MICROBIOLOGICAL QUALITY OF EDIBLE LAND SNAILS FROM SELECTED MARKETS IN GHANA

BY

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DECLARATION

With the exception of duly acknowledge references, this thesis has been the product of my own investigations and has not been presented for another degree elsewhere.

This study was carried out at the Department of Microbiology, University of Ghana Medical School (UGMS) under the joint supervision of Rev. Prof. Patrick F. Ayeh-Kumi and Dr. Simon K. Attah.

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"We hereby declare that the preparation and presentation of this thesis was done in accordance with the guidelines on supervision of thesis laid down by the University Of Ghana School of Graduate studies"

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Supervisor

DEDICATION

This work is dedicated to my mother, Dadzie Yaa Felicia.

Your principle of universal respect, love and care for all, irrespective of their religious nor racial difference, has thus brought me this far. Your incessant motivation, support and belief in the need for higher education is unmatched. Your investment in me cannot be overlooked. God richly bless you, "Eno Maame Apiema."

You are greatly appreciated.

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In the arm of flesh there is failure, yet, in trusting God Almighty, oasis show up in the desert. Thanks be to God for His grace and mercy for the successful completion of this work.

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ABSTRACT

Background

Snail meat has served as a delicacy and reliable source of protein for man over the years. Despite the increasing price of snails, there is a corresponding increase in demand worldwide. Snails are currently being exploited for other purposes than just food. Russia and Japan are amongst some of the countries that use live snail (slime) for beauty therapy. In Ghana, almost every ethnic group consumes snails. There are undocumented reports of varied uses of the snail haemolymph as a cure for ailments such as tuberculosis, whooping cough, stroke, hypertension and asthma amongst other uses. The slime on the other hand, when applied to skin with burns is said to improve skin cell regeneration as well as enhancing smoothness of skin. These consumers may have positive results for their health needs but are ignorant of the possible microbial pathogen content. The aim of the study was to assess the microbiological quality of the edible land snails.

This is to draw awareness to the general public regarding the health hazards they expose themselves to in consuming the snails and their products particularly in the raw state.

Method: 150 snails (75 Achatina achatina and 75 Achatina fulica) were purchased from a total of 9 markets distributed in four regions of Ghana. Bacteriological, parasitological and mycological analysis was carried out on four different specimens (haemolymph, slime, hepatopancreas and faecal matter) of the snails. Standard plate count (SPC) and enterobacteriaceae count (EC) were done. Isolation and biochemical identification of bacteria were done. For the parasitological analysis, wet and stained (centrifuged and uncentrifuged) specimens were observed microscopically at X40 magnification. Stains carried out included Giemsa, Ziehl-Neelsen, iodine and trichrome. Mycological analysis

was done by streaking specimens on Saboraud agar and subsequent nigrosin and Lactophenol cotton blue staining done for identified fungal agents.

Results: Thirty-two different bacteria including *Salmonella spp, Klebsiella spp, Shigella spp, Yersinia spp* and *Clostridium tetani* were isolated. *Giardia spp, Balantidium coli* and trophozoites and cysts of unidentified parasites were isolated. Fungal agents isolated included *Candida spp, Cryptococcus neoformans, Aspergillus spp,* and *Rhizopus spp*. A statistically significant result was observed for the Standard Plate count and Enterobacteriaceae count with regards to the snail specimens and the markets where they were bought from, source of snail, and region of purchase. During the study, bacteria load count ranges (10^2-10^6) obtained were within and above the levels set for most foods hence by that comparison can be inferred to be microbiologically safe. The risk therefore with the administration of the unprocessed specimens orally or otherwise is of immense public health concern bearing in mind the pathogenic microbes isolated.

Conclusion: From the study, both pathogenic and non-pathogenic (including opportunistic) microbial agents were isolated from snails obtained from the Ghanaian markets. The consumption of the snails particularly their use in the raw state is not a safe practice owing to the isolation of these organisms from them.

LIST OF ABBREVIATIONS

µm	micrometre
AA	*Achatina achatina*
ACC	Aerobic Colony Count
AF	*Achatina fulica*
AMA	Accra Metropolitan Assembly
APW	Alkaline Peptone Water
CDC	Centres for Disease Control
CFU	Colony Forming Unit
EC	Enterobacteriaceae Count
FM	Faecal Matter
g	Gram
GHP	General Hygienic Practices
Ha	Haemolymph
HACCP	Hazard Analysis Critical Control Point
HP/HPF	Hepatopancreas
KMA	Kumasi Metropolitan Assembly
KOH	Potassium hydroxide
LCB	Lactophenol Cotton Blue
mg	Milligram
MIU	Motility- Indole- Urea
mL	millilitre
mm	millimetre

P	P- value (<5% indicates statistical significance; event not due to chance)
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar
S	Slime
SDA	Sabouraud Dextrose Agar
SF	Selenite F
SPC	Standard Plate Count
TAA	Trimeric Auto-transporter Adhesins
TCBS	Thiosulphate Citrate Bile Salts Sucroce agar
TFTC	Too Few To Count
TMA	Tema Metropolitan Assembly
TNTC	Too Numerous To Count
TSS	Toxic Shock Syndrome
ZN	Ziehl-Neelson

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CHAPTER ONE

1.1 Introduction

Food is essential for a healthy life. Good food provides the body with carbohydrates, proteins, fats and oils, water, minerals and vitamins. Proteins especially are very important for life processes. Sources of proteins (including animals and plants) are from legumes, nuts, meat and eggs amongst others. Snail meat has been documented to be a good and high source of protein, iron and calcium and it is known to contain almost all the amino acids needed by humans (Ajayi *et al*., 1978; Imevbore and Ademosun, 1988; Ademola *et al*., 2004; Cobbinah *et al.*, 2008).

They belong to the class Gastropoda. Gastropods characteristically move on their belly and are often enclosed in a spirally coiled shell. The word "snail", generally includes sea snails, land snails and freshwater snails. The "Giant African land snail" is the common name used to describe any of the three snail species native to Africa. The three, are: the giant East African snail (*Achatina fulica*), the giant Ghana tiger snail (*Achatina achatina*) and margies (*Archachatina marginata*). Snails belong to the phylum Mollusca, members of which are soft bodied animals that often possess hard shells for protection (Cobbinah *et al.*, 2008; Hodasi, 1995).

Snail meat has been consumed by humans worldwide since pre-historic times (Cobbinah *et al.*, 2008). The calcareous shell of the snail when burnt, ground into powder, mixed with oil and has been applied to boils by many indigenes in Africa as a form of treatment. The slime is currently being used for beauty therapy in Siberia (Russia). Snail products such as the haemolymph are also being exploited as a cure for various ailments. The invasive land snail *Achatina fulica*, is a species known to carry parasites and harbours a dense and

metabolically active microbial community, the diversity and composition of which is however unknown (Cardoso *et al*., 2012). A mollusc is usually the first intermediate host for all flukes. Human parasitic agents (*Schistosome spp, Angiostrongylus spp and Fasciola hepatica* amongst others) known to infect aquatic species of molluscs (genus *Bulinus*, *Biomphalaria*, *Oncomelania*, etc) as part of the life cycle however have not been documented in the edible snails mentioned above. Angiostrongyliasis or eosinophilic meningoencephalitis is another human disease most commonly caused by the parasitic nematode (roundworm) *Angiostrongylus cantonensis* (the rat lungworm). The indirect life cycle of the rat lungworm requires an intermediate host (usually a mollusc) for the development of various larval stages.

1.2 Snails

Snails are invertebrates ranging from the size of a palm nut or even less to an adult fist or a little larger. They can be found in swamps, ditches, water bodies, forests, gardens and farm lands. Snail preserves were established in Rome in 50 B.C. The typical life span of a snail is 9 - 15 months but some species may live up to 2 to 4 years. Snails aestivate if the temperature is too high (often more than 30°C) or if the air humidity is too low (less than 70-75% relative humidity). They hibernate if the temperature drops below 5°C (Cobinah *et al.*, 2008).

1.2.1 Origin of the name *molluscan* and genus *Achatina*

The word mollusc is derived from the Latin word *molluscus* (from *mollis*) meaning soft.

The words "gastro" and "pod" from “gastropod,” mean “stomach,” and “foot” respectively. The name “*Achatina*” is from the Greek word 'achates', for agate. Agate (Figure 1.1) is a fine-grained variegated chalcedony (a cryptocrystalline variety of the

silica mineral quartz) (http://www.arnobrosi.com/snails/etymology.html). The colour of Agate ranges from grayish to milky-white. Agate contains opaque, dark-coloured inclusions arranged in stripes and branches to form fern-like and moss-like images (encyclopaedia britanica, 2013). The class Gastropoda is next only to the class Insecta in terms of numbers and diversity (Bouchet *et al*., 2005). The snails of the genus *Achatina* have the characteristic "agate" colourful stripes and branches.

