microbiome found in the digestive system of one species, *H. nigra*. The streak-plate method was utilized to isolate the pure cultures of the samples and were placed onto Mueller-Hinton (MHA) agar, Mannitol salt agar (MSA), and Eosin-methylene blue (EMB) agar. The researchers also prepared test tubes to be used for SIM media, and 3% Hydrogen peroxide for the Catalase test, which will be the primary methods of biochemical characterization. Colony morphology was performed on streaked plates' colonies, alongside gram-staining in order to determine the characteristics of the bacteria present in the isolates. Afterwards, the researchers performed putative identification through phenotypic traits and metabolic profile in order to further classify the isolates. The results show that *H. nigra* samples' isolates contained a wide variety of bacteria. Through morphological characterization, bacteria from the *Bacillaceae* family were the most prevalent in the isolates. With biochemical characterization, it was also determined that bacteria from the *Bacillus* and *Eschericia* genera were the most prevalent in the isolates. These findings would be vital for future research that delves into the bacteria found in the microbiome of *H. nigra*, especially if antibacterial screening is conducted. These bacteria can also be explored and characterized further through 16S rRNA gene sequencing. For a broader idea of the bacteria present in the microbiome, future research should consider the utilization of *H. nigra* samples from different source locations.

CHAPTER 1

INTRODUCTION

Background of the Study

Humans are a species so dependent on culture and technology that cultural adaptation has replaced biological adaptation (Stock, 2008). Unlike other organisms, they are not as susceptible to changing themselves in order to adapt; instead, they are capable of changing and molding their environment and its ecosystems to what they deem suitable enough for them to live comfortably. A notable example of humans changing the environment lies in the creation of antibiotics, an invention that has led to countless saved and extended lives. Lancini et al. (1995) define antibiotics as microbial metabolites that inhibit the growth of other microorganisms. Antibiotics, also known as antibacterials, commonly comprise medications that destroy or slow down the growth of bacteria. When taken appropriately, antibiotics can be potent medications that combat the most common infections and have the potential to even save lives (Felman, 2019). Antibiotics gave way to many modern medical procedures to become possible such as treating cancer, transplanting organs, and conducting open-heart surgeries (Hutchings et al., 2019). Dr. Paul Ehrlich won the Nobel Prize in 1908 for his discovery of the first anti-infective small molecule, *arsephenamine* (Salvarsan), which he found could treat syphilis, a highly infective sexually transmitted disease. The drug was commercially successful, but it actually had potentially fatal side effects (Bennett et al., 2019). Nevertheless, this discovery served as the blueprint for future antibiotics, with Alexander Fleming's discovery of penicillin, the first true antibiotic, back in 1928 serving as the origin of the age of antibiotic discovery (Hutchings et al., 2019).

Bacteria are prokaryotic organisms that evolve and adapt according to their environment like other beings. When humans introduced antibiotics that hinder their ability to reproduce and grow, it sparked the fire for survival. Alexander Fleming, in his 1945 Nobel Prize acceptance speech, stated that "Then there is the danger that the ignorant man may easily underdose himself and, by exposing his microbes to non-lethal quantities of the drug, make them resistant." It is a statement that has since withstood the test of time. Humanity started to mass-produce antibiotics in the 20th century, exposing bacteria to antibiotic selection pressures, leading to the rapid increase in resistance seen in many pathogens today (Larsson, 2014). Since then, a decline in novel antibiotics alongside the development of antibiotic resistance in many human pathogens has led to the current antimicrobial resistance crisis (Hutchings et al., 2019). Today, an increasing number of pathogens are resistant to not only penicillin and its derivatives but also to all other available antibiotics (Michael et al., 2014). Wenzel (2004) claimed that the medical field is currently threatened by the convergence of three extremely unfavorable conditions: "High levels of antibiotic resistance among important pathogens, an uneven supply of novel classes of antibiotics, and a dramatic reduction in the number of pharmaceutical companies engaged in the discovery and development of antiinfective agents." These result in a crisis that needs more attention – the drying up of the antibiotic pipeline, a crisis delving into the scarcity of effective antibiotics alongside the lack of research that goes into their discovery and development (Wenzel, 2004).

Luepke and Mohr (2017) stated that most antibiotics that are used today were discovered and introduced commercially by the end of the 1960s, with only a few antibiotics developed after that had unique mechanisms. Thus, a dire need for novel antibiotics was born. Antibiotics are commonly created by and from soil microorganisms (Britannica Encyclopedia, 2022). Like the land, the oceans are a hotspot for the tremendous biodiversity of microorganisms, however, marine microorganisms potentially offer the production of metabolites not observed in terrestrial microorganisms (Kasanah & Hamann, 2004). Marine environments are also an untapped source of microorganisms used for antibiotic research. *H. nigra* is an echinoderm with advanced regenerative properties and holds different kinds of organisms in its intestinal tract. Pagán-Jiménez et al. (2019) states that adult holothurians (and other echinoderms) have a diverse range of microorganisms in their intestines, including bacteria, viruses, protozoa, and fungi. The interest in these invertebrates has only been increasing due to their production of different high-value bioactive molecules (Bordbar et al., 2011; Pereira et al., 2014). Studies suggest that invertebrate intestines happen to be a site for antibiotic compounds, with one from Agamennone et al. (2019) stating that bacteria isolated from the gut microbial communities in *Folsomia candida*, a species of springtail, display inhibitory activity against various pathogens.

Sea cucumbers have long been used as tonics and traditional medicine with an amazing profile of high-value nutrients and protein (Mohammadizadeh et al., 2013). One study is from Hidayati et al. (2021) who found *Bacillus tequilensis*, a fibrinolytic protease-producing bacterium isolated from the fermented gut of *H. scabra*. According to Plotieau et al. (2013), bacteria have been found in the guts of several holothurians, particularly deep-sea species, and it is widely assumed that they constitute major components of holothurians' diet. As a result, they are excellent candidates with antimicrobial potential. In the case of *holothurians*, different studies have also shown that they have a unique microbiota, but their role as antimicrobial producers has been seldom studied. *Holothurians,* specifically *H. nigra* and *H. scabra,* are selective deposit feeders that detect and preferentially ingest bacteria-rich sediments. The foregut of *H. scabra* could even act as a reservoir where some bacteria such as *Vibrio* proliferate (Plotieau et al., 2013). In addition to serving as a source of nutrients, digested bacteria may also benefit holothurians by contributing to midgut digestion through the action of their enzymes. Due to this, the researchers aim to explore the bacterial content of the microbiome in the *H. nigra* intestines to serve as a preliminary survey for further studies, especially in the field of antibacterial research.

In line with this, certain steps need to be accomplished in order to isolate and characterize the bacteria present in the microbiome of the *Holothuria nigra* digestive system. The microbiome from samples of *H. nigra* first needs to be isolated from the intestines, and variations between the isolated microorganisms may alter the bacteria to be discovered. The isolates will undergo different processes and be inoculated into different media and observed afterward for characterization.

Objectives of the Study

The researchers aim to explore the bacteria found in the microbiome of *Holothuria nigra*'s digestive system. In order to do so, the group will be following these specific objectives:

1. Isolate pure cultures of the culturable bacteria from the *H. nigra* digestive system swabs.
2. Determine the morphological characterization of the isolates through colony morphology and gram-staining.
3. Establish the biochemical characterization of the isolates using MSA, EMB Agar, SIM Media, and Catalase test.

Significance of the Study

In the past, with antibiotics not being created yet, what people consider simple illnesses now were extremely lethal and had high mortality rates. Take Tuberculosis (TB) for example. It was one of the most dreaded illnesses in the world and the leading cause of mortality in the United States throughout the late nineteenth and early twentieth century. At the turn of the century, it was estimated that 450 Americans died from Tuberculosis (TB) every day, most of whom were between the ages of 15 and 44. The illness was so prevalent and severe that it was said to be death itself. Even though *mortality* from tuberculosis in Western Europe and North America had declined substantially from its peak around 1800, 100 years later it was still significant: 194/100,000 in the United States, making it the third most common cause of death after cardiovascular diseases and influenza–pneumonia (Murray, 2004).

Sepsis induced by *Staphylococcus aureus*, particularly methicillin-resistant *S. aureus* (MRSA), is generally recognized as another major healthcare-related concern by the general public and the medical community. According to published data, mortality from infections linked with *S. aureus bacteraemia* can range from 2.5% to 40%. The mortality rate is known to fluctuate greatly depending on the patient's age, clinical presentation, and comorbidities (Turnidge et al., 2009).

Infections and illnesses caused by antibiotic-resistant bacteria have expanded dramatically in recent years and are now a major source of morbidity and mortality globally, particularly in wealthy nations. *Methicillin-resistant S. aureus*, are multidrug-resistant organisms or MDROs. In the future decades, *S. aureus*, *vancomycin-resistant enterococci*, and third-generation *cephalosporin*-resistant Gram-negative bacteria are predicted to outnumber cancer as the leading causes of mortality (Zipperer et al., 2016). Despite the pressing need for novel antibiotics that are effective against resistant bacteria, only a small number of compounds are in development, the vast majority of which are congeners of already employed antibiotic families. Nonetheless, innovative ways for cultivating previously uncultured potential antibiotic producers or activating dormant biosynthetic gene clusters have lately resulted in the identification of completely new antimicrobial substances with beneficial characteristics.

Nowadays, we consider colds, fevers, and other bacterial infections as easily treatable, but the antibiotic resistance crisis would make it exponentially more difficult for current antibiotics to sustain the health of populations worldwide. As bacteria continue to mutate and evolve to fight against antibiotic resistance, the discovery of novel antibiotics should still be prioritized due to bacteria being the leading cause of illnesses.

Conceptual Framework

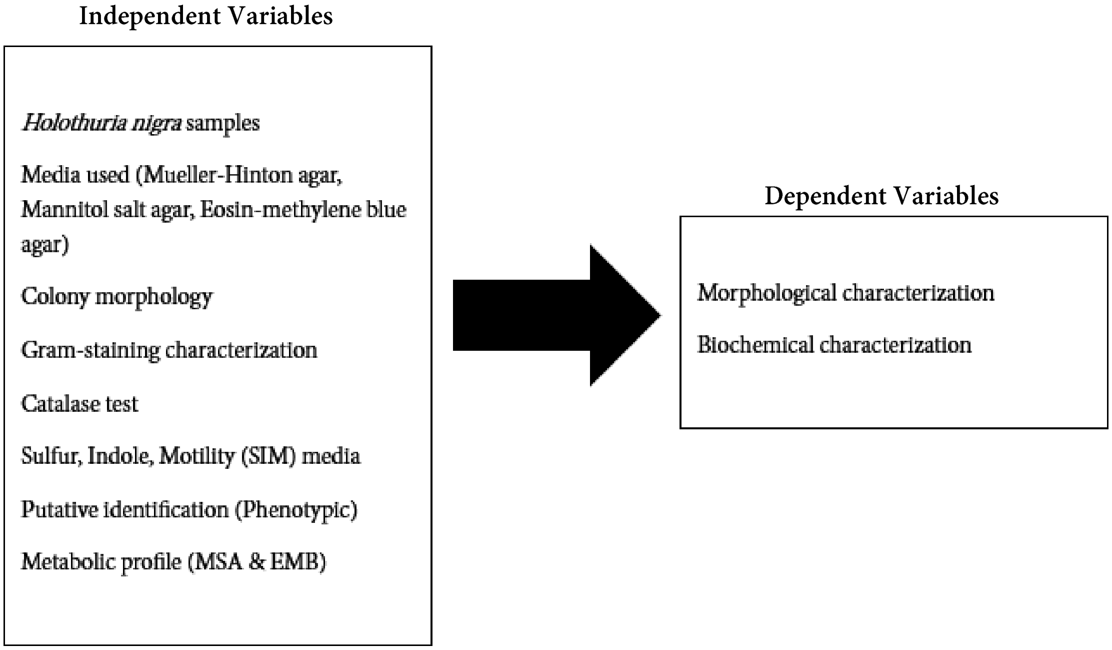


Figure 1. *The framework illustrates the relationship between the independent variables (methods and media used) and the dependent variable (characterization results)*

Scope and Limitations

The study will focus on the specific species of Holothuroidea, commonly known as sea cucumbers, *Holothuria nigra*, found in the waters of Calatagan, Batangas. One major factor that this paper does not take into consideration is the samples' source location. The samples only being gathered in this one general area may serve as a hindrance as the results may not be true for *H. nigra* in other areas due to unknown environmental factors that may have affected the gathered samples' bacterial content. In order to somewhat alleviate this issue, the researchers, as much as possible, gathered *H. nigra* samples from four stations around one general area on the coast of Verde Island. The researchers predict that obtaining samples from separate stations would aid in coming up with more accurate results in terms of the bacteria present in the microbiome due to the possible appearance of discrepancies between the results of the four samples throughout the conducting of the methodology.

The study shall be primarily conducted within the Colegio San Agustin - Biñan Senior High School laboratories. The equipment and media to be used, apart from the samples themselves, shall be procured from the laboratory unless there is a shortage, wherein the researchers would simply provide the materials needed. As this study serves as a preliminary survey of the bacteria present in the *H. nigra* digestive system, it is a descriptive study, therefore, no hypothesis will be made about the study's results. Similarly, with the study involving only qualitative figures, there would be no statistical analysis, as there would exist no data to analyze quantitatively.

The most fundamental limitation that influenced how the researchers would handle and continually revise the study is the lack of time. Originally planned to perform antibacterial screening on the isolates, the researchers instead chose to settle for simply isolating and characterizing the microbiome of *H. nigra*, which would serve as a preliminary assessment to determine if *H. nigra* is worth exploring further in terms of antibacterial research. The researchers were also unable to have multiple trials and replicate for the study as gathering the samples takes a lot of time and resources.

Another limitation lies in the limited number of *H. nigra* samples that will be gathered for the experiment, with the researchers only gathering four samples to be used for the entirety of the research procedures. Due to a limitation in resources and time, the study's results may not be as accurate as compared to a study with a larger sample size. Alongside the limitation in resources comes a limitation in experience which may hinder the researchers from being able to properly utilize the methodologies employed to produce desired results. This is most evident in the lack of experience the researchers have with the proper dissection of living organisms, namely, sea cucumbers.

Alongside that is the lack of experience with the preparation and utilization of agar and other media, namely Mueller-Hinton Agar (MHA), Mannitol Salt Agar (MSA), Eosin-Methylene

Blue Agar (EMB), Sulfur, Indole, Motility (SIM) Media, and Catalase test (Hydrogen peroxide). This is most evident in how the researchers were unable to perform the proper Indole portion of the procedure for SIM Media, as only the Sulfur and Motility portions were done properly. For the entire methodology, the researchers utilized available media from the laboratory's internal inventory, instead of preparing the media themselves using raw materials.

This paper does not take into account the environmental factors that may have affected the bacteria present in the samples' microbiomes. It also does not delve into screening the isolates for antibacterial activity, as the study is primarily concerned with exploring and characterizing the microbiome of the *H. nigra* digestive system. The researchers would not be looking for specific bacteria in particular; however, they would attempt to identify what bacteria are present across the different samples' isolates. This information would serve as a good foundation for future studies that involve the microbiome of the *H. nigra* digestive system.

All these limitations certainly hinder the final results of the study from being 100% accurate. However, the researchers believe that this study's results may provide a solid foundation for antibacterial research on various Holothuroidea species, especially if the results are indicative of possible effective antibacterial activity.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Antibiotic Resistance

Ever since antibiotics became available to the general public, they have been widely used. It has been regarded as a miracle, a cure-all that can kill bacteria without causing damage to the cells of those receiving treatment. However, during the past few decades, bacteria have become more common to resist not just one but several antibiotics, making them exceedingly difficult to manage. Crucial bacterial pathogens are becoming more resistant to standard antibiotic treatments, and multidrug-resistant bacteria are emerging at an alarming rate. Tackling bacterial infections and the associated illnesses is challenging. According to MacGowan & Macnaughton (2017), Species of particular concern are the 'ESKAPE pathogens' (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa and Enterobacter spp.*) which are especially relevant to nosocomial infections, as well as other *Enterobacteriaceae* (e.g. *E. coli*), *Mycobacterium tuberculosis* and *N. gonorrhoeae.* It will be necessary to devise new therapeutic methods and alternative antimicrobial treatments due to the lack of efficient medications, ineffective preventative measures, and the small number of new antibiotics in the clinical pipeline. In fact, the World Health Organization (WHO) has named antibiotic resistance as one of the three most important public health threats of the 21st century.

Multidrug resistance patterns in Gram-positive and Gram-negative bacteria, specifically, have resulted in difficult-to-treat, if not untreatable, illnesses with standard antimicrobials. Because many healthcare settings lack early identification of pathogenic bacteria and their antimicrobial susceptibility patterns in patients with bacteremia and other severe illnesses, broad-spectrum antibiotics are administered extensively and often inappropriately. As it stands nowadays, antibiotics, unlike most other drugs, tend to lose potency over time due to the development and spread of antibiotic resistance among bacterial pathogens. So far, no antibiotic class has escaped this persistent process. However, the period passed between the antibiotic's introduction into clinical practice and the formation of resistance has varied, as may the dynamics of resistance diffusion. According to Rossolini et al. (2014), the accumulation of resistance traits to various antibiotic classes, resulting in isolates with multidrug-resistant (MDR) phenotypes, has gradually reduced the available treatment choices for some diseases. Despite the fact that resistance is linked with lower fitness/virulence, certain MDR strains maintain a remarkable propensity for cross-infection and spreading in the clinical context and can suffer fast epidemic dissemination (the so-called high-risk MDR clones). Resistance to some Gram-negative infections may involve most or perhaps all available antimicrobial drugs, resulting in extensively drug-resistant (XDR) or totally drug-resistant (TDR) phenotypes that mimic pre-antibiotic circumstances.

The urgent need for novel antibiotics

The importance of natural products in drug development has changed dramatically over the last 30 years, with large pharmaceutical corporations showing a substantial reduction in engagement by the mid-1990s. Nonetheless, ambitious academics, mainly in collaboration with business, filled the void left by bigger research and development activities (Molinski et al., 2008). Antibiotics had revolutionized medicine, allowing the treatment of illnesses that were historically deadly and permitting treatments that were previously inconceivable. Their availability has established a level of health certainty that earlier generations did not have. This guarantee, which is currently taken for granted, will be eroded if resistance development continues to outpace antibiotic research, as it has in the previous decade (Livermore, 2004). Additionally, resistance is an unavoidable Darwinian result of antibiotic use, though its frequency varies depending on 1) the regimens and extent of use; 2) the effectiveness of infection control; and 3) random factors such as the initial escape of resistance genes to mobile DNA and into biologically "fit" strains. No antibiotic is immune to all forms of resistance, and if one were, it would likely shift the microbial ecology in favor of more resilient opportunistic pathogens capable of filling the vacated niche. Antibiotic overuse develops stronger bacteria, as several pathogens such as *S. Aureus* and *multidrug-resistant Tuberculosis* had already begun to be resistant to their counterparts that are penicillin and Isoniazid, respectively. When microorganisms grow resistant to antibiotics, treating the ailment becomes more complicated and expensive. The inability to cure significant bacterial infections poses a substantial hazard to public health. Antibiotics, after all, are substances that are meant to kill bacteria by attacking various components of their structure or cellular machinery.