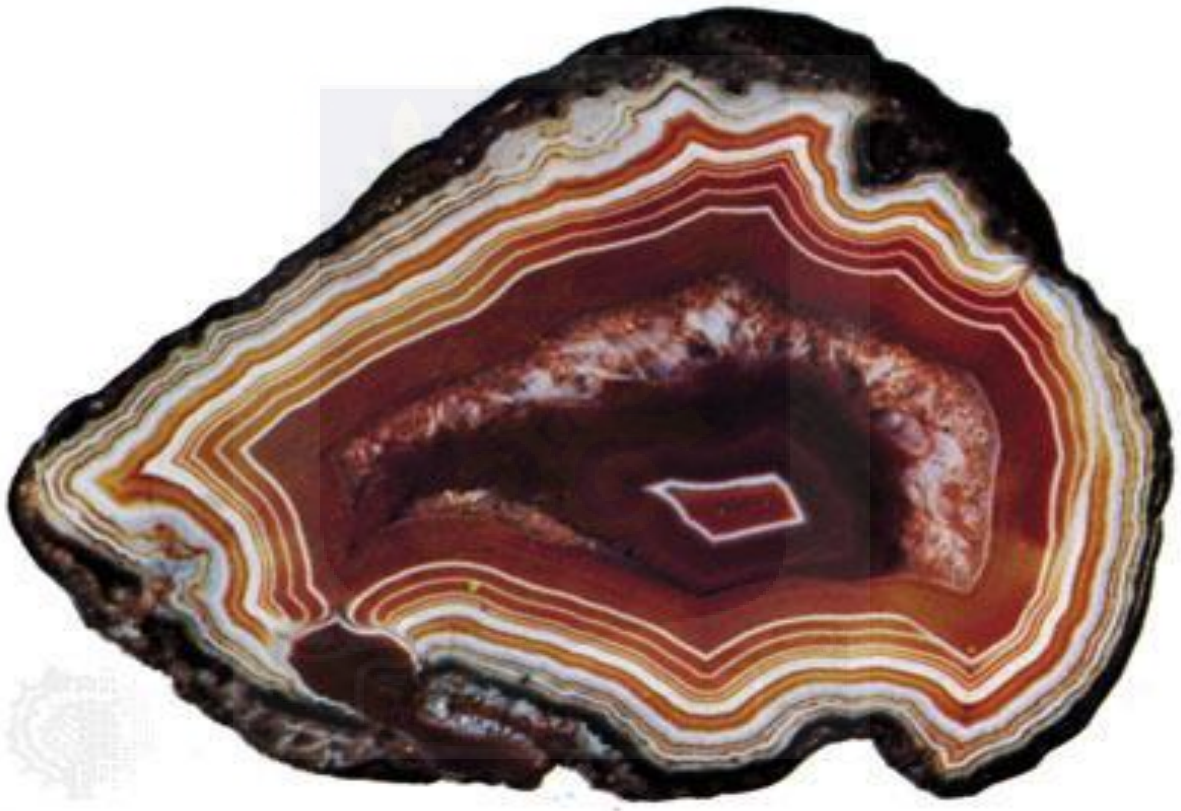


Figure 1.1: A picture of Agate showing the colourful stripes and rings

1.2.2 Classification

The two snails (*Achatina achatina* and *Achatina fulica*) of importance in this research belong to the kingdom Animalia, phylum Mollusca, class Gastropoda, order Pulmonata, family Achatinidae, genus *Achatina* and species a*chatina* and *fulica* respectively. The phylum Mollusca comprises four major groups which are the univalves (class Gastropoda), the bivalves (class Bivalvia), the chittons (class Polyplacophora) and the cephalopods (class Cephalopoda) (Bouchet *et al*., 2005).

1.2.3 Distribution

The giant African Snails are pulmonate, nocturnal, hermaphroditic gastropods of the family Achatinidae. According to Hodasi (1995), the snails occur in sub-Saharan Africa, ranging from the Gambia in the West to the Lake Chad region in the East and that they extends southwards to the Orange River in South Africa. Monney (1994), showed that in Ghana, two species of the *Achatina* genus, namely *Achatina achatina* and *Achatina fulica*, and two species of the A*rchachatina* genus, namely *Archachatina degneri* and *Archachatina marginata* are known to occur.

1.2.4 Morphology

Molluscs are eucoelomates (they possess a true coelom which is a body cavity with mesoderm lining all sides) with a bilateral body symmetry, cephalization, triploblastic (endoderm, mesoderm and ectoderm) structure. They have a complete mouth-to-anus digestive system. Molluscs are not metameric (not segmented). A snail basically comprises of two parts: the body and the shell. The body has three divisions: the head, the foot and the visceral mass. The head is not well demarcated and carries two pairs of

retractable tentacles. One pair of the two tentacles contains the eyes in the knobbed end. The long, muscular foot (generally used for locomotion) occupies almost the entire ventral surface and is not clearly demarcated from the rest of the body. There is a shallow longitudinal groove along the centre of the foot. The hump-shaped visceral mass is housed in the shell above the foot. This contains the digestive, reproductive and respiratory organs. The skin over the visceral hump, the mantle, secretes a large calcareous shell made up of 98% calcium carbonate. In most species the shell accounts for about a third of the body weight. The shell is a protective casing into which the snail withdraws its body whenever danger is looming.

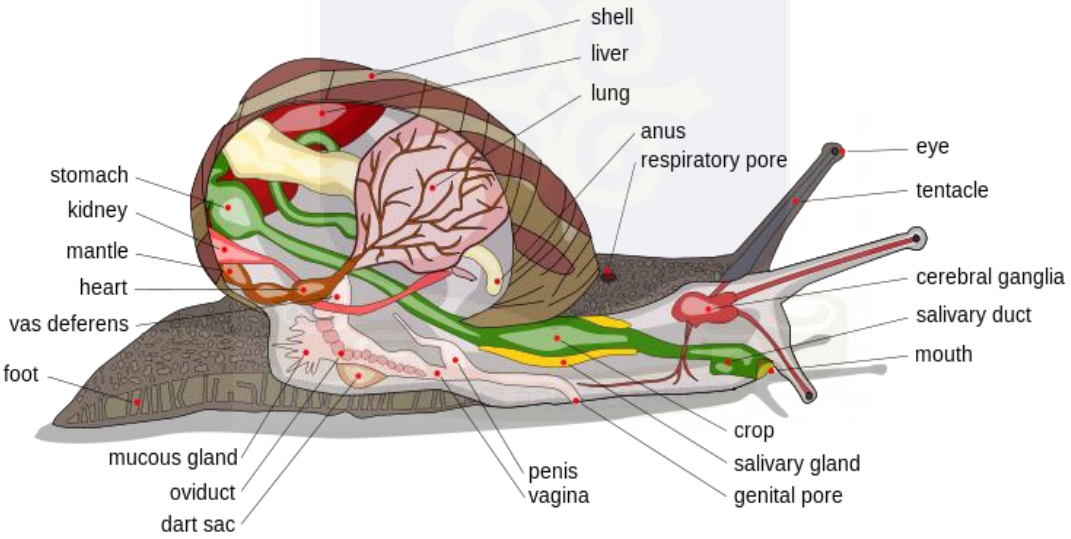


Figure 1.2: A labeled diagram of the anatomy of a snail.

Source: (http://www.google-gastropod.org/morphology_snails.htm)

1.3a

1.3b

Figures 1.3a showing *Achatina fulica* and 1.3b: *Achatina Achatina* in a basket.

1.2.5 Circulation

The circulatory system is a system of organs that work together to allow the circulation of blood and lymph which transports or ensures the even distribution of gases, nutrients, hormones, water as well as ions. The system consists of a pump (the heart), tubes ("blood vessels"), and a fluid (the "blood"). Snails have posterio-dorsally placed heart, the haemolymph as blood, and the haemocoele as the blood vessels (encyclopaedia britannica, 2013). Land snails are terrestrial pulmonates characterized by the ability to breathe air, by virtue of having a pallial lung (pulmonary sac formed by mantle) instead of a gill. They have an open circulatory system as opposed to the closed circulatory system of vertebrates. In an open circulatory system, the haemolymph is not entirely confined to the haemocoele. This system does not possess separate systems for blood and lymph or interstitial fluid. This open circulatory system of the land gastropod is also called a haemolymphatic system. The open circulatory system simply involves a haemocoele (a fluid filled cavity) which bathes the organs of the snail directly with oxygen and nutrients while collecting and circulating metabolic waste.

The posterio-dorsally placed heart is enclosed in a pericardium typically consisting of a ventricle and two posterior auricles. When the heart beats, it pulls oxygenated haemolymph within the pallial lungs toward the heart. This part of the mollusc circulatory system is closed, and as the blood moves from the veins toward the heart, it passes through a large, thin-walled atrium suspended in the pericardial cavity and surrounded by pericardial fluid. Metabolic wastes diffuse across the wall into the fluid, and are then filtered by the metanephridium that opens into the pericardial space.

1.2.5.1 The haemolymph

The "blood" of the snail is technically called the "haemolymph." This fluid has the function of transporting nutrients to cells, thermoregulation, ionic balance, general homeostasis and pH regulation. The haemolymph fills the haemocoele (extracellular spaces containing haemolymph) of the snail and bathes surrounding cells. The haemolymph is composed of water, organic compounds and inorganic salts (magnesium, calcium, sodium, potassium and chlorides). The organic compounds include hormones, carbohydrates, proteins (amino acids), and lipids. The haemolymph has a primary oxygen transporter molecule called haemocyanin. The haemolymph has a colourless to bluish appearance depending on its state of oxygenation.

Snails use various innate mechanisms involving cell-mediated and humoral reactions such as soluble haemolymph factors that eliminate invading pathogens. This is because the snails appear to lack an adaptive immune system. There are various free-floating haemocytes, within the haemolymph as part of the immune system. Studies done by Lockyer *et al*. (2008) revealed the presence of certain alleles of cytosolic copper/zinc superoxide dismutase suggesting an example of the presence of an innate immune system.

1.2.5.2 Haemocyanin

Haemocyanin is a copper-based respiratory pigment (Morton, 1958). They are metalloproteins containing copper rather than iron that is found in haemoglobin. Haemocyanin contains two copper atoms that reversibly bind a single oxygen molecule (O_2). Oxygenation causes a colour change between the colourless Cu^{+} deoxygenated form and the blue Cu^{2+} oxygenated form (Dolashka *et al*., 1996).

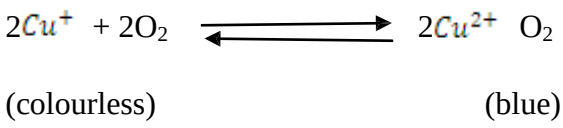


Figure 1.4: A chemical equation of the reversible combination of copper with oxygen

Haemocyanins are second only to haemoglobin in frequency of use as an oxygen transport molecule. Haemocyanins have a relatively larger size (400 to 20,000 kDa) compared to haemoglobin (65kDa) (Waxman, 1975). Due to their large size, they are not bound to haemocytes as observed in haemoglobin (haemoglobin in red blood cells found in vertebrates have a small size which would easily clog and damage blood filtering organs such as the kidney if not bound in cells) but are instead suspended directly in the haemolymph. The free floating nature helps offset its low efficiency as an oxygen transporter (Waxman, 1975; Strobel, 2012).

Haemocyanins are made up of many individual subunit proteins which contain active sites (haemoglobin has four subunits). Each subunit weighs about 75kDa and is arranged in chains or bundles in weights exceeding 1500kDa. The copper atoms of haemocyanin are bound as prosthetic groups coordinated by histidine residues, unlike haemoglobin which carries its iron atoms in porphyrin rings (heme groups). Studies on the amino acid sequence determinations of a number of haemocyanins by Avissar (1986), indicated the presence of a common single stretch of six amino acid residues, "His-His-Trp-His-Trp-His". This stretch is believed to be the likely complex to carry the binuclear copper responsible for the binding of oxygen.

1.2.6 The hepatopancreas

The hepatopancreas is an organ with glandular and enzymatic roles. It is the digestive tract of gastropods. It provides the functions of the liver (hepato-) and pancreas in mammals. Thus, it is involved in food digestion (digestive enzymes secretion). The hepatopancreas is a centre of lipid metabolism and storage of lipids in gastropods (Böer, 2006).

1.2.7 The slime

Snails are observed to produce slime trails. Slime is a mucus secretion from special glands situated in the muscular foot of the snail. This secretion plays a role in adherence of the snail to surfaces. The slime contains approximately 91 to 98% weight of water, depending on the species, as well as small amounts of high molecular weight glycoproteins (Denny, 1984). It also aids in lubrication and movement of the snail. A snail releases different kinds of mucus depending on the way it is stimulated. When the stimulation is normal the slime is viscous (sticky) but if the snail is disturbed continuously or even violently, it releases clear foamy secretions.

1.2.8 The shell

The shell is an external skeleton which serves not only for muscle attachment, but also for protection from predators and from mechanical damage. The mantle edge secretes the shell. The shell consists mainly of three layers: the outer layer (the periostracum) made of organic matter, a middle prismatic layer, and an inner layer consisting of laminated calcite, often nacreous (Hayward, 1996). The periostracum consists of conchiolin, the prismatic layer is made of columnar calcite (calcium carbonate), and the nacreous layer is composed of calcium carbonate and proteins.

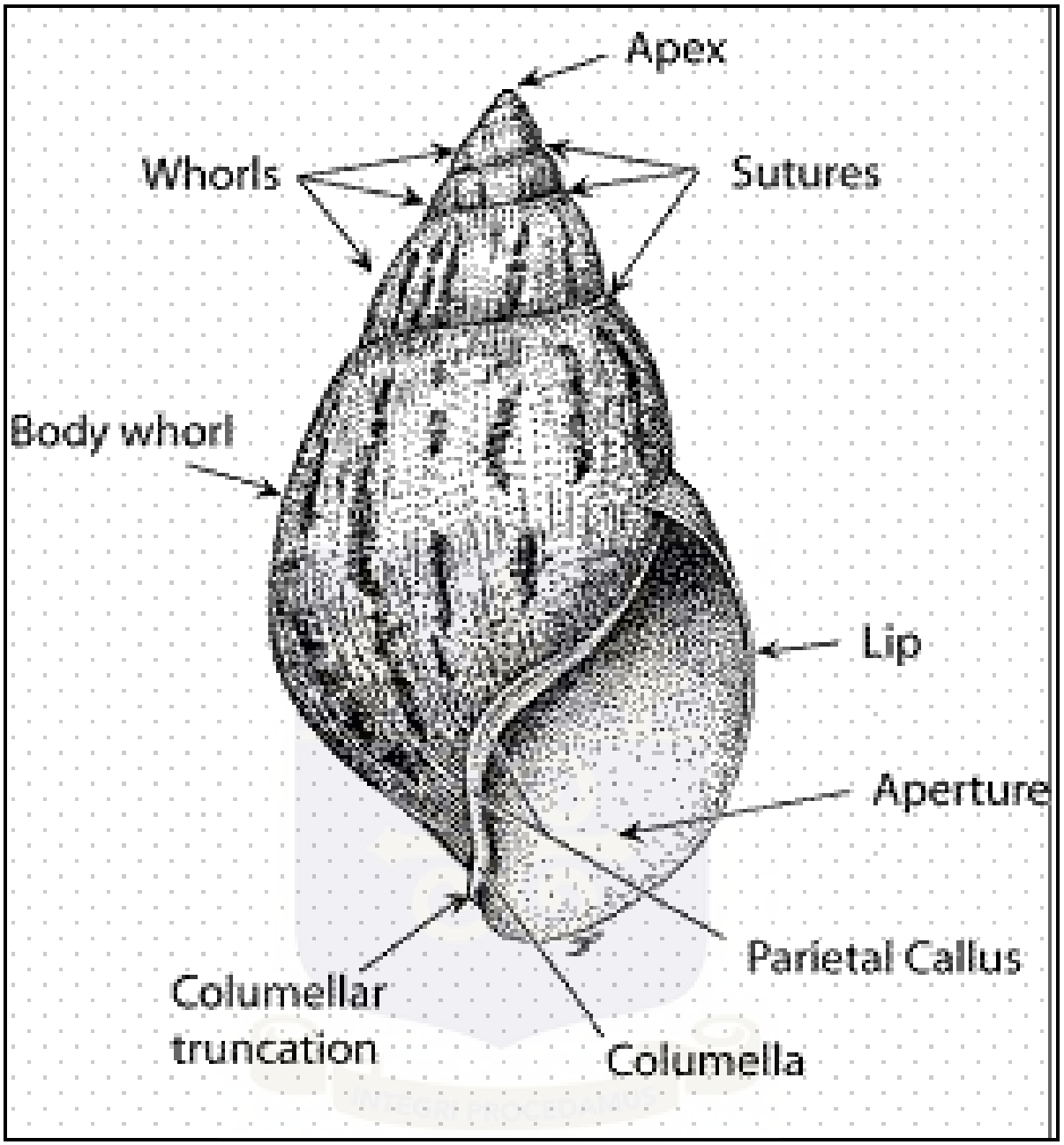


Figure 1.5: A labeled diagrammatic illustration of the achatinid shell

(http://www.columbia.edu/itc/cerc/danoff-burg/invasion_bio/inv_spp_summ/Achatina_fulica.htm**)**

1.3 Microbial Community in Snails

1.3.1 Possible microbes associated with edible land snails

Land snails are ubiquitous creatures found on farms, forests and in homes. In some communities with poor sewage disposal systems, snails are often found at the sewage

disposal sites. The diverse environments in which these snails are found make them susceptible host to a vast range of micro-organisms. Thus, they could harbour parasites, bacteria, viruses as well as fungi.

1.3.1.1 Bacterial agents

Pseudomonas sp has been isolated and found to be the cause of intestinal infections that may spread rapidly amongst dense snail populations (Cobbinah, 2008). Studies done by Cardoso *et al*. (2011) using molecular techniques revealed that the crop fluid samples showed a higher abundance of proteobacteria while faecal samples were dominated by *bacteroidetes* and *firmicutes*, abundant microorganisms in the faeces of warm-blooded animals including humans. These can cause a wide variety of symptoms especially in persons with compromised immune systems.

Bacteriological studies (Akinboade *et al.*, 1980; Akpavie *et al.*, 2000) indicate that various potentially pathogenic bacteria inhabit different organs and tissues including the haemolymph of the African giant snail. The presence of pathogenic bacteria in the snails is a health threat to consumers.

Studies done by Efuntoye *et al.* (2011) on the enterotoxigenicity of *Staphylococcus aureus* isolated from the intestines of snails revealed the presence of *S. aureus* in snails. A study by Bukola *et al.* (2011), on the microbiological composition of snails also indicated the presence of *Proteus sp, Streptococcus sp, Shigella flexneri, Staphylococcus aureus, E. coli, Klebsiella aerogenes, Citrobacter sp, Bacillus subtilis, Bacillus cereus, Aeromonas sp., Micrococcus liteus, Salmonella typhi, Vibrio parahaemolyticus and Vibrio cholera.*

The fungi isolates were *Aspergillus niger, Fusarium oxysporum* and *Cryptococcus sp.* This, thus, poses a health risk to unsuspecting consumers.

Adagbada *et al.* (2011) reported isolation from *Achatina achatina* and *Archachatina marginata, Salmonella sp, Shigella sp, Yersinia sp, Aeromonas sp, Vibrio sp, Pseudomonas sp, Enterobacter sp, Proteus sp, Staphylococcus sp, Klebsiella sp and E. coli*. Studies done by Ebenso *et al.* (2012) isolated bacteria such as *Salmonella, Vibrio* and *Escherichia coli* from the edible land *snail Archachatina marginata.*

1.3.1.1.1 *E. coli*

E. coli is a facultative, anaerobic, Gram-negative rod that lives in the intestinal tracts of animals. Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. *E. coli* consistently inhabits the human intestinal tract, and is an organism that predominantly lives in the human GI tract; however, it constitutes a very small proportion of the total bacterial content. The regular presence of *E. coli* in the human intestine and faeces has led to tracking the bacterium in nature as an indicator of faecal pollution and water contamination. As such, it is taken to mean that, wherever *E. coli* is found, there may be faecal contamination and the possible presence of intestinal parasites in humans.

Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination (CDC, 2012). The harmless strains are part of the normal flora of the gut, which can benefit their hosts by producing vitamin K (Bentley & Meganathan, 1982), and by preventing the establishment of pathogenic bacteria within the intestine (Reid, 2001).

1.3.1.1.2 *Staphylococcus aureus*

Staphylococcus aureus can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis, folliculitis, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections.

Staphylococcus can cause food poisoning when the food is contaminated through mishandling and inadequate refrigeration. Other sources of food contamination include the equipment and surfaces on which food is prepared. These bacteria multiply quickly at room temperature to produce a toxin that causes illness. *Staphylococcus* is killed by cooking and pasteurization (food safety, 2013).

1.3.1.1.3 *Bacillus anthracis*

Bacillus anthracis is the causative agent of anthrax. Humans acquire the disease directly from contact with infected herbivores or indirectly via their products (Lee *et al*., 2007). *Bacillus* species are rod-shaped, endospore-forming facultatively anaerobic, Gram-positive bacteria. The many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment including the soil.

The common disease presentations are cutaneous, pulmonary, and gastrointestinal. The cutaneous form is caused by handling contaminated materials, whilst the pulmonary form is caused by inhalation. Skin abrasions facilitate the entry of spores in local lesions where they germinate and develop into gelatinous oedema. Herbivorous animals become infected when they ingest the spores from the soil. When humans contact infected animals

(including the flesh, bones, hides, hair and excrement), they may become infected as well. Anthrax is almost never transmitted between humans (Todar, 2012).

1.3.1.1 .4 *Clostridium tetani*

These are the causative agents of tetanus. They are widely distributed in the soil and faeces of animals. Tetanus is a neurological disease. *C. tetani* releases an exotoxin (tetanospasmin) which blocks the release of neurotransmitters from the presynaptic membrane of inhibitory interneurons of the spinal cord and brainstem of mammals that regulate muscle contraction. This leads to continuous muscle contraction primarily in the neck and jaw muscles (lockjaw). If the infection is left untreated, it will eventually lead to respiratory failure and death. Mortality rates for tetanus are relatively high.

1.3.1.1.5 *Yersinia enterocolitica*

Yersinia enterocolitica infection causes the disease yersiniosis. Yersinosis is a zoonotic disease occurring in humans and animals such as cattle, deer, pigs, and birds. Many recover from the disease and become asymptomatic carriers (Collins, 1996). It infects the host by sticking to the cells of the host using Trimeric Auto-transporter Adhesins (TAA).

Acute *Y. enterocolitica* infections usually lead to mild self-limiting enterocolitis or terminal ileitis in humans. Symptoms may include watery or bloody diarrhoea and fever. After oral uptake yersiniae replicate in the terminal ileum and invade the Peyer's patches. From here, yersiniae can disseminate further to the mesenteric lymph nodes causing lymphadenopathy. This condition can be confused with appendicitis and is therefore called

pseudoappendicitis. In immunosuppressed individuals, yersiniae can disseminate from the gut to the liver and the spleen and form abscesses.

1.3.1.1.6 *Bacillus cereus*

Bacillus cereus is an endemic, soil-dwelling, Gram-positive, rod-shaped, beta-haemolytic bacterium. Some strains are harmful to humans and cause foodborne illness (Ryan, 2004). *Bacillus cereus* is a facultative anaerobe, and like other members of the genus *Bacillus*, it can produce protective endospores. Its virulence factors include cereolysin and phospholipase C.