However, pathogens can eventually overcome antibiotics through the concept of *Survival of the Fittest*, thus mutations occur. It is when bacteria are first exposed to an antibiotic, those in which that are most susceptible to the antibiotic die quickly, thereby leaving any surviving bacteria to pass on their resistant characteristics to succeeding generations. Because bacteria are so common, random mutation of bacterial DNA results in a wide range of genetic alterations. Bacterial defense mechanisms against antibiotics can be developed through mutation and evolution. Some bacteria, for example, have evolved biochemical "pumps" that may remove an antibiotic before it reaches its target, whilst others have evolved enzymes that inactivate the antibiotic. According to D'Andrea et al. (2019), if adequate measures are not implemented soon, the "antibiotic resistance issue" might return humanity to a "pre-antibiotic period" in the near future. Unfortunately, the slow progress of antibiotic discovery and development over the previous few decades has resulted in a scarcity of novel compounds for clinical application, aggravating the impact of antibiotic resistance on morbidity and death rates. In this situation, many current healthcare techniques associated with a greater risk of infection and requiring antibiotics (e.g., anti-cancer therapy, solid organ and stem cell transplants, or prosthetic device implantation) are significantly jeopardized.

Marine invertebrate microbiome as a potential source of antibacterial compounds

Natural products, especially those from terrestrial plants and microorganisms, have long been a traditional source of therapeutic compounds (for example, morphine from poppies, cardiotonic digitalis glycosides from foxgloves and penicillins from fungus). Modern pharmaceutical discovery efforts owe much to natural products. Indeed, pharmacologically active molecules derived from plants and microorganisms are a significant source of novel experimental drugs. However, interest in marine natural products delayed advancements in technology (mostly scuba diving) to harvest the source species. Nonetheless, by the late 1950s, the thought of pharmaceuticals from the water had piqued the imagination of some (Molinsky et al., 2008). Marine ecosystems have received significantly less attention compared to the terrestrial environment, which has been the pharmaceutical industry's focus for more than 50 years for the production of antibacterial metabolites. It is now understood that the richness of organisms in marine habitats dwarfs that of tropical rainforests. Protozoans, jellyfish, anemones, flatworms, roundworms, bryozoans, clams, squid, copepods, annelids, sea stars, sea cucumbers, corals, sponges, and algae are just a few of the many species found in the ocean (Sverdrup et al., 1942). Most marine creatures also house particular populations of microorganisms on their surfaces or inside the boundaries of their tissues. Similar to this, the various microbial communities found in ocean sediments have largely gone unnoticed as a source of new bacteria that produce antibiotics. Without a doubt, studying uncharted environments, like the world's seas, is necessary to meet the urgent demand for novel antibacterials. When marine resources are used for producing novel antibacterials, supply shortages would be the biggest challenge that would prevent the development of antibiotics made from marine natural resources. Since it is quite impossible to collect unlimited amounts of marine animals or plants, the isolation and cultivation of symbiotic microorganisms are of great importance for a sufficient and constant supply of bioactive compounds. Symbiotic marine microorganisms which can be cultivated will facilitate future development of marine natural product leads if the compound can be produced by an associated microorganism (Kobayashi & Ishibashi, 1993). Another idea that stems from research is the fact that metabolites generated from sponge-associated microbes, primarily bacteria, fungus, and algae, have demonstrated potential use in medicine, cosmeceuticals, environmental protection, and industry, alongside the discovery of Sponge bacteria-derived natural compounds having anticancer, antibacterial, antifungal, and antiviral activities. Attempts to re-identify the origin of known and future sponge-sourced natural products would provide further light on the functions and relevance of bacteria inside marine sponges (Amelia et al., 2022).

Holothuriidae harbors a diverse microbiome

The genome of microbial life forms inhabiting a live host, as well as their interactions with the host, is referred to as the microbiome. Sea cucumbers are repeatedly exposed to a wide variety of microbes – bacteria, fungi, and viruses, for example, and these may possibly be detrimental to these organisms. As a result, their existence is contingent on excellent antimicrobial production mechanisms. Recent extensive research has shown sea cucumber extracts to have multiple biological activities, including antimicrobial, anticancer, and antioxidant properties. Its extracts are known to contain an immense range of secondary metabolites such as triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, cerebrosides, lectins, peptides, glycoprotein, glycosphingolipids, cytotoxins, and essential fatty acids (Bordbar et al., 2011). Many of these naturally occurring compounds are of particular interest for potential antibiotic discovery as well as constituents of innovative leads and mass-produced products for a wide range of industrial uses, including medicines, agrochemicals, functional foods, and nutraceuticals.

Streak-Plate Method

In order to isolate the pure cultures from a sample, the streak plate method will be utilized. According to Katz (2008) from the American Society for Microbiology, the purpose of the streak plate is to obtain isolated colonies from an inoculum by creating areas of increasing dilution on a single plate, wherein isolated colonies represent a clone of cells, being derived from a single precursor cell. When culture media is inoculated using a single isolated colony, the resulting culture grows from that single clone. By the end of this method, pure cultures will be isolated from the main colony. From there, an inoculating loop will be used to streak in a separate agar plate in four quadrants. Throughout this method, the inoculating loop undergoes flaming in order to continually sterilize it and prevent unwanted contamination of the agar plates.

Stab Culture (Agar Stab)

For SIM Media, a different kind of method is utilized in order to inoculate the sample. Stab cultures refer to agar plates created by pouring agar medium into a test tube and allowing it to solidify. An opening is created in the agar, and the medium is introduced either using a needle or a pipette. This technique is frequently employed for shipping cultures or temporarily storing them.

Mueller-Hinton Agar (MHA)

According to Mueller & Hinton (1941), Mueller-Hinton agar, an agar-based growth medium utilized in microbiology, is employed for cultivating bacterial isolates and assessing their susceptibility to antibiotics. The medium was originally formulated in 1941 by microbiologists John Howard Mueller and Jane Hinton while they were affiliated with Harvard University. Mueller-Hinton agar consists of various constituents, including beef extract, acid hydrolysate of casein, starch, and agar to solidify the mixture. It is important to note that the specific composition of Mueller Hinton agar can vary depending on the manufacturer and intended application, but generally, the medium is nutrient-rich and devoid of inhibitors that could impede bacterial growth.

Mannitol Salt Agar (MSA)

According to Shields & Tsang (2006), Mannitol salt agar (MSA) is a widely employed growth medium in microbiology that serves both as a selective and differential medium. It promotes the growth of specific bacteria while inhibiting the growth of others. MSA contains a high concentration (around 7.5–10%) of salt (NaCl), which acts as an inhibitor for most bacteria. As a result, MSA selectively favors certain Gram-positive bacteria, such as Staphylococcus, Enterococcus, and Micrococcaceae, that can tolerate high salt concentrations, while inhibiting the growth of most Gram-negative bacteria.

Additionally, MSA serves as a differential medium for identifying mannitol-fermenting staphylococci. It consists of mannitol, a sugar alcohol, and phenol red, a pH indicator used to detect acid produced by mannitol-fermenting staphylococci. Staphylococcus aureus, for example, produces yellow colonies with yellow zones on MSA, while other coagulase-negative staphylococci produce small pink or red colonies without any color change in the medium. When an organism can ferment mannitol, it produces an acidic byproduct that causes the phenol red in the agar to turn yellow.

The primary purpose of MSA is the selective isolation of presumptive pathogenic (pp) Staphylococcus species, aiding in their identification and differentiation.

Eosin-Methylene Blue (EMB) Agar

According to Lal & Cheeptham (2007), Eosin-methylene blue (EMB) agar exhibits selectivity towards gram-negative bacteria while inhibiting the growth of gram-positive bacteria. It serves as a valuable tool for isolating and distinguishing various types of gram-negative bacilli and enteric bacilli, commonly referred to as coliforms and fecal coliforms, respectively. When lactose-fermenting bacteria are present in the medium, they form colonies with distinct colors, while non-lactose-fermenting bacteria produce colorless colonies.

EMB agar finds utility in water quality testing as it helps differentiate coliforms and fecal coliforms, which can serve as indicators of potential pathogenic microorganism contamination in water samples. Additionally, EMB agar aids in the differentiation of organisms belonging to the colon-typhoid-dysentery group. Escherichia coli colonies exhibit a metallic sheen with a dark center, Aerobacter aerogenes colonies possess a brown center, and non-lactose-fermenting, gram-negative bacteria appear pink when grown on EMB agar.

Colony Morphology

Colony morphology refers to the visual characteristics exhibited by a bacterial colony on an agar plate. The observation of colony morphology plays a crucial role in the microbiology laboratory for the identification of microorganisms. To accurately assess the distinctive features such as shape, size, color, surface appearance, and texture, colonies must be adequately separated from one another. Growing on agar surfaces, microorganisms form colonies whose appearance helps clinicians and researchers to identify genera or even species. (Sousa et al., 2013).

Gram-Staining Characterization

According to Tripathi & Sapra (2020), Gram-staining is an essential technique in the field of microbiology. It was named after the Danish bacteriologist Hans Christian Gram, who first introduced it in 1882 to identify pneumonia-causing organisms. Typically, gram staining is the initial test performed and involves the application of crystal violet or methylene blue as the primary dye. Organisms that retain the primary color and appear purple-brown under a microscope are classified as Gram-positive. Conversely, organisms that do not retain the primary stain appear red under a microscope and are referred to as Gram-negative.

The first step in gram staining involves staining the slide with crystal violet dye. This is followed by the addition of iodine to fix the dye, forming a complex known as crystal violet-iodine that prevents easy removal of the dye. Subsequently, a decolorizer, often a mixture of ethanol and acetone, is used to remove the dye. The fundamental principle of gram staining lies in the ability of the bacterial cell wall to retain the crystal violet dye during the decolorization process. Gram-positive microorganisms have a higher peptidoglycan content in their cell walls, while gram-negative organisms have a higher lipid content.

Sulfur, Indole, Motility (SIM) Media

According to Visser & Mossel (1985), SIM (Sulfide-Indole-Motility) is a semi-solid medium specifically designed to facilitate the differentiation of Enterobacteriaceae, particularly Salmonella and Shigella, based on their ability to produce hydrogen sulfide, split indole from tryptophan, and exhibit motility. The presence of sodium thiosulfate and ferric ammonium sulfate enables the detection of hydrogen sulfide. Sodium thiosulfate serves as a substrate for enzymatic reduction, and the resulting colorless hydrogen sulfide gas reacts with ferric ammonium sulfate, forming ferrous sulfide. This insoluble black precipitate causes the medium to darken.

The determination of indole production is feasible due to the presence of tryptophan in the pancreatic digest of casein within the medium. Tryptophan, an amino acid, can be oxidized by certain bacteria to produce three major end products: indole, pyruvic acid, and ammonia. The addition of specific aldehydes to the medium forms colored end products, enabling the detection of indole. Kovac’s Reagent, which contains p-dimethylamino benzaldehyde, reacts with indole to generate a distinctive pinkish-red end product that is easily visible.

The semi-solid nature of the medium facilitates the detection of motility. Motile organisms are able to grow and spread outward from the central stab line, indicating their ability to move within the medium.

Catalase Test

According to Reiner (2010), the catalase test is employed to detect the presence of the enzyme catalase in bacteria. It plays a crucial role in distinguishing catalase-positive Micrococcaceae from catalase-negative Streptococcaceae. While its primary purpose is to differentiate between genera, it also proves useful in the identification of specific gram-positive bacteria, such as Aerococcus urinae (positive) and Aerococcus viridians (negative), as well as Gram-negative organisms like Campylobacter fetus, Campylobacter jejuni, and Campylobacter coli (all positive) from other Campylobacter species. Some studies have even reported its value in the presumptive differentiation of certain Enterobacteriaceae.

Moreover, the catalase test is valuable for distinguishing between aerobic and obligate anaerobic bacteria, as anaerobes generally lack the catalase enzyme. Within this context, the catalase test aids in differentiating catalase-negative aerotolerant strains of Clostridium from catalase-positive Bacillus strains.

Putative Identification

Putative identification of a specific family and genus of bacteria refers to the preliminary or tentative identification of bacterial isolates based on characteristic features, such as phenotypic traits or genetic markers, without employing extensive confirmatory tests (Weyant et al., 2003). This initial identification can provide valuable insights into the taxonomic placement of the bacteria and guide further investigations. Phenotypic identification methods involve assessing various metabolic and physiological properties of bacteria, such as colony morphology, Gram staining, and biochemical tests (Weyant et al., 2003). These tests can provide clues about the family and genus of the bacteria.

Metabolic Profile

Mannitol Salt Agar (MSA) is a selective and differential medium commonly used to isolate and differentiate Staphylococcus species based on their ability to ferment mannitol (Leboffe & Pierce, 2017). The interpretation of color changes in MSA can provide valuable information about the metabolic characteristics of bacterial isolates. If the bacterial growth on MSA appears pink or red, it indicates that the organism is a mannitol non-fermenter (Leboffe & Pierce, 2017). This result suggests that the organism may belong to a different genus, such as Micrococcus or non-pathogenic Staphylococcus species. On the other hand, if the bacterial growth turns yellow, it indicates mannitol fermentation (Leboffe & Pierce, 2017). This color change is due to the acid production resulting from the fermentation process. Staphylococcus aureus, a pathogenic species, is known to ferment mannitol, producing acid and causing the color change to yellow (Leboffe & Pierce, 2017).

Eosin Methylene Blue (EMB) Agar is another selective and differential medium used for the isolation and differentiation of Gram-negative enteric bacteria (Atlas, 2010). The interpretation of color changes on EMB agar can provide insights into the lactose fermentation ability of the bacterial isolates. If the bacterial growth on EMB appears colorless, it indicates that the organism does not ferment lactose and is a non-lactose fermenter (Atlas, 2010). This result suggests the possibility of non-pathogenic bacteria such as Proteus or Salmonella species. On the other hand, if the bacterial growth on EMB agar turns pink to purple, it suggests that the organism is a lactose fermenter (Atlas, 2010). This color change occurs due to the production of acid during lactose fermentation. Pathogens such as *Escherichia coli* and *Klebsiella pneumoniae* often produce this characteristic color change (Atlas, 2010).

CHAPTER 3

METHODOLOGY

Research Design

With the main goal being the characterization of microbiome, the researchers have decided on a specific research design in mind. Thus, this paper follows a descriptive research design, due to characterization falling under this specific type of research design. Due to time constraints as mentioned in the Scope and Limitations, the study underwent only one trial and replicate. The main location wherein the study's procedure was conducted was within the campus premises of Colegio San Agustin - Biñan, a Catholic, private school approximately located at Southwoods Interchange, Brgy. San Francisco, Biñan City, Laguna, Philippines. The samples of the *Holothuria nigra* specimen utilized were gathered from the general area and coasts of Calatagan, located in the province of Batangas, Philippines. The researchers have decided on a time interval for when the study should be enacted based on two factors: 1) The amount of time left for the study to be conducted in the A.Y. 2022-2023; and 2) The limitations and challenges brought upon by the researchers' respective schedules as students. Thus, the first two quarters of 2023 were dedicated to the data-gathering procedures, which ensured that enough time was left to analyze and perform the needed characterization of the samples' isolates. All the equipment that was utilized throughout the study was provided by the Senior High School Science Laboratories, alongside the media that was required to be prepared before characterizing. If the laboratory encounters a shortage, the researchers were the ones who provided distilled water, laboratory gloves, tissues, aluminum foil, a fire-starting device such as a lighter, etc.

1.0 Collection and verification of *Holothuria nigra* specimen

The study focuses on the specific species of Holothuroidea, *Holothuria nigra*, found in the waters of Calatagan, Batangas, Philippines. The researchers decided to gather fresh samples from Calatagan due to word of mouth stating a large number of sea cucumbers spread out in the area. The researchers would verify the specimen's species based on the primary characteristic distinguishing *H. nigra* from other *Holothuria* species, as the exterior color they possess is black, or a shade similar to it.

The samples only being gathered in this one general area may serve as a hindrance, as the results of the paper may not be very accurate for *H. nigra* in other areas due to the possibility of certain environmental factors affecting the bacteria found in the microbiome of the gathered samples. The researchers gathered *H. nigra* samples from four different stations around the general coast of Calatagan.

1.1 Preparation of the agar media

The first part of the methodology accounted for the preparation of the agar medium to be done prior to the extraction of the sample. Due to the procedure needing a few hours at least, the researchers had to dedicate almost an entire day to preparing the said marine agar medium. Mueller-Hinton Agar from the laboratory was utilized as the medium. Throughout the entire methodology, a constant supply of MHA agar plates was needed for the initial culturing, subculturing, and isolation of the pure cultures. This was done through the use of 500 mL Erlenmeyer flasks, with 11.7 g dissolved in 300 mL distilled water for each flask. For the initial preparation of MHA plates to swab the dissected samples in, two 500 mL Erlenmeyer flasks were used, which would total the amount of agar used to 23.4 g dissolved in 600 mL distilled water. In pouring agar onto a Petri plate, the mouth of the plate was first sterilized using an alcohol burner in order to prevent contamination.

1.2 Dissection and extraction

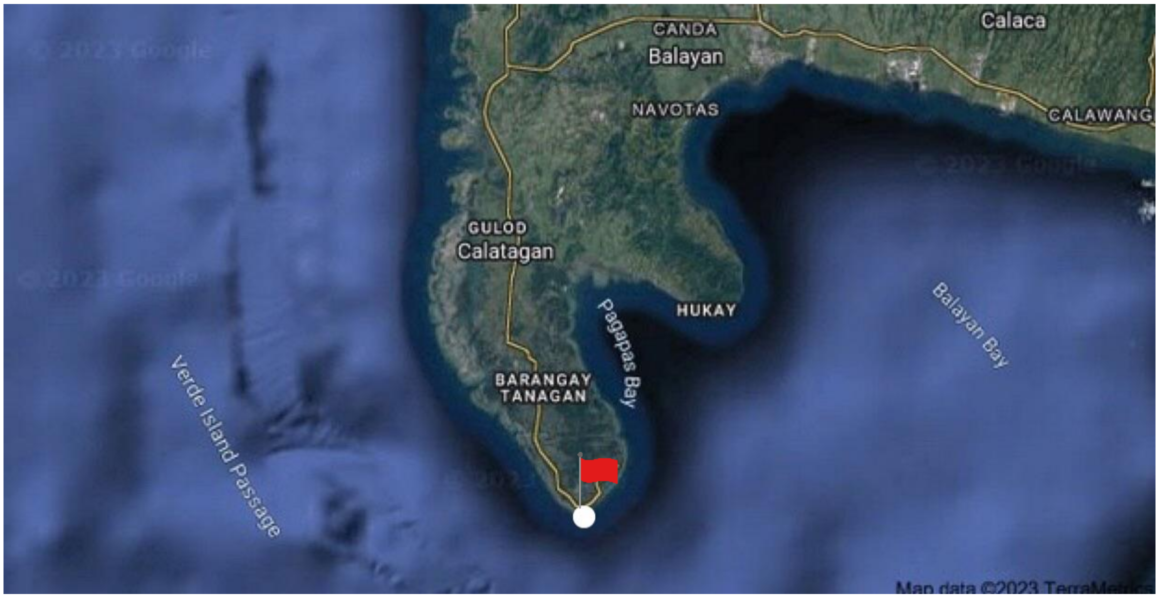


Figure 2. Satellite image of Calatagan, Batangas (courtesy of Google Maps)

As the *H. nigra* specimen was to be gathered in coastal areas, the researchers traveled to a beach of Calatagan to survey the area and look for the possible specimen in four different stations. Prior to traveling to the extraction site, a dissection kit along with other required apparatus such as applicator sticks were procured from the Senior High School Laboratory, as the researchers planned to conduct the dissection procedure on the same site where the sea cucumbers will be found.

The researchers conducted a gut content analysis for each of the specimen and discovered the presence of seawater and sand for each one. No other foreign object was found in each of the samples' digestive systems. The researchers hypothesize that, with *H. nigra* actively ingesting sand or marine sediment, it may be a factor in the bacteria present in its guts. Utilizing a basis for a sea cucumber's anatomical structure from Savalli (2023), the researchers were able to identify the different parts of the guts they will be swabbing, namely the stomach and the intestines.