1.3.1.2 Fungal agents

Fungal isolates from a study done by Bukola *et al.* (2011), on the microbiological composition of snails also indicated the presence of *Aspergillus niger, Fusarium oxysporum* and *Cryptococcus spp.*

1.3.1.2.1 *Aspergillus niger*

A. niger is one of the causative agents of aspergillosis. Inhalation of large amounts of spores can be deadly in the debilitated. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane.

1.3.1.3 Viral agents

No particular viral infections have been documented in snails either as transmitted by the snails or as a disease causing virus in them.

1.3.1.4 Parasites

It has been established that the water snail species serve as intermediate hosts of some trematodes, notably the *Schistosoma spp*, *Fasciola hepatica* and *Angiostrongylus cantonensis* (the causative agent of the rat lungworm disease which completes its life cycle in the land snail species following ingestion of infected rat faeces).

1.3.1.4.1 *Schistosoma spp*

This parasite is a trematode that belongs to the subclass Digenea and the order prosostomata. The species known to infect man include *Schistosoma mansoni, S. haematobium, S. intercalatum, S. japonicum, S. matheii, S. bovis* and *S. Mekongi* (Chitsulo *et al*., 2000; Cheesbrough, 2005). The disease, schistosomiasis, is endemic in 74 countries of the world. It is estimated that around 200 million people are infected and that between 500 and 600 million persons are at risk (Cheesbrough, 2005). These infections persist because of ignorance, poverty, poor hygienic practices and the unavailability of good sanitary facilities.

1.3.1.4.2 *Angiostrongylus cantonensis*

The giant African snails are carriers of the rat parasite, *Angiostrongylus cantonensis*. Man contracts this disease (angiostrongyliasis) by ingesting improperly cooked snail meat or by handling live snails and transferring some of the mucus (slime) to the human mucus membranes (such as those in the eyes, nose and mouth). The parasite undergoes development in man to cause a form of meningitis with eosinophilia; eosinophilic meningoencephalitis (Cheng *et al.*, 2011).

1.3.1.4.3 *Fasciola spp*

Fascioliasis, also known as liver fluke disease is caused by any of the two trematode species of Fasciola (*F. hepatica and F. gigantica*). Man gets infected upon ingestion of raw (aquatic) vegetation such as lettuce and green salad contaminated with encysted metacercariae. *Fasciola sp* have a life cycle that occurs partly in an intermediate freshwater snail host (*Lymnaea sp*) and a definitive host (sheep, goat or cattle).

The undifferentiated ova of the parasites develop into a miracidia under moist conditions in 9 – 15 days at 22 – 25 ºC. The miracidia which hatch out of the eggs live for only eight hours and can move in a film of moisture on damp pastures. Further development takes place after free living miracidium penetrates an amphibious snail. More species of *Lymnaea* have been indicated as capable of acting as intermediate hosts for *Fasciola*. In the snail, the miracidium metamorphoses into a sporocyst, rediae, daughter rediae and cercariae. The cercariae emerge from the snail and encyst on water cress, grass, tree barks or soil. When ingested by a definitive host the metacercariae excyst in the duodenum. The disease has acute and chronic phases. The chronic phase occurs when the mature fluke enters the bile duct and symptoms pertaining to obstruction of the bile duct or inflammation of the duct occurs (WHO, 2012).

1.3.1.4.4 *Fasciolopsis spp*

This disease, fasciolopsiasis, is caused by the parasite *Fasciolopsis buski* which was previously called *Distoma buski*. The parasite is also known as the giant intestinal fluke of man. Its definitive hosts are man, pig and dog. The molluscan hosts are of the genus *Segmentina*. The eggs are passed in the faeces of the definitive hosts. These eggs hatch in 3 to 7 weeks in water to release the ciliated miracidia, which penetrate the snail host. In the snail, the miracidia develop into sporocysts, then into rediae, daughter rediae

and cercariae. The cercariae emerge from the snails and get converted into metacercariae on the outer covering of water chestnuts. Human beings get infected by eating contaminated raw water plants especially when peeling off the outer layers with their teeth.

1.3.1.4.5 *Paragonimus spp*

This is a chronic infection of the lungs caused by the trematodes of the genus *Paragonimus*. The commonest infective species is *Paragonimus westermani*. Man gets infected by eating raw or poorly cooked crab or crayfish in the gills or muscles within which the metacercariae of the parasite are found. The parasite passes its life cycle in 3 hosts: one definitive and two intermediate hosts. The definitive hosts are mammals, domestic animals, tigers and leopards. The intermediate hosts include a first host which is a freshwater snail of the genus *Melania* in South East Asia. The second intermediate hosts are crabs and crayfishes.

In West Africa, *Potadoma* and *Semisulcospira* serve as hosts. The second host is either a freshwater crayfish or crabs (Potamon or Sudanonautes). The golden brown operculate eggs reach the bronchioles from where they are coughed up and excreted in sputum or swallowed and passed out in faeces. On reaching water the miracidia hatch out and penetrate into fresh water snails (e.g. *Semisulcospira libertina, S. amaurensis, Thiara, Potadoma, Melania*). In the snails the miracidia develop into sporocysts followed by two generations of rediae and 3 months later give rise to very short tailed cecariae (micro-cercous Xiphidiocercariae). These emerge from the snail and swim in water and can survive for 24-48 hours. If they find freshwater crabs they enter them.

The crab species include *Potamon, Sesarma, Eiocheir* and *Sudanonautes* or the freshwater crayfish, *Astacus*. They penetrate and encyst in the gills or muscles as metacercariae. The freshwater crustaceans can probably become infected by ingesting unencysted cercariae in the water or even inside infested snails. The disease is insidious beginning with a non-specific cough that becomes chronic and is productive of blood tinged sputum known as endemic haemotypsis. Patients also experience pleural pain and dyspnoea. Depending upon secondary bacterial infection there may be pneumothorax and pleural adhesion. Lesions in the brain can lead to seizures.

1.3.1.4.6 *Clonorchis spp*

Clonorchiasis is caused by *Clonorchis sinesis*. Humans are the principal definitive hosts, but dogs and other fish eating canines act as reservoir hosts. Two intermediate hosts are required to complete its life cycle. The first intermediate host is a snail and the second is a fish. The eggs passed in faeces contain the ciliated miracidia. They do not hatch in water, but only when ingested by suitable species of operculate snails such as *Parafoassarulu, Bulinus or Alocinma* species. The cercariae escape from these snails and swim about in water, waiting to get attached to the second intermediate host, suitable freshwater fish of the carp family. They then shed their tails and encyst under the scales or in the flesh of the fish to become in about 3 weeks the metacercariae which are the infective stages. Human infection occurs when such a fish is eaten raw or improperly processed. Frozen, dried or pickled fish may act as source of infection. Infection may also occur through fingers or cooking utensils contaminated with the metacercariae during the preparation of the fish for cooking.

1.3.1.4.7 *Eurytrema pancreaticum*

This is a related fluke which is commonly present in the pancreatic duct of cattle, sheep and monkeys. The life cycle involves intermediate snail hosts of the *Lymnaea spp*. Occasional human infection has been noticed in China and Japan.

1.3.1.4.8 *Balantidium coli*

B. coli, a ciliated protozoan causes balantidiasis (Ramachandran, 2009). *Balantidium coli* is the only ciliated pathogenic protozoan known to infect humans. Balantidiasis is a zoonotic disease and is acquired by humans via the faeco-oral route from the normal host, the pig (in which it is asymptomatic).

1.4 Health implications of micro-organisms in raw snail haemolyph, slime and other products

From the literature reviewed, numerous studies have been carried out using snails (*Helix aspersa, Lymnaea spp, A. marginata, A. achatina, A. fulica*, etc) and their various products such as eznymes, hormones, proteins, and serum-factors. Snail meat has been consumed and still is a highly relished delicacy. The snail promises to be of a great valuable use in medicine. Not many studies, however, have been conducted on the slime and haemolymph especially to assess the presence of microbes pathogenic to man. The microbes isolated, can cause acute and or chronic diseases. They are implicated in diseases such as food poisoning, sepsis, endocarditis, diarrhoea, and pneumonia. No studies however exist in Ghana on the microbial content of snail products. The presence of the mentioned microorganisms poses numerous health hazards to unsuspecting consumers especially considering the raw use of snail products. There is the need to carry out a study in this respect.

1.5 Problem Statement

Snails are a delicacy and serve as a popular, seasonal food widely distributed in Ghana. The belief that these edible snails are harmless and harbour no pathogens has aroused an international use of the snails for various purposes. Ghana and Nigeria are amongst the West-African countries known to be the highest consumers of snails. Several undocumented reports indicate the use of snail and snail products for acclaimed medicinal purposes in Ghana. The haemolymph in the giant African snails prevalent in the country has been associated with various medicinal claims. The consumption of the haemolymph are for purposes such as an aphrodisiac, cure for tuberculosis, foot rot, whooping cough, stroke, anaemia and the application of the slime as a dermatotherapeutic agent amongst other uses. Extensive literature search indicate that, little or limited information is currently available on the microbiological quality of edible snails in Ghana and worldwide.

Studies conducted by Akinboade *et al.,* 1980 and Akpavie *et al.,* 2000, have shown that various pathogenic bacteria inhabit different tissues including the haemolymph of the African giant land snails. Similar studies done by Efuntoye *et al.* (2011) on the enterotoxigenicity of *Staphylococcus aureus* isolated from the intestines of snails revealed the presence of *S. aureus* confirmed positive with coagulase, DNAase, phosphatase and enterotoxin in snails. A study by Bukola *et al.* (2011), on the microbiological composition of snails also indicated the presence of *Proteus spp, Streptococcus sp, Shigella flexneri, Staphylococcus aureus, E. coli, Klebsiella aerogenes, Citrobacter spp, Bacillus subtilis, Bacillus cereus, Aeromonas spp, Micrococcus liteus, Salmonella typhi, Vibrio parahaemolyticus and Vibrio cholera.* Fungi isolates found were *Aspergillus niger, Fusarium oxysporum* and *Cryptococcus sp.*

1.6 Justification

The giant land snail meat is a reliable source of protein. The demand for the meat continues to increase despite the corresponding increase in the price. The snail, aside its use as a food source, has been used for new and indigenous medicinal purposes. The direct application of uncooked snail haemolymph into wounds, onto foot regions in cases of suspected foot rot, and the consumption of the fluids for curative purposes amongst other uses poses a serious health threat to users. It is necessary to assess this risk considering the potential human pathogens isolated from these snails and their implication in disease states. The health profession is gradually migrating from the ideals of curative medicine to preventive alternatives. Many preparations (most especially the traditional indigenous preparations) which have not been proven to be scientifically effective are being used by many worldwide for medicinal reasons. To the users or consumers, these preparations may work as expected, but the risks of possible microbial pathogen content are often overlooked.