During the process of dissection, the researchers also conducted the isolation of the microbiome by swabbing into the prepared agar plates which was then used for the subculturing procedure. Based on the desired results, the researchers preferably used only one *H. nigra* specimen in each of the four different stations around the site. After the dissections, the carcasses were then disposed of using proper procedures.

2.0 Isolation and subculturing

Prior to arrival on the site, the researchers have already prepared cotton swabs, ~12-15 plastic plates alongside the prepared MHA agar plates, and a cooler. This is due to the prepared agar only having a short shelf life, and so the researchers planned to maximize the allotted time by doing these first steps of the methodology almost simultaneously. The isolation was done in a 1:1 ratio, with the researchers swabbing three different sectors of the *H. nigra* digestive system: the intestine, stomach, and the general area onto one agar plate each. Afterward, the same process was done for the next three specimens found on the other three stations. This is to increase the accuracy of the kinds of bacteria present and to increase the probability of culturing plentiful samples of bacteria. Once a plate had been swabbed, it was immediately labeled, closed, and sealed once again with parafilm. These were stored in the cooler and brought back to the campus.

After some time, once the bacteria that grew on the agar plates were enough, the researchers started with the process of isolation and subculturing. The researchers chose 10 sample plates out of the gathered 12 to be utilized for the rest of the study. These plates are labeled with the prefix “MART”, the initial of the part of the digestive system, alongside which *H. nigra* sample the part is from (e.g. MART-I1 [Intestine-1st Sample]). The labels are I for the Intestine, S for the Stomach, and R for a General Area of the Digestive System. The chosen sample plates are as follows: MART-I1 (Control), MART-I2, MART-I3, MART-I4, MART-S2, MART-S3, MART-S4, MART-R2, MART-R3, and MART-R4, which, in total, equaled to 10 sample plates.

While waiting for the bacteria in the plates to propagate, the researchers prepared MHA, alongside Mannitol Salt Agar (MSA), and Eosin-Methylene Blue (EMB) Agar. Like MHA, they were also prepared in 500 mL Erlenmeyer flasks. MSA was prepared with 33.8 g dissolved in 300 mL distilled water, while EMB Agar was prepared with 11.288 g dissolved in 300 mL distilled water.

The researchers utilized the four-quadrant streak-plate method in order to streak the chosen samples onto plates of newly-prepared MHA, MSA, and EMB Agar following a 1:3 ratio - 1 sample plate is to 1 MHA, 1 MSA, and 1 EMB Agar plate. MHA allows for the propagation of a wide variety of bacteria, and it was then used for characterization. MSA and EMB, on the other hand, are selective growth media that allow only a few organisms to grow, and these plates had a part later on in biochemical characterization. Between each streaking, the inoculating loop was sterilized using an alcohol burner, utilizing the method of flaming. This is the final step in isolating and subculturing the samples, as, through this step, the researchers already obtained the pure cultures to be used for the next methods.

3.0 Preparation of biochemical characterization media

The researchers utilized 30 sterilized test tubes which were used for SIM Media. The SIM Media was prepared with 9.5575 g dissolved in 250 mL distilled water in two 500 mL Erlenmeyer flasks, which totals to about 19.115 g dissolved in 500 mL distilled water. The stab culture method was performed using the 10 MHA streaked plates on the SIM media. Afterwards, the test tubes were incubated in the incubator at room temperature (37°C) for two days.

Around this time, the researchers have also prepared the 3% Hydrogen peroxide (H2O2) needed for the Catalase test. They employed the use of 10 microscopic glass slides to place 2-4 drops of Hydrogen peroxide in. The 10 MHA plates were then swabbed onto the glass slides, following a 1:1 ratio.

It is important to take note that every piece of equipment that may transfer or may be a site for bacterial propagation used by the researchers in conducting the methodology was covered with paper and sterilized in the laboratory oven at 170°C for 1 hour and 45 minutes. This includes petri plates, test tubes, and the like. In order to ensure that no contamination affects the accuracy of the study's results, the pouring of media, streaking, and stabbing was done in a biosafety cabinet. After the streaking and stabbing methods, the plates were incubated in the incubator at 37°C for two days.

4.0 Colony morphology

The first aspect of the study's characterization is colony morphology. This was done on all 30 plates of MHA, MSA, and EMB Agar. The streaked plates' colonies were then observed with the naked eye based on the following physical characteristics: Form, Margin, Texture, Size, and Color. This specific method of colony morphology was derived from Sousa et al. (2013).

5.0 Gram-staining characterization

The researchers employed the use of Gram-staining characterization on the 10 MHA plates as they hold the most potential for bacterial diversity compared to the two other selective agar media. 10 microscopic glass slides were procured alongside a gram-staining kit from the laboratory. From there, the researchers performed the gram-staining procedure on the streaked MHA samples. They used the paper of Tripathi & Sapra (2020) as a reference in performing this method. Afterward, the Gram-stained slides underwent an examination under a microscope, first starting with the X40 objective, then moving to the X100 objective after evaluation. The researchers then looked out for areas with a singular thick cell, as large, thick areas often give inaccurate results.

6.0 Putative identification

The putative identification of a specific family and genus of bacteria refers to the preliminary or tentative identification of bacterial isolates based on characteristic features, such as phenotypic traits or genetic markers, without employing extensive confirmatory tests (Weyant et al., 2003). This initial identification can provide valuable insights into the taxonomic placement of the bacteria and guide further investigations. Phenotypic identification methods involve assessing various metabolic and physiological properties of bacteria, such as colony morphology, Gram staining, and biochemical tests (Weyant et al., 2003). These tests can provide clues about the family and genus of the bacteria.

7.0 Metabolic profile

To determine metabolic profiling of MSA and EMB agar, the following steps were followed by the researchers: the colonies on the MSA agar plate were observed for characteristics such as color. The presence of prevalent pale yellowish colonies indicated potential mannitol fermentation. The color change indicates potential mannitol fermentation, as acid production from fermentation results in a shift from reddish to yellow due to the phenol red pH indicator (Shields & Tsang, 2006). Meanwhile, the colonies on the EMB agar plate were examined for color, with the presence of dark yellow colonies on the EMB agar plate indicating weak lactose fermentation (Lal & Cheeptham, 2007).

CHAPTER 4

RESULTS AND DISCUSSION

1.0 Isolation of pure cultures

Objective one seeks to isolate the pure cultures of the *H. nigra* digestive system microbiome. Utilizing the streak-plate method, the researchers isolated the pure cultures from a sample plate on 1 MHA, 1 MSA, and 1 EMB agar plate, with a 1:30 ratio. Throughout the entire isolation process, the flaming method was utilized in order to consistently rid the equipment used of possible contaminants. Therefore, through the isolation of pure cultures of the microbiome in the digestive system of *H. nigra* samples, objective one is addressed.

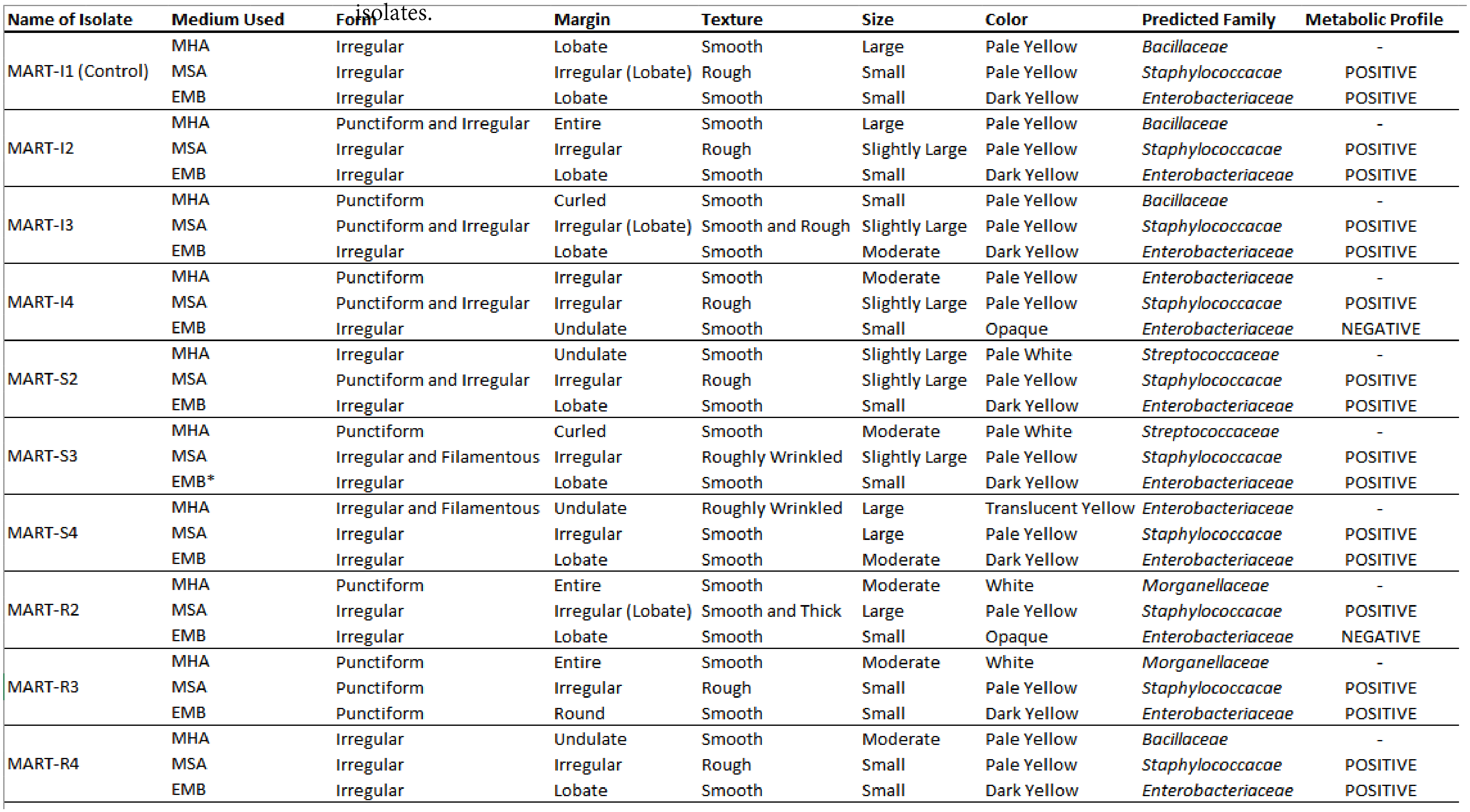
1.1 Sterility control

Prior to isolating the pure cultures of the samples, the researchers incubated their prepared agar plates in the incubator at room temperature (37°C) to determine if the plates were contaminated. It was determined that the incubated agar plates did not show any signs of contamination, and will therefore serve as accurate growth sites, representing the bacteria present in the microbiome of the *H. nigra* digestive system.