It is well documented that some species of snail serve as intermediate hosts in the life cycle of parasites such as *Schistosoma sp, Fasciola sp, Opisthorchis sp* and *Paragonimus sp* amongst others. Reports by WHO (2009) implicate food borne pathogens especially *Salmonella sp* and *E. coli* to be responsible for an estimated 200,000 deaths. The snail is observed to habit filthy environments such as sewage and it is also known to ingest rotten materials.

A pilot study carried out by the Department of Microbiology (UGMS) indicated a high contamination of snails with *Staphylococcus sp, Streptoccocus sp, E. coli, Cryptococcus neoformans* and *Candida sp. Giardia lamblia* and *Balantidium coli* were found.

Human diseases caused by some of the above highlighted pathogens include pneumonia, diarrhoea, septic arthritis, osteomyelitis, sepsis, endocarditis, toxic shock syndrome, food poisoning, staphylococcal scalded-skin syndrome. Thus, this poses a health risk to unsuspecting consumers. There is also no documentation on the microbiological assessment and quality of edible snails on the Ghanaian market.

The above mentioned organisms are known to cause acute or chronic fatal ailments. Thus, the edible snails could serve as an unthought-of reservoir for the uninterrupted transmission of human pathogens. In the light of this and with the foreknowledge of the uses of these snail products, it is paramount to undertake this microbiological study. The consumption or alternative use of the raw snail haemolymph, slime and other parts poses a danger to consumers considering the pathogens isolated from them. The need to analyse these fluids is necessary to establish the microbiological safety of edible snails in Ghana considering the emerging acclaimed therapeutic values ascribed to the snail haemolymph and its other products mentioned. Furthermore the sick (including the immuno-compromised) are vulnerable subjects for the trial of these traditional remedies. This research will make available pertinent information and serve as a basis for further research especially on the efficacy of the snail products as therapeutic agents for the ailments mentioned. Also, it will serve as a basis for health policy makers to advise the populace on the inappropriate use of snail products for medicinal purpose.

1.7 Hypothesis

1.7.1 Null Hypothesis:

- Microbes that inhabit edible land snails (*Achatina achatina and Achatina fulica*) on the Ghanaian market are non- pathogenic and are of low bacteria loads.

1.8 Aim and Objectives

1.8.1 Aim

To assess the microbiological quality of edible snails from selected markets in Ghana.

1.8.2 Objectives

- To isolate bacteria, fungi and parasites associated with the giant land snails from selected markets in Ghana.
- To note any significant correlation between the snail type and microbial density.
- To identify possible sources of these pathogens.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Global/ National Issue of Snails

Food is any material which consists essentially of protein, carbohydrate, and fat used in the body of an organism to sustain growth, repair, and vital processes and to furnish energy (encyclopaedia britanica, 2013). It is usually of plant or animal origin. Food serves as a notable vehicle for transmission of microbial diseases. Snails have served various purposes in the world ranging from its use as food (snail meat) [figures 2a and 2b], use of slime for medicinal purposes, and the haemolymph as a cure to many ailments and medical pathologies, the hepatopancreas as a source of enzymes for medical therapy, and the shell as a source of calcium carbonate in animal feed amongst others. The snail and its shell when sold serves as a source of income for many worldwide. Also, the shell has aesthetic and religious value (used by fetish priest and priestesses). Snails, despite their positive impact in the lives of many, however serve as reservoirs for pathogens.

2.1.1 Nutritional value

Snails, like shellfishes are highly nutritional balanced food (Ifon and Umoh, 1987). The meat of snails is highly relished and considered a delicacy in the West African zones, especially in Nigeria and Ghana (Agbelusi and Ejidike, 1992; Cobbinah *et al.*,, 2008). It is high in protein (12-16%), iron (45-50) mg/kg and a good source of calcium. Snails have a low fat and low cholesterol content compared to other protein sources like poultry and pigs. According to Cobbinah (1997), the meat of *A. marginata* has 12–60% protein, 0.05–0.8% fat and 45–50 mg/kg iron content. Also snails have almost all the amino acids needed by humans (Cobbinah *et al.*, 2008). Ajayi (1978) reported that snails have low lipid and saturated fatty acids, which have important health implication and may be beneficial to

hypertensive patients and others who do not take fatty foods. In European countries like France, cooked land snails are an expensive and sought out meal. Escargot is a dish of cooked land snails, usually served as an appetizer in France and in French restaurants.

2.1a

Figure 2.1a: A plate of served escargot with special tongs and fork

Source: **(**http://en.wikipedia.org/wiki/Escargot**)**

2.1b

Figure 2.1b: Homemade soup containing snail meat

Source: **(**http://www.wikipedia.org/wiki/cooked_snails**)**

2.1.2 Medicinal value

The medicinal use of snails dates back to the time of Hippocrates who proposed the use of snail mucus against many human ailments. The use of helicine snails in medicine has been traced by Cadart (1955) from the earliest of times and reported that the mucus of *Helix pomatia* aids or enhances the action of penicillin. Various preparations or formulations of snail and/or their products are used to treat pain, abscesses and other wounds. According to the book written by Cobbinah *et al*. (2008), snails had also been used for symptoms associated with tuberculosis and nephritis. In a book written by Cobbinah *et al*. (2008), he makes mention of a recent study showing that the glandular substances in edible snail meat cause agglutination of certain bacteria, which could be of value in fighting a variety of ailments, including whooping cough. Edible snails also play an important role in folk medicine. The therapeutic properties of snails were thought to come from the holy attributes accorded it (Cranga, 1991).

In Ghana, the bluish liquid (haemolymph) obtained when the meat has been removed is believed to be good for infant development. The high iron content of the meat is considered important in treating anaemia (Okafor, 2001; Cobbinah *et al*., 2008). In the past, it was recommended for combating ulcers and asthma. At the Imperial Court in Rome, snail meat was thought to contain aphrodisiac properties and was often served to visiting dignitaries in the late evening (Okafor, 2001).

The Food and Drug Administration (FDA) of America demonstrated the presence of a substance, ziconotide (SN xii), a synthetic peptide (drug) in snail venom in 1999. Pre clinical and clinical studies of this new drug showed a reduction of pain intensity by 53% (Webster, 2001). Formulations made from *Helix pomatia* are now confirmed to cure whooping cough and chronic bronchitis. The fluids from snails are touted to have

antiseptic and fluidizing properties. About 30 enzymes have been isolated from the digestive mucus, pancreostomach, muscle and lymph fluid. Mucolytic and bacteriolytic properties of snails are noted as well as the antispasmodic activities, musculotropic effects and sedative properties (Bonnemain, 2005).

Ogunsanmi *et al*., (2003), reported that the haemolymph of *Archachatina marginata* contain albumin/globulin ratios similar to those of some mammals like the pangolin and sheep. Studies done by Salawu (2011), indicated the presence of triglycerides and cholesterol in the haemolymph of *Archachatina marginata*. Enzymes, reported to be present in the snail's haemolymph in the same study by Salawu (2011) included Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate transaminase. From the study, Sodium (Na+), potassium (K+), calcium (Ca2+), magnessium (Mg2+), zinc (Zn2+) and bicarbonate (HCO3-) are among some of the ions found in the haemolymph of both the wild and domestic snails.

Bose and Bhattacharya (2000) reported that C-reactive protein was a normal component of *Achatina fulica*. The molecular weight of this protein was reported to be 400kDa with high absorbance in a light wavelength region of (200-230) nm.

According to Abere and Lameed (2008), a special form of calcium phosphate extracted from snails has been implicated in the cure of some kidney diseases, tuberculosis, diabetes, asthma, heart diseases and circulatory disorders. Snails have also been implicated in the reduction of pain and loss of blood during labour as well as in the treatment of small pox (Akinnusi, 1998). Taylor (1900), discussed the possible curative powers of snails in ridding the body of tuberculosis and recommended a method of puncturing the snail shell

to enable a patient suck the oozing liquor. Clapp (1902), similarly referred to slugs in coconut milk as a cure for asthma.

Negligin, a curative substance extracted by the Institute of Medicine in Japan from the giant snails was proven to be orthocalcium phosphate (Pangga, 1949). This chemical is claimed to cure kidney disease, tuberculosis, anaemia, diabetes, asthma, urticaria, circulatory disorders, improve constipation and hemorrhoids, prevent influenza and to restore virility and vitality (Pangga, 1949). This chemical is thought to perpetuate beauty by reversing the skin ageing process. Currently, in Russia, massage using the slime of an African Giant snail is practiced. It is believed that the slime plays a role in the regeneration of the skin, eliminating wrinkles, scars and traces of burn marks (Jimenez, 2012). Figures 2a and 2b show pictures depicting the current use of snail or snail extracts. Figures 2.3a and 2.3b show the improved effect of using Elicina (a snail extract cream) for treatment of burns and other conditions in Chile (Gónzalez, 2000).

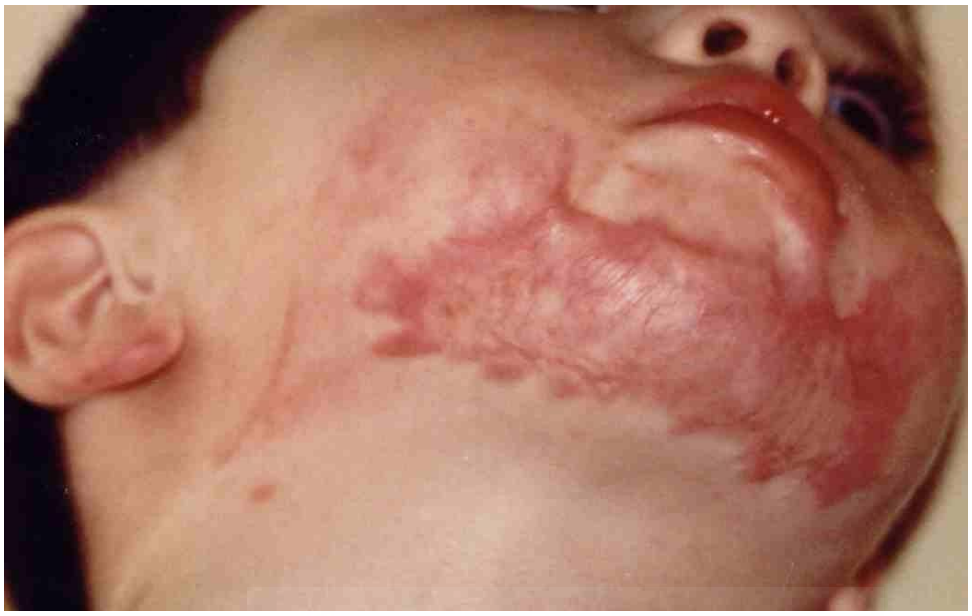
The slime is also recommended especially for singers and those in need of hormonal injections. Pons *et al*. (1999) demonstrated that the broncho-relaxant effect of helicidine was related to the release of E2 prostaglandins and inhibited by pre-treatment with indomethacine, a cyclooxygenase inhibitor. Snail slime is technically known as "Helix Aspersa Müller Glycoconjugates." It is a thick complex mixture of proteoglycans, glycosaminoglycans, glycoprotein enzymes, hyaluronic acid, copper peptides, antimicrobial peptides and trace elements including copper, zinc, and iron. Snails also possess high concentrations of glycosaminoglycans and mucopolysaccharide in the slime.