2.0 Colony morphology

Objective two seeks to determine the morphological characterization of the isolates. This section will explore the colony morphology aspect of this objective.

Table 1. Colony morphology, putative identification, and metabolic profile of the isolates.



Name of Isolate	Medium Used	Form	Margin	Texture	Size	Color	Predicted Family	Metabolic Profile
MART-I1 (Control)	MHA	Irregular	Lobate	Smooth	Large	Pale Yellow	*Bacillaceae*	-
	MSA	Irregular	Irregular (Lobate)	Rough	Small	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Lobate	Smooth	Small	Dark Yellow	*Enterobacteriaceae*	POSITIVE
MART-I2	MHA	Punctiform and Irregular	Entire	Smooth	Large	Pale Yellow	*Bacillaceae*	-
	MSA	Irregular	Irregular	Rough	Slightly Large	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Lobate	Smooth	Small	Dark Yellow	*Enterobacteriaceae*	POSITIVE
MART-I3	MHA	Punctiform	Curled	Smooth	Small	Pale Yellow	*Bacillaceae*	-
	MSA	Punctiform and Irregular	Irregular (Lobate)	Smooth and Rough	Slightly Large	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Lobate	Smooth	Moderate	Dark Yellow	*Enterobacteriaceae*	POSITIVE
MART-I4	MHA	Punctiform	Irregular	Smooth	Moderate	Pale Yellow	*Enterobacteriaceae*	-
	MSA	Punctiform and Irregular	Irregular	Rough	Slightly Large	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Undulate	Smooth	Small	Opaque	*Enterobacteriaceae*	NEGATIVE
MART-S2	MHA	Irregular	Undulate	Smooth	Slightly Large	Pale White	*Streptococcaceae*	-
	MSA	Punctiform and Irregular	Irregular	Rough	Slightly Large	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Lobate	Smooth	Small	Dark Yellow	*Enterobacteriaceae*	POSITIVE
MART-S3	MHA	Punctiform	Curled	Smooth	Moderate	Pale White	*Streptococcaceae*	-
	MSA	Irregular and Filamentous	Irregular	Roughly Wrinkled	Slightly Large	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB*	Irregular	Lobate	Smooth	Small	Dark Yellow	*Enterobacteriaceae*	POSITIVE
MART-S4	MHA	Irregular and Filamentous	Undulate	Roughly Wrinkled	Large	Translucent Yellow	*Enterobacteriaceae*	-
	MSA	Irregular	Irregular	Smooth	Large	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Lobate	Smooth	Moderate	Dark Yellow	*Enterobacteriaceae*	POSITIVE
MART-R2	MHA	Punctiform	Entire	Smooth	Moderate	White	*Morganellaceae*	-
	MSA	Irregular	Irregular (Lobate)	Smooth and Thick	Large	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Lobate	Smooth	Small	Opaque	*Enterobacteriaceae*	NEGATIVE
MART-R3	MHA	Punctiform	Entire	Smooth	Moderate	White	*Morganellaceae*	-
	MSA	Punctiform	Irregular	Rough	Small	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Punctiform	Round	Smooth	Small	Dark Yellow	*Enterobacteriaceae*	POSITIVE
MART-R4	MHA	Irregular	Undulate	Smooth	Moderate	Pale Yellow	*Bacillaceae*	-
	MSA	Irregular	Irregular	Rough	Small	Pale Yellow	*Staphylococcacae*	POSITIVE
	EMB	Irregular	Lobate	Smooth	Small	Dark Yellow	*Enterobacteriaceae*	POSITIVE

Figure 3. The table displays the characteristics in colony morphology of each sample for each medium used. Through these characteristics, the bacteria's family is predicted. Interpretation of EMB and MSA are also indicated.

Legend:

MHA – Mueller-Hinton agar

MSA – Mannitol salt agar

EMB – Eosin-methylene blue (agar)

* signifies that the plate and agar were not in the best condition, therefore the results may be inaccurate.

Figure 4. Growth of colonies in MHA, MSA, and EMB agars, respectively.

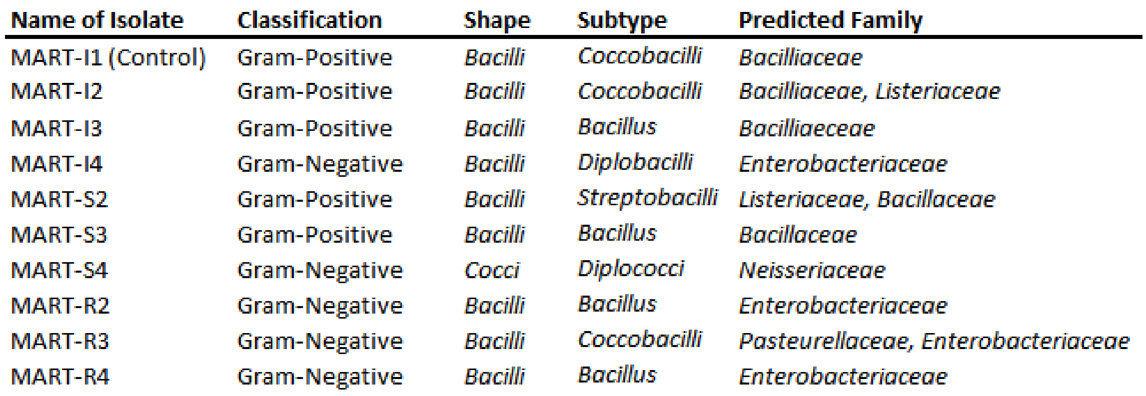
The results presented in the table above show the characteristics of each isolate per agar used in their respective plates. Starting with the MSA, the most significant characteristic that they possess is the irregularity of forms and margins above the agar. There is also a consistent color of a pale yellowish growth that all of the MSA plates have, with the majority of it measured to a relatively large size and observed with a rough texture. The MHA isolates, however, have been shown to grow in irregular and punctiform shapes, as its margins are mixed in-between different classifications with prevailing entire or undulate variations. The majority of the MHA isolates have been observed with a smooth texture, with moderate to slightly large surface area, and a prevalent pale yellow color to it. Lastly, EMB is observed with the least characteristics visible to the human eye, as prevalent irregular forms and lobate margins are seen above the agar. At the same time, it has the most consistent findings of characteristics with all plates observed with moderate to small surface area, smooth textures, and prevalent dark yellow shades of colony. Based on these characteristics, the researchers predict that the bacteria present in the MHA isolates may belong to the *Bacillaceae, Enterococcaceae, Streptococcaceae,* and *Morganellaceae* families. In the selective growth media, however, the bacteria present in the MSA isolates belong to the *Staphylococcaceae* family, while the bacteria present in the EMB agar isolates belong to the *Enterobacteriaceae* family.

The isolates grown on MSA display irregular form and margin above the agar. This irregularity suggests that these microorganisms may possess varying metabolic capabilities. The metabolic profile observed on MSA agar primarily revolves around the fermentation of mannitol. Microorganisms capable of fermenting mannitol produce acid as a metabolic byproduct, causing a color change in the pH indicator phenol red from reddish to yellow. The presence of pale yellowish colonies on MSA agar indicates the potential fermentation of mannitol by the isolates, demonstrating their metabolic capability in utilizing mannitol as a carbon source. The metabolic profile of EMB agar revolves around the fermentation of lactose. Lactose-fermenting microorganisms produce acid as a metabolic byproduct, resulting in a color change in the medium. The appearance of dark yellow colonies on EMB agar suggests the potential metabolic capability for weak lactose fermentation. The dark yellow color indicates less acid production as a byproduct of weak lactose fermentation. This may be due to lesser metabolic activity or efficiency in lactose utilization by the microorganisms on EMB agar. Therefore, this metabolic profiling of the MSA and EMB agar through observation of color changes addresses one aspect of the biochemical characterization of objective three.

3.0 Gram-staining characterization

This is the continuation of the previous section that seeks to address the morphological characterization of the isolates. In this section, the Gram-staining characterization findings will be explored.

Table 2. Gram-staining characterization of the isolates in Mueller-Hinton agar alongside the shape and predicted family.



Name of Isolate	Classification	Shape	Subtype	Predicted Family
MART-I1 (Control)	Gram-Positive	*Bacilli*	*Coccobacilli*	*Bacilliaceae*
MART-I2	Gram-Positive	*Bacilli*	*Coccobacilli*	*Bacilliaceae, Listeriaceae*
MART-I3	Gram-Positive	*Bacilli*	*Bacillus*	*Bacilliaeceae*
MART-I4	Gram-Negative	*Bacilli*	*Diplobacilli*	*Enterobacteriaceae*
MART-S2	Gram-Positive	*Bacilli*	*Streptobacilli*	*Listeriaceae, Bacillaceae*
MART-S3	Gram-Positive	*Bacilli*	*Bacillus*	*Bacillaceae*
MART-S4	Gram-Negative	*Cocci*	*Diplococci*	*Neisseriaceae*
MART-R2	Gram-Negative	*Bacilli*	*Bacillus*	*Enterobacteriaceae*
MART-R3	Gram-Negative	*Bacilli*	*Coccobacilli*	*Pasteurellaceae, Enterobacteriaceae*
MART-R4	Gram-Negative	*Bacilli*	*Bacillus*	*Enterobacteriaceae*

Figure 5. The table displays the MHA isolates' characterization in gram-staining. In 10 isolates, half are gram-positive, while the other half are gram-negative bacteria. The physical shape of the bacteria under a microscope was observed, and from it, a deeper classification was predicted.

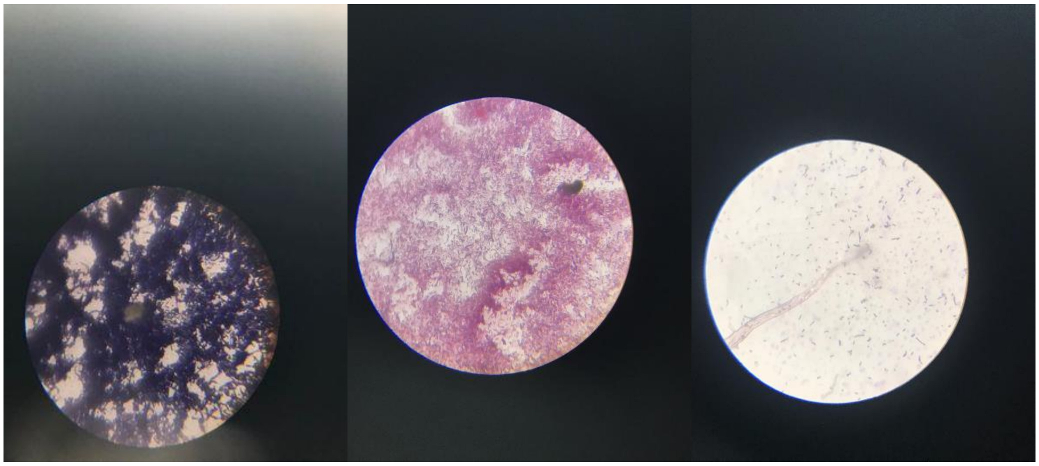


Figure 6. Gram-stained microscopic slides belonging to MART-S2, MART-I4, and MART-S3, respectively.

Findings in conducting the Gram-staining characterization in the 10 MHA plates resulted in classifying which isolates are Gram-positive and Gram-negative bacteria, alongside identifying the shape of each bacterium per microscopic slide, which was then used as the basis for labeling the present genus of bacteria in each slide for the following tests. In characterizing the isolates using Gram-staining, a more accurate inference can be made about what family the bacteria belong to. Based on the findings, the MHA isolates may belong to the *Bacilliaceae, Listeriaceae, Enterobacteriaceae, Neisseriaceae,* and *Pasteurellaceae* families. Therefore, Tables 1 and 2 present findings that address the morphological characterization of the isolates through colony morphology and gram-staining characterization.

4.0 Catalase test

Objective three seeks to address the biochemical characterization of the isolates. This section explores one aspect of it, namely, the catalase test through 3% Hydrogen peroxide (H_2O_2).

Table 3. Catalase test of the isolates in Mueller-Hinton agar indicating a positive or negative reaction to 3% Hydrogen peroxide alongside the predicted genus.

Name of Isolate	Result	Predicted Genus
MART-I1 (Control)	+	*Bacillus, Listeria, Corynebacterium*
MART-I2	+	*Bacillus, Listeria*
MART-I3	+	*Bacillus*
MART-I4	+	*Eschericia*
MART-S2	+	*Bacillus, Listeria*
MART-S3	+	*Bacillus, Acintobacter*
MART-S4	-	*Neisseria*
MART-R2	+	*Acintobacter, Eschericia*
MART-R3	+	*Eschericia, Pasteurella*
MART-R4	+	*Moraxella, Neisseria, Citrobacter, Eschericia*

Figure 7. The table displays the results of the MHA isolates that underwent the catalase test. The findings show that there was only one isolate, MART-S4, that did not positively react to 3% Hydrogen peroxide. With more of the isolates' characteristics being discovered, the researchers were able to classify the bacteria down to the genus level.

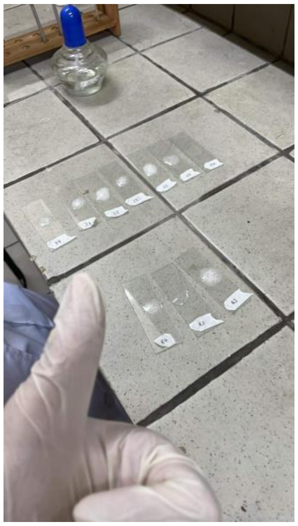


Figure 8. Catalase test procedure and results

The purpose of the catalase test for the research is to determine the microbes' capacity to break down hydrogen peroxide by creating the enzyme catalase. The outcome of the method resulted in having all but MART-S4 to be catalase +. These findings led the researchers to further classify the isolates' families into genera as displayed in the table above.

5.0 Inoculation in SIM media

The final aspect of biochemical characterization explored in this study utilizes the Sulfur, Indole, Motility (SIM) media. As stated in the scope and limitations, however, only the sulfur and indole characteristics were tested.

Table 4. Results of the isolates in Mueller-Hinton agar after inoculation in SIM media alongside the predicted genus.

Name of Isolate	Sulfur	Motility	Predicted Genus
MART-I1 (Control)	+	+	*Bacillus*
MART-I2	+	+	*Listeria*
MART-I3	+	+	*Bacillus*
MART-I4	+	+	*Eschericia*
MART-S2	+	+	*Listeria, Bacillus*
MART-S3	+	+	*Bacillus*
MART-S4	+	+	*Neisseria*
MART-R2	+	+	*Eschericia*
MART-R3	+	+	*Eschericia*
MART-R4	+	+	*Eschericia, Citrobacter*

Figure 9. The table displays the results of the MHA isolates that were inoculated in SIM media. The findings show that all the isolates had a positive result for both sulfur and motility. With this being the final procedure, the displayed predicted genera are the final results for the study.

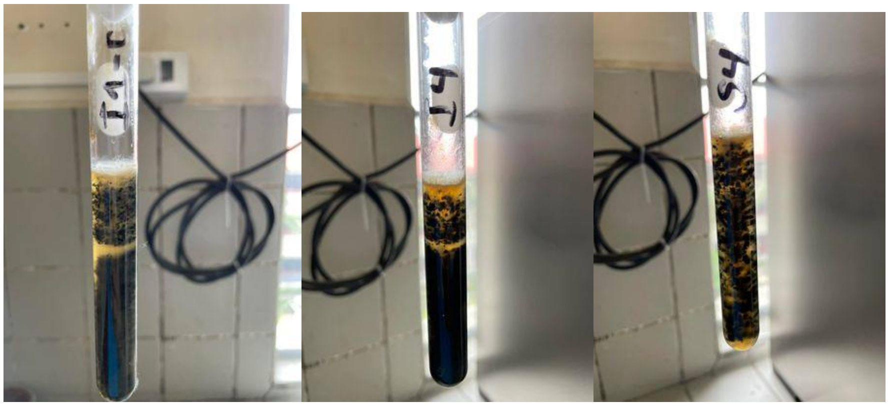


Figure 10. SIM media inoculation results for MART-I1 (Control), MART-I4, and MART-S4, respectively.

Sulfide, Indole, Motility (SIM) media is then prepared for conducting the motility test of the various bacilli that can be differentiated based on their capacity to generate sulfide gas (H_2S), make indole from tryptophan, and move into the medium. Excluding the process of producing indole, all isolates were observed with results of a positive H_2S test, as well as a positive motile reaction. A positive result in sulfur is indicated by a black precipitate forming in the test tube. A positive result in motility is indicated by growth in the stab following the line of inoculation. Based on the findings, the genera of the bacteria were determined as displayed in the table. Therefore, alongside the results from the Catalase test, with the biochemical characterization complete, objective three is addressed.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

This study's main objective is to characterize the bacteria present in the microbiome of the *H. nigra* digestive system as a preliminary survey. *H. nigra* was chosen for this study as holothurians have a diverse microbiome due to their being prone to consuming bacteria-rich marine sediments. Morphological and biochemical characterization was performed on the isolates, and the isolates were able to be characterized based on their characteristics and how they interacted with the tests.

With all this in mind, the following are concluded after conducting the study:

1. The *H. nigra* samples' isolates contained a diverse variety of bacteria.

2. Through morphological characterization, bacteria from the *Bacillaceae* family are the most prevalent in the isolates.

3. Through biochemical characterization, it was determined that bacteria from the *Bacillus* and *Escherichia* genera are the most prevalent in the isolates.

Recommendations

Based on the research design, the related literature, the results obtained, and the conclusion of this study, the following recommendations were drawn:

1. Further characterize the bacteria through 16S rRNA gene sequencing.
2. Characterize the bacteria found in *H. nigra* samples from different sources.
3. Perform antibacterial screening on *H. nigra* microbiome based on the preliminary screening results of this study.