Figure 2.2a: Use of snail slime for beauty therapy in Siberian city (Russia)

Source: (http://www.msnbc.com/snailslime/beauty).

Figure 2.2b: Use of snail slime for beauty therapy in Siberian city (Russia)

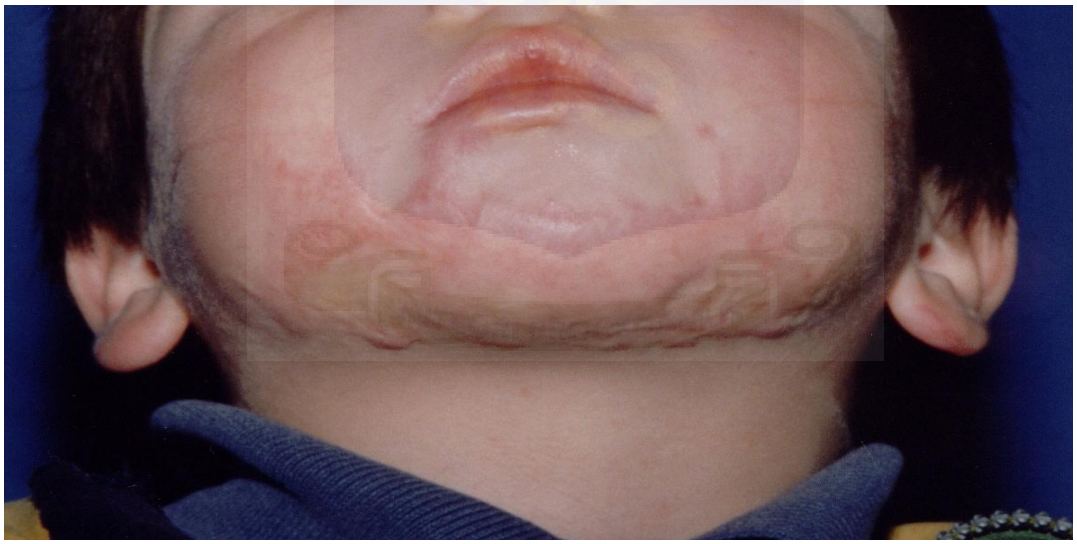
Source: (http://www.msnbc.com/snailslime/beauty).



2.3a

Before treatment with Elicina cream

Figure 2.3a: Results of applying Elicina snail extract cream on a burns patient



2.3b

After treatment with Elicina cream

Figure 2.3b: Results of applying Elicina snail extract cream on a burns patient

2.1.3 Scientific research by environmentalists

Atomic energy agencies, have also utilized snails in qualitative and quantitative analysis. Scientists use snail shells in their researches. For example, atomic energy researchers expose shells to atomic rays to test the effects of radiation (Cooley, 1971). Heavy metal contamination of the environment has been an issue of great concern. Fluoride contamination is one of such great concerns. It has been shown that exposure of vertebrates to low levels of fluoride, if sufficiently long, results in the accumulation of fluoride, ultimately associated with musculo-skeletal symptoms and metabolic disorders (Machoy *et al.* 1995; Zakrzewska, 1995).

The adenylate energy cycle in snail muscle has been shown through a study to simultaneously reduce with an increasing exposure of the snails to fluoride. Fluoride contamination of the environment has a toxic effect on plants, animals and humans (Rac *et al.*, 2005). It has been realized that snails serve as a good bio-indicator of environmental contamination. Snails as well as other molluscs, have thus over the years been used for the monitoring of heavy metals in the ecosystem. This has been done by collecting molluscs from the rural areas (Dwojak and Zakrzewska, 1998; Jurkiewicz- Karnkowska, 1998).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Study Site and Population

Samples for the study were collected randomly from selected markets in four regions of Ghana namely, Greater Accra, Volta, Ashanti and Eastern regions. Markets within the Greater Accra region from where sampling was done included the Makola Market, the Tema trade fair market (TEXPO), the Tema Station Market, the Mini-Market of the Korle-Bu Police Barracks vicinity and the Circle Odawnaa Market. The Asigame (literally meaning "the big market") was the market where the sampling was done in the Volta region. Sampling in the Eastern region was done in the Akropong Mandela Market and the Aburi Market. In the Ashanti region sampling was done in the Kumasi Kejetia Market. Samples were collected from three vendors in the Makola Market and from one vendor each from the remaining markets bringing the total number to 10.

3.2 Study Design

The study, which is a cross sectional qualitative one, was designed to detect the presence of microbes in edible land snails. A simple random sampling technique was used employed in the research. Snails were purchased from consented and confirmed *Achatina achatina and Achatina fulica* snail vendors.

3.3 Target Population and Sample Size

The snails used for the study were of the edible species of *Achatina achatina* and *Achatina fulica*. One hundred and fifty specimens each of haemolymph, slime and hepatopancreas

and 50 specimens of faeces, totaling 500 specimens were obtained from 150 snails (half of this number were *A. achatina* and the remaining half were *A. fulica*).

3.3.1 Inclusion and Exclusion criteria

3.3.1.1 Inclusion criteria

- Live and intact shelled *Achatina achatina* and *Achatina fulica* species obtained from consented and confirmed vendors were used for the study.

3.3.1.2 Exclusion criteria

- Snails that were excluded from were those that were dead or those with broken shells and species other than *Achatina achatina* and *Achatina fulica*.

3.4 Specimen collection, transport and storage

Informed consent was obtained from individual snail vendors and a unique number assigned to each specimen. The live snails were bought and transported into sterile transparent plastic bags (Nasco WHIRL-PAK). Each plastic bag had one snail only. The bags were further perforated to allow enough air-flow for respiration by the achatinids. The snails were then placed in an ice chest (partially open). The achatinids were then transported to the parasitology laboratory at the Department of Microbiology (UGMS). All samples were processed the same day they were collected.

3.4.1 General Specimen extraction process

The shell of each snail was washed thoroughly using phosphate buffered saline (PBS) and disinfected with 70% alcohol before extraction. Two different methods of extraction were devised and used for the extraction process. These methods were; a meticulous de-shelling method and a "pierce-and-pull" method. The de-shelling process involved using a disinfected laboratory pestle, sterilized surgical apparatus (scissors and forceps), to reveal the organs. The "pierce and pull" method, on the other hand, involved the use of a sterilized pointed-piercing device and sterilized surgical scissors. In the "pierce-and-pull" method, the piercincg device was used to pierce the foot (meat) of the snail and pulled out of the shell in a winding order. This method resulted in the expulsion of the haemolymph into the shell.

The haemolymph in both methods were pulled with a sterile syringe into sterilized standard 25 ml glass bottles. The slime was treated thus, but with the use of the plunger only. The hepato-pancreas was cut using a scissors. The faecal matter was picked up with the pair of forceps into the sterilized standard 25 ml bottle. Thus, all specimens were collected into appropriately labeled bottles. Ten grams of the hepato-pancreas was macerated (using a mortar and pestle) in 20 ml of phosphate buffered saline (PBS) and making a 1:10 dilutions by filling the preparation up to 100 ml.

The different stains used include iodine, Methylene blue, Malachite green, Haematoxylin and Eosin, Safranin, Lactophenol Cotton Blue, Giemsa, Ziehl-Neelson, Gram and Eosin. The specimens obtained were cultured for the presence of bacteria and fungi. Below are four diagrammatic illustrations of how the parasitological, bacteriological and fungal processing was done.

3.5 Specimen Analysis

3.5.1 Methods for bacteriological analysis

3.5.1.1 Standard Plate Counts (SPC)/ Aerobic colony count (ACC)

Standard Plate Count (SPC), also known as Aerobic colony count ACC) is a common microbiological test used for monitoring microbial quality of various products. The process indicates the number of bacterial colonies growing on a non-specific solid nutrient agar (medium) after a given period of incubation (Extension Advisory Team, 2013). The working area (especially the leveled work bench) was disinfected to rid the environment of dust sterile test tubes and petri dishes for the study were appropriately labeled. 9ml sterile phosphate buffered saline was added to 1ml of the slime, haemolymph, faeces, and hepatopancreatic fluid respectively in a sterile test tube to give a total volume of 10 ml which gives a 1:10 dilution. Furthermore, a five-fold serial dilution was made and examined by means of the pour plate method. One (1ml) millimetre of diluted sample from each dilution to be analyzed was transferred into each corresponding labeled petri dish using a pipette. Twenty five (25) millilitres of cooled molten agar (PCA) was poured over the sample in the petri dish (1ml), giving a total of twenty six (26) millilitres. In order to ensure that the sample mixes thoroughly with the medium, it was swirled both clockwise and anticlockwise three times after which it was allowed to set on a flat-top bench. All platings were done in duplicates. Solidified plates were then incubated at 37°C for 18-24 hours. After incubation, counts were made using a colony counting device that would allow viewing of individual colonies. All discrete colonies were counted where possible and expressed in colony forming units per gram (cfu/g) for the hepatopancreas (solid) and colony forming units per millilitre (cfu/ml) for the fluid specimens (slime. haemolymph and faecal matter).

3.5.1.2 Enterobacteriaceae Count (EC)

A sample of 0.1 ml from each dilution was transferred using a pipette and spread over twenty five (25ml) millilitres of solidified MacConkey agar using an L-rod dragaski spreader. The plates were then incubated aerobically at 37°C for 18-24 hours. After incubation, colony counting was done using a colony counting device that allowed viewing of individual colonies. All discrete colonies were counted where possible and expressed in colony forming units per gram (cfu/g) for the hepatopancreas (solid organ) and colony forming units per millilitre (cfu/ml) for the fluid specimens (slime, haemolymph and faecal matter).

3.5.1.3 Isolation and identification of organisms

Approximately 5ml of each specimen was centrifuged at 11,000 rpm for 30 minutes in a refrigerated centrifuge and decanted. A loop full of the sediment was then inoculated into Selenite F (SF) broth and Alkaline Peptone Water (APW) which are enriched media for the growth of *Salmonella spp.* and *Shigella spp*, and *vibrio spp* respectively. The inoculated SF and APW were incubated for 18-24 hours after which it was sub-cultured unto Salmonella/Shigella agar (SSA) and Thiosulphate-citrate-bile sucrose agar(TCBS) respectively. Another wire loop full of the sediment was also inoculated unto MacConkey agar for the detection of *Salmonella, Shigella, Escherichia coli, Klebsiella spp, Citrobacter spp, Enterobacter spp*, and other *Enterobacteriaceae.*

The specimens were plated onto Blood Agar (to capture other organisms, as well as gram positive organisms) and Chocolate Agar identification of other pathogenic bacteria. Other selective media were used to aid in a reliable diagnosis. These selective media included Kings media (selective for *Pseudomonas spp*), *Bacillus cereus* agar (selective for *Bacillus cereus*), reinforced Clostridial agar (selective for *Clostridium spp*) as well as Salmonella and Shigella Agar (SSA; selective for *Salmonella spp* and *Shigella spp*). All

incubations were done at 37^0 C under aerobic conditions and others were incubated anaerobically for the isolation of anaerobes. In cases of mixed growth, purity plating was carried out and suspected colonies were further identified using standard biochemical methods.

3.5.1.4 Purity plating

To obtain the pure culture, a pool was made on MacConkey agar and blood agar using a loopful of the inoculum obtained from an isolated colony on the culture with mixed growth. Streaking was then done from the pool using the four dimensional method by flaming the loop in-between each streak to obtain isolated colonies to obtain parallel overlapping strokes. It was then incubated at 37°C under aerobic or anaerobic conditions for 18-24 hours. The process was done repeatedly for each distinct bacterium colony observed in the mixed growth. The morphology and biochemistry of the pure bacteria strain was then studied.

3.5.1.5 Identification of organisms

Bacterial isolates were identified using biochemical methods and microscopy. The Gram stain was the first step used for organism identification. This was done to identify presence of Gram positive organisms from Gram negative organisms. When the Gram stain reaction showed the presence of Gram positive organisms, a catalase test was done to differentiate *Staphylococcus aureus* (catalase positive) from *Streptococcus spp* (catalase negative). A coagulase test was further carried out to differentiate *Staphylococcus aureus* from other *Staphylococcus spp*. When the Gram stain reaction showed the presence of Gram negative organisms, an oxidase test was done to differentiate enterobacteriaceae (oxidase negative, e.g; *Klebsiella spp*) from non-enterobacteriaceae (oxidase positive, e.g; *Pseudomonas spp*). Both oxidase positive and negative organisms were subjected to

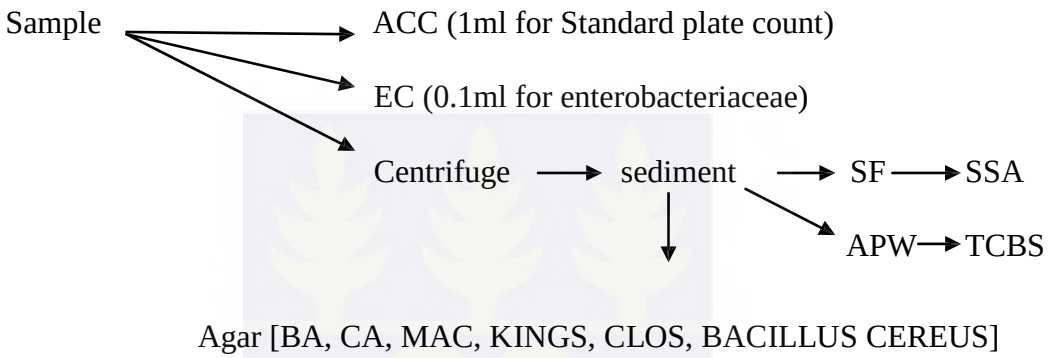
further biochemical tests such as Urea, Indole, Citrate and Triple Sugar Iron (TSI) for confirmation of species. Motility of the bacteria was observed microscopically. The Motility-Indole-Urea (MIU) media was used on other occasions (this medium shows all three reactions for results; motility, urea reaction and indole reaction). The MiniBact-E panel of biochemical tests was employed in the identification and confirmation of bacterium species.

3.5.1.6 Minibact-E kit

This biochemical test kit identifies bacteria species based on a panel of reactions. It is based on the utilization or colour changes (upon addition of reagents) observed from the reaction of bacteria with the following compounds (substrates); hydrogen sulphide (H_2S), nitrate, phenylalanine, indole, lysine, ornithine, malonate, urea, voges-proskauer, esculin, β-galactosidase, saccharose, arabinose, adonitol, inositol and sorbitol. Double distilled water (approximately 4.5ml) in a sterilized bijou bottle was inoculated with a colony of bacteria from the purity plate. The mixture was vigorously shaken to obtain a homogenous solution of the bacteria inoculum. The same was done for all isolated colonies to be identified. The MiniBact-E kits (with 16 wells) were taken out of the refrigerator and brought to room temperature. The reagents for the test were also brought to room temperature.

Using a micropipette, two hundred microlitres (200µl) of the homogenous bacteria inoculum were transferred from the mixture in the bijou bottles into each of the sixteen (16) wells of a labeled column (designated for a particular specimen). The same was done for each bacterial isolate to be identified, and incubated for four hours at 37°C (that is, the substrates/compounds and bacteria inoculum). Reagents were then added to designated columns in the wells containing nitrate, phenylalanine, indole and voges-proskauer. A

colour chart was used to read the reactions to determine positivity or negativity of the samples. An appropriate number in triplets (in the order of 421) was generated. The summation of the numbers in each triplet codon gives a distinct set of six (6) numbers from the readings of the sixteen (16) wells. The final six numeral (example; 20000 for *Shigella spp* and 002200 *Yersinia pseudotuberculosis*) is thus used to identify the bacteria isolate.



- Biochemical tests
- Stain: Grams stain, ZN stain, Spore stain (Bacillus, Clostridium)

Figure 3.1 A diagrammatic illustration of the bacteriological processing

3.5.1.7 Gram stain

A drop of the specimens was placed on clean, grease free glass slide. A smear was made using a well flamed wire loop. The smear was allowed to air dry and then stained with the Gram's reagent. The smear was flooded with crystal violet (60 seconds) and poured off, then flooded with Gram's iodine (60 seconds), and rapidly decolorized after washing the iodine with distilled water. The smear was counterstained using neutral red (60 seconds), subsequently washed off with distilled water and allowed to air dry. A drop of immersion oil was added to the smear and observed using the x100 objective.

3.5.1.8 Catalase test

A drop of the catalase reagent was placed on a clean grease free glass slide. Using a clean glass coverslip, a pure colony of bacteria was added to the catalase reagent. A positive reaction was indicated by a continuous bubble formation which is evidence of the presence of the catalase enzyme in the bacterium.

3.5.1.9 Coagulase test

Suspensions of Staphylococci from culture were made on the two ends of a clean glass slide. One end was labeled as "test" and the other as "control". The test suspension was treated with a drop of citrated plasma and mixed well. The observation of agglutination or clumping of cocci within 5-10 seconds was taken as positive.

3.5.1.10 Oxidase test

A drop of the oxidase reagent was placed on a clean grease free glass slide. Using a clean glass coverslip, a pure colony of bacteria was added to the oxidase reagent. A positive reaction was indicated by a continuous bubble formation which is evidence of the presence of the oxidase enzyme in the bacterium.

3.5.1.11 Spore stain

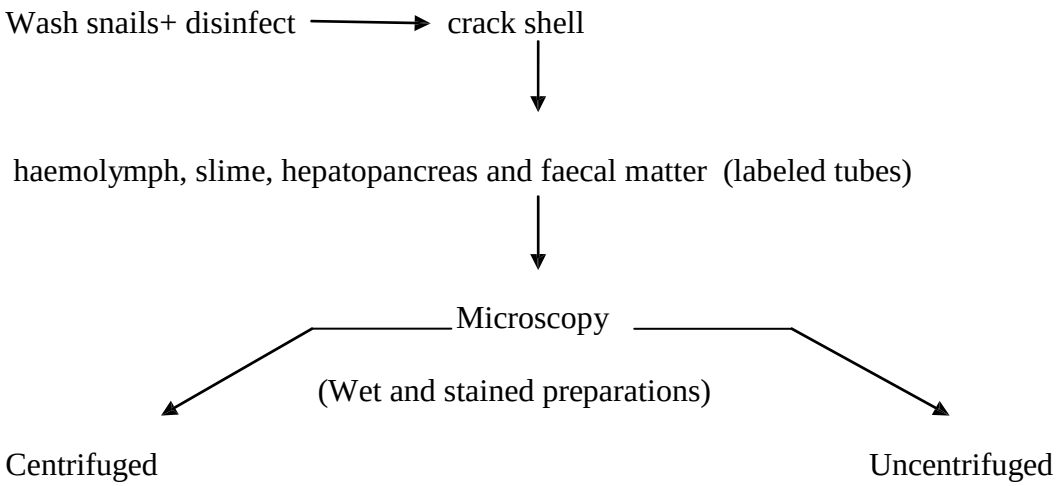
The spore stain is done to observe spores in sporulating cells which Gram stain may not reveal distinctly. In this process, a smear was prepared from the bacteria colony and allowed to air dry and then heat fixed. The smear was flooded with malachite green stain and heat was applied till vapour was observed. Sulphuric acid was then used to flood the smear and washed off in one (1) minute. Carbol-fuchsin was then added to the smear for a minute, washed off, and the smear allowed to dry. The spore stain was observed at x100 for the presence of spores.

3.5.1.12 Ziehl-Neelson stain

In the staining process, the smear prepared from the bacteria was flooded with carbol-fuchsin. The dye was heated (to aid the penetration of the dye into organism) for few minutes till vapour was observed. The stained smear was washed off with sulphuric acid and counterstained with methylene blue (malachite green was used on other occasions). The acid fast bacilli retained the red colour of carbol-fuchsin, whilst, the non-acid fast bacilli appeared blue or green (for methylene blue and malachite green use respectively).

3.5.2 Methods for parasitological analysis

Wet preparations (centrifuged and uncentrifuged) and stained preparations (centrifuged and uncentrifuged) were examined microscopically. Using a Pasteur pipette, a drop of the sample each unstained (uncentrifuged and centrifuged) was place on a separate clean grease free glass slide. A coverslip was added and observed under low and high power of the light microscope. The same was done for specimens that were stained (centrifuged and uncentrifuged); this was done on separate glass slides. Stains used included Iodine, Methylene blue, Giemsa, Haematoxylin and Eosin, Malachite green, cold Ziehl-Neelson, and Eosin.



Stains: Iodine, Methylene blue, Giemsa, Haematoxylin and Eosin, Malachite green, cold Ziehl-Neelson, and Eosin.