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APPENDICES

APPENDIX A

Measurements used:

- MUELLER HINTON AGAR

11.7 g dissolved in 300 ml distilled water (per 500 ml of erlenmeyer flask) so 23.4 g, 600 ml

- MANNITOL SALT AGAR BASE

33.8 g, 300 ml (one 500 ml erlenmeyer flask)

- EMB AGAR

11.288 g, 300 ml (one 500 ml erlenmeyer flask)

- SIM MEDIUM (30 test tubes)

9.5575 g, 250 ml (2 erlenmeyer 500 ml flask) so 19.115, 500 ml

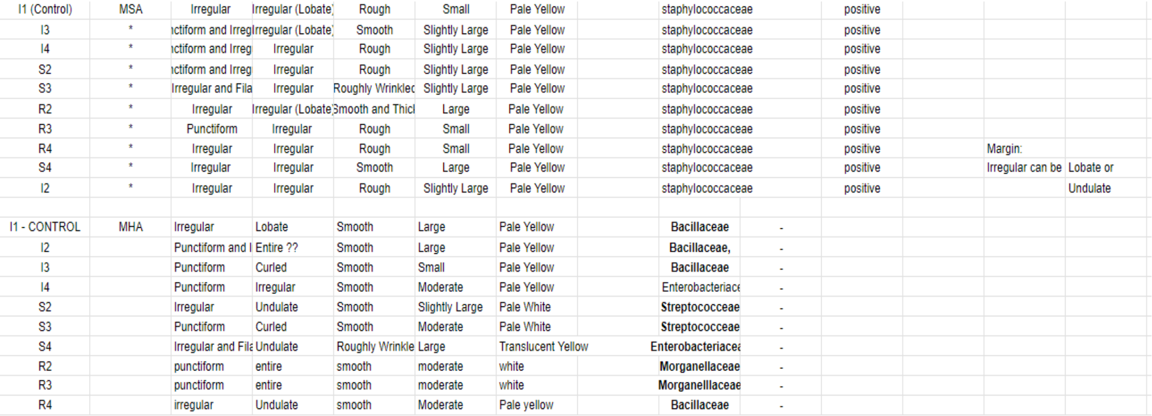
- LYSINE IRON AGAR (30 test tubes)

9.14 g, 250 ml (2 erlenmeyer 500 ml flask) so 18.28, 500 ml

*Incubation period is 2 days at 37 degrees celsius both will be implemented after stabbing and after streaking of isolates.

APPENDIX B

Raw data images:



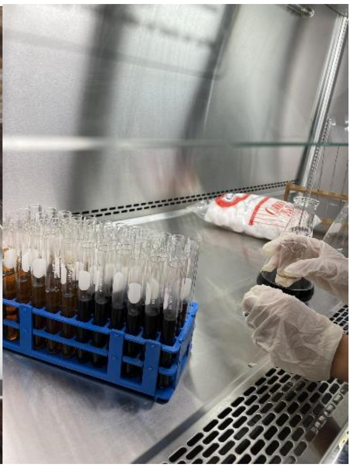
I1 (Control)	MSA	Irregular	Irregular (Lobate)	Rough	Small	Pale Yellow		staphylococcaceae		positive			
I3	*	ıctiform and Irreg	Irregular (Lobate)	Smooth	Slightly Large	Pale Yellow		staphylococcaceae		positive			
I4	*	ıctiform and Irreg	Irregular	Rough	Slightly Large	Pale Yellow		staphylococcaceae		positive			
S2	*	ıctiform and Irreg	Irregular	Rough	Slightly Large	Pale Yellow		staphylococcaceae		positive			
S3	*	Irregular and Fila	Irregular	Roughly Wrinklec	Slightly Large	Pale Yellow		staphylococcaceae		positive			
R2	*	Irregular	Irregular (Lobate)	Smooth and Thicl	Large	Pale Yellow		staphylococcaceae		positive			
R3	*	Punctiform	Irregular	Rough	Small	Pale Yellow		staphylococcaceae		positive			
R4	*	Irregular	Irregular	Rough	Small	Pale Yellow		staphylococcaceae		positive		Margin:	
S4	*	Irregular	Irregular	Smooth	Large	Pale Yellow		staphylococcaceae		positive		Irregular can be	Lobate or
I2	*	Irregular	Irregular	Rough	Slightly Large	Pale Yellow		staphylococcaceae		positive			Undulate
I1 - CONTROL	MHA	Irregular	Lobate	Smooth	Large	Pale Yellow		**Bacillaceae**	-				
I2		Punctiform and I	Entire ??	Smooth	Large	Pale Yellow		**Bacillaceae,**	-				
I3		Punctiform	Curled	Smooth	Small	Pale Yellow		**Bacillaceae**	-				
I4		Punctiform	Irregular	Smooth	Moderate	Pale Yellow		Enterobacteriace	-				
S2		Irregular	Undulate	Smooth	Slightly Large	Pale White		**Streptococceae**	-				
S3		Punctiform	Curled	Smooth	Moderate	Pale White		**Streptococceae**	-				
S4		Irregular and Fila	Undulate	Roughly Wrinkle	Large	Translucent Yellow		**Enterobacteriacea**	-				
R2		punctiform	entire	smooth	moderate	white		**Morganellaceae**	-				
R3		punctiform	entire	smooth	moderate	white		**Morganelllaceae**	-				
R4		irregular	Undulate	smooth	Moderate	Pale yellow		**Bacillaceae**	-				

I1 - CONTROL	EMB	irreg	lobate	smooth	small	dark yellow	done	enterobacteriaceae		positive
I2		irreg	Lobate	Smooth	Small	Dark yellow	done	enterobacteriaceae		positive
I3		Irref	lobate	smooth	moderate	Dark yellow	done	enterobacteriaceae		positive
I4		Irreg	Undulate	Smopth	Small	Opaque	done	enterobacteriaceae		negatvie
S2		irregular	lobate	smooth	Small	Dark yellow	done	enterobacteriaceae		positive
S3	sira							enterobacteriaceae		positive
S4		Irreg	lobate	smooth	moderate	dark yelloe	done	enterobacteriaceae		positive
R2		Irregular	lobate	smooth	small	Opaque	done	enterobacteriaceae		negative
R3		Punctiform	round	smooth	Small	dark yellow	done	enterobacteriaceae		positive
R4		irregular	Lobate	Smooth	Small	dark yellow	done	enterobacteriaceae		positive
GRAM STAINING		shapes	sub types	family / genus						
I1 - CONTROL	POSITIVE	bacilli	coccobacilli	Bacilliaceae						
I2	POSITIVE	bacilli	coccobacilli	Bacilliaceae, Listeriaceae						
I3	POSITIVE	bacilli	bacillus	Bacilliaceae						
I4	NEGATIVE	bacilli	diplobacilli	Enterobacteriaceae						
S2	POSITIVE	bacilli	streptobacilli	Listeriaceae, Bacillaceae						
S3	POSITIVE	bacilli	bacillus	Bacillaceae						
S4	NEGATIVE	cocci	diplococci	Neisseriaceae						
R2	NEGATIVE	bacilli	bacillus	Enterobacteriaceae						
R3	NEGATIVE	bacilli	coccobacilli	Pasteurellaceae, Enterobacteriaceae						
R4	NEGATIVE	bacilli	bacillus	Enterobacteriaceae						

APPENDIX C

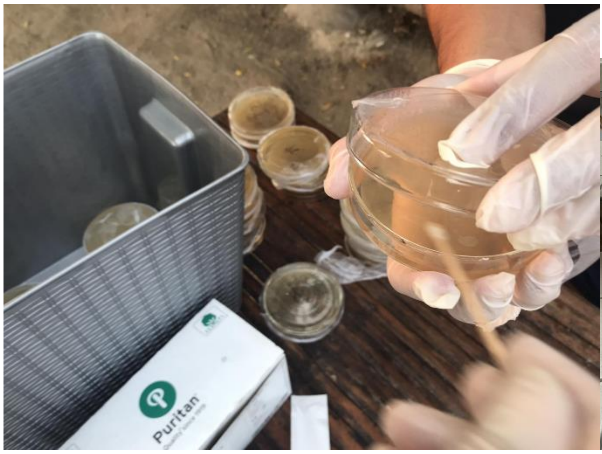
Photodocumentation:

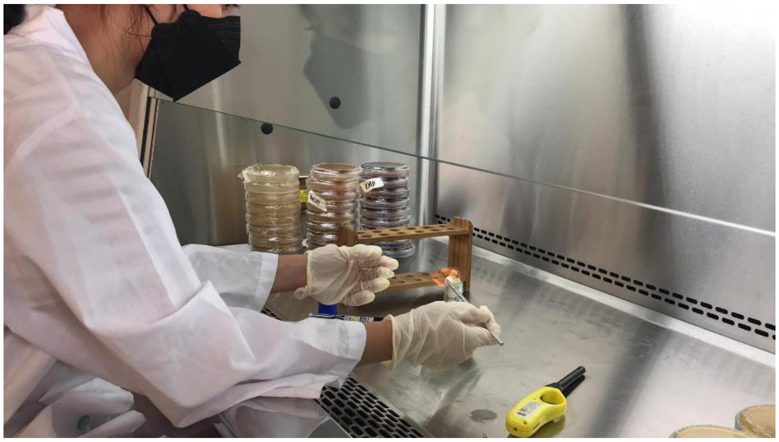

OKADA

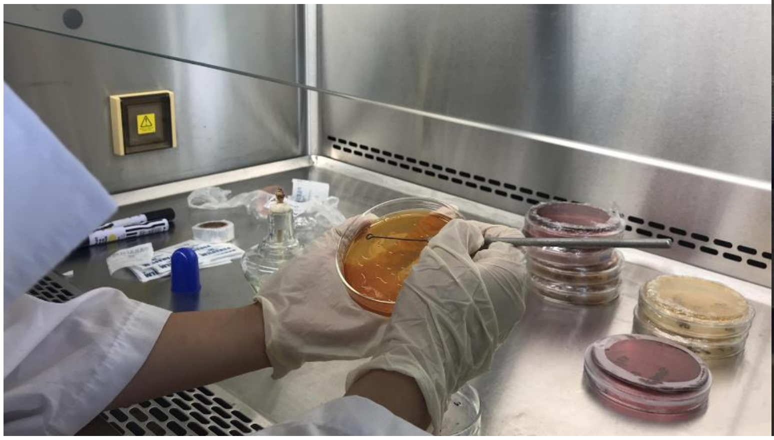


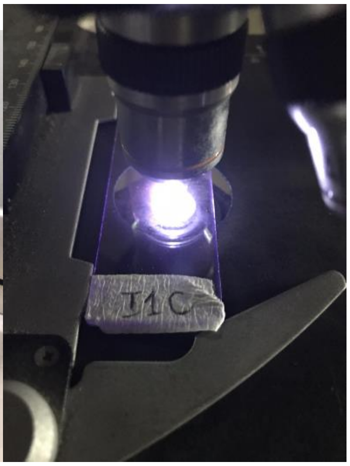

EMB
MSA
MHA


OKADA


Puritan







J1C