Figure 3.2 A diagrammatic illustration of the parasitological processing

3.5.3 Methods for mycological analysis

3.5.3.1 Direct microscopy

Using a Pasteur pipette, a drop of the specimen was placed on a clean grease free glass slide. A drop of 10% KOH was added to aid clearing of debris in the samples and examined by microscopy

3.5.3.2 Culture

The samples were cultured using Sabouraud agar (with the antibacterial agent Chloramphenicol incorporated into the media to inhibit bacterial growth). Using a wire loop, a pool was made on the Saboraud agar and streaking was done in the four

dimensional pattern. The inoculated agar were incubated at 25ºC for about one week and observed daily for growth. A wet mount preparation was done from the culture colonies. Lactophenol Cotton Blue staining was done for moulds, and Nigrosin staining done for yeasts and *Cryptococcus neoformans*.

3.5.3.3 Mould identification

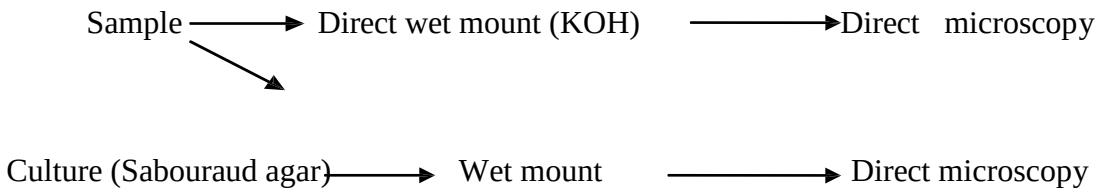
3.5.3.3.1 Lactophenol cotton blue (LCB) mount preparation procedure

A drop of Lactophenol Cotton Blue (LCB) was placed on a clean grease free glass slide using a Pasteur pipette and a small quantity of the culture (mould) was transferred unto the glass slide with the LCB using transparent cellophane tape. This was gently placed on the preparation to avoid trapping air bubbles. The sample was then examined under the light microscope using the low power (10X) and high power (40X) objectives. The morphological features were carefully observed to arrive at the detection of yeast.

3.5.3.4 Yeast and *Cryptococcus neoformans* identification

3.5.3.4.1 Nigrosin (negative stain) preparation procedure

A drop of Nigrosin stain was placed on a clean grease free glass slide and a small quantity of the culture/yeast colony was transferred onto it. The inoculum were emulsified with the Nigrosin stain and allowed to air-dry. The negatively stained preparation was observed with the x100 objective using a light microscope. The morphological features were carefully observed for the identification of yeast and *Cryptococcus neoformans*.



❖ Stain: Nigrosin stain, LCB stain

Figure 3.3 A diagrammatic illustration of the mycological processing

3.6 Wet and stained specimen image capturing

Although stained slides were preserved in the many cases, snapshots were taken to enable electronic viewing of pathogens in stained slides (smears) of specimen. The light microscopes used did not have an image capturing device attached hence, at the appropriate objective lenses used [x100 for immersion oil; and x40 for wet preparations with a coverslip (stained and unstained)], a digital camera or phone camera (Samsung galaxy pocket and Blackerry Z100) was used to zoom further to obtain pictures.

CHAPTER FOUR

RESULTS

4.1 Demographic Data

A total of 11 snail vendors from 9 markets were involved in this work. These vendors were from 4 regions (i.e. Greater Accra, Ashanti, Eastern and Volta) in Ghana. A structured questionnaire used in the study (Appendix B) revealed information on the gender, age, educational status, source of snails, reasons for reduced snail numbers in the country, and the use of snail products for medicinal and other purposes from the vendors. Majority (10) of the vendors were above 35 years with only 1 vendor being 19 years of age. All the 11 vendors were females. Of the total number, 7(63.6%) have had no formal education, 3 (27.3%) admitted having completed the Junior High School level of education and 1(9.1%) had completed the Senior High School level of education.

All the vendors sold in relatively dirty environments. (Dirty, in this context made specific reference to proximity of the vendor to heaped rubbish, a public place of convenience, observation of flies on the snails or in the environment, as well as muddy floors). The presence of muddy water and proximity of rubbish to the vendors' stand was a common scene. The snails were packaged in a sack and put in a wooden carton. Thus, the packaging did not prevent snails from coming into contact with each other. Some snails, observed to have fallen unto the untidy floors were picked back by the vendors unto the lots displayed for sale. All the 11 vendors sampled did not deny understanding the possibility of cross-contamination considering the way snails were displayed for sale. Yet, they absolutely were of the view that, cooking will kill any pathogen present, hence the need to ignore the untidy manner in which they were packed together. "They live on the ground and we pick them up," one vendor said.

Figure 4.1a: Showing a vendor extracting the meat from the snail shell to obtain the haemolymph

Figure 4.1b: Showing a snail vendor on a market day.

Concerning the 9 markets involved in the study, 5 (55.6%) were in the Greater Accra region, 2 (22.2%) were in the Eastern region, 1(11.1%) was in the Volta region and 1 (11.1%) was in the Ashanti region. The five markets in the Greater Accra region however accounted for 90 (60%) of the snails purchased for the study. The Eastern, Ashanti and Volta regions accounted for 60 (40.0%) of snails purchased, of which each accounted for 20 (13.3%).

Regarding the source of the snails, a total of 11 sources were mentioned. These sources included Abidjan (Cote D'Ivoire), Kukurantumi, Nkurakan, Abetifi, Axim, Ho, Adawso-Suhum, Asenewa, Agbogbloshie, Dodowa and Kadjebi. Of the above mentioned sources, the Greater Accra Region had snails from 7 (63.6%) sources [Abidjan, Kukurantumi, Abetifi, Kadjebi, Adawso-Suhum and Dodowa] sources, the Ashanti region had snails from 2 (18.2%) sources [Abidjan and Axim], the Volta Region had snails from 2 (18.2%) sources [Abidjan and Ho] and the Eastern region had snails from 4 (36.4%) sources [Asenewa, Agbogbloshie, Adawso-Suhum and Nkurakan].

Concerning vendor and snail source, Abidjan served as a source for 9 (81.8%) vendors, Agbogbloshie served as a source for 2 (18.2%) vendors, Nkurakan served as a source for 2 (18.2%) vendors, Adawso-Suhum served as a source for 2 (18.2%) vendors whilst Kukurantumi, Kadjebi, Abetifi, Dodowa, Ho, Asenewa and Axim served as a source for individual vendors who admitted to obtaining snails from these towns.

Of the 150 snails used for the study, half were *Achatina achatina*, and the remaining half *Achatina fulica.* The snails were purchased from a total of 11 snail vendors from 9 specific markets in 4 regions of Ghana. The 9 markets included the Makola, Odawnaa, TEXPO, Korle-Bu, Tema station, Asigame, Kejetia, Mandela and Aburi markets.

4.2 Enumeration of microbes

A total of 1,289 microbes (bacteria, fungi and parasites) were identified in the specimens (the haemolymph, slime, hepatopancreas and faecal matter) of the snails. Of this number, bacteria accounted for 85.7% (1,105), fungi, 11.1% (143) and parasites, for 3.2% (41).

4.2.1 Bacteria load

The null hypothesis tested is that the microbes that inhabit edible land snails (*Achatina achatina and Achatina fulica*) on the Ghanaian market are non- pathogenic and are of low bacteria loads.

Table 4.1a: Total number of bacteria isolated from the specimen of the two Achatinid species.

	Bacterial agents	***A. achatina* (AA) Number of samples infected with bacteria isolates**				***A. fulica* (AF) Number of samples infected with bacteria isolates**				**Total samples**
		Ha	**S**	**HP**	**FM**	**Ha**	**S**	**HP**	**FM**	
1	*Klebsiella pneumonia*	9	16	3	1	12	22	7	8	78
2	*Klebsiella oxytoca*	2	9	2	4	7	2	6	0	32
3	*Klebsiella ozonae*	3	0	2	0	1	4	3	0	13
4	*Klebsiella rhinoscleromatis*	0	0	0	0	0	2	0	0	2
5	*Erwinia herbicola*	13	25	3	4	12	7	17	3	84
6	*Enterobacter cloacae*	8	9	3	2	12	12	5	0	51
7	*Enterobacter gergoviae*	0	0	0	0	1	0	0	0	1
8	*Enterobacter aerogenes*	5	8	0	5	3	13	8	1	43
9	*Enterobacter spp*	3	7	5	5	2	4	1	3	30
10	*Shigella spp*	7	4	3	2	5	9	3	0	33
11	*Salmonella spp*	5	2	1	0	4	6	3	0	21
12	*Morganella morganii*	7	11	7	2	2	5	7	2	43
13	*Yersinia enterocolitica*	13	9	5	2	8	11	7	3	58
14	*Yersinia pseudotuberculosis*	9	8	4	0	5	7	6	2	41
15	*Yersinia spp*	3	1	1	1	1	2	0	0	9
16	*Escherichia coli*	3	4	0	2	6	7	6	0	28
17	*Citrobacter spp*	8	12	3	4	11	15	6	6	65
18	*Citrobacter freundii*	10	13	7	6	9	15	10	8	78

Note: Ha; Haemolymph, S; Slime, HP; Hepatopancreas, FM; Faecal matter.

Table 4.1b: Total number of bacteria isolated from the specimen of the two Achatinid species.

	Bacterial agents	***A. achatina* (AA)** **Number of samples infected with bacteria isolates**				***A. fulica* (AF)** **Number of samples infected with bacteria isolates**				**Total samples**
		Ha	**S**	**HP**	**FM**	**Ha**	**S**	**HP**	**FM**	
1	*Citrobacter koseri*	9	7	5	7	7	12	6	4	57
2	*Providencia stuartii*	0	3	7	0	3	8	5	1	27
3	*Providencia rettgeri*	2	2	0	3	1	0	2	1	11
4	*Proteus spp*	0	0	2	0	3	4	0	0	9
5	*Hafnia alvei*	1	0	0	0	0	2	0	0	3
6	*Staphylococcus spp*	5	7	2	3	13	8	3	1	42
7	*Staphylococcus aureus*	6	3	2	2	8	5	2	1	29
8	*Streptococcus spp*	5	3	8	1	5	3	8	5	38
9	*Streptococcus pneumoniae*	7	2	3	0	6	0	1	0	19
10	*Bacillus cereus*	3	3	5	3	5	6	1	0	26
11	*Clostridium spp*	13	7	7	4	4	12	3	2	52
12	*Clostridium tetani*	6	5	1	1	4	3	2	0	22
13	*Pseudomonas spp*	3	2	2	0	5	4	0	1	17
14	*Bacillus spp*	11	6	1	3	5	9	4	4	43

Note: Ha; Haemolymph, S; Slime, HP; Hepatopancreas, FM; Faecal matter

Clostridium tetani, a Gram positive rod, is responsible for the disease; tetanus. It is seen in the sporulating stage in figure 4.2. The characteristic "racket" or "drum-stick" appearance is evident in the Gram stain as the bulging terminal end (terminal spore). The snails thus, were infected from soil with the agent.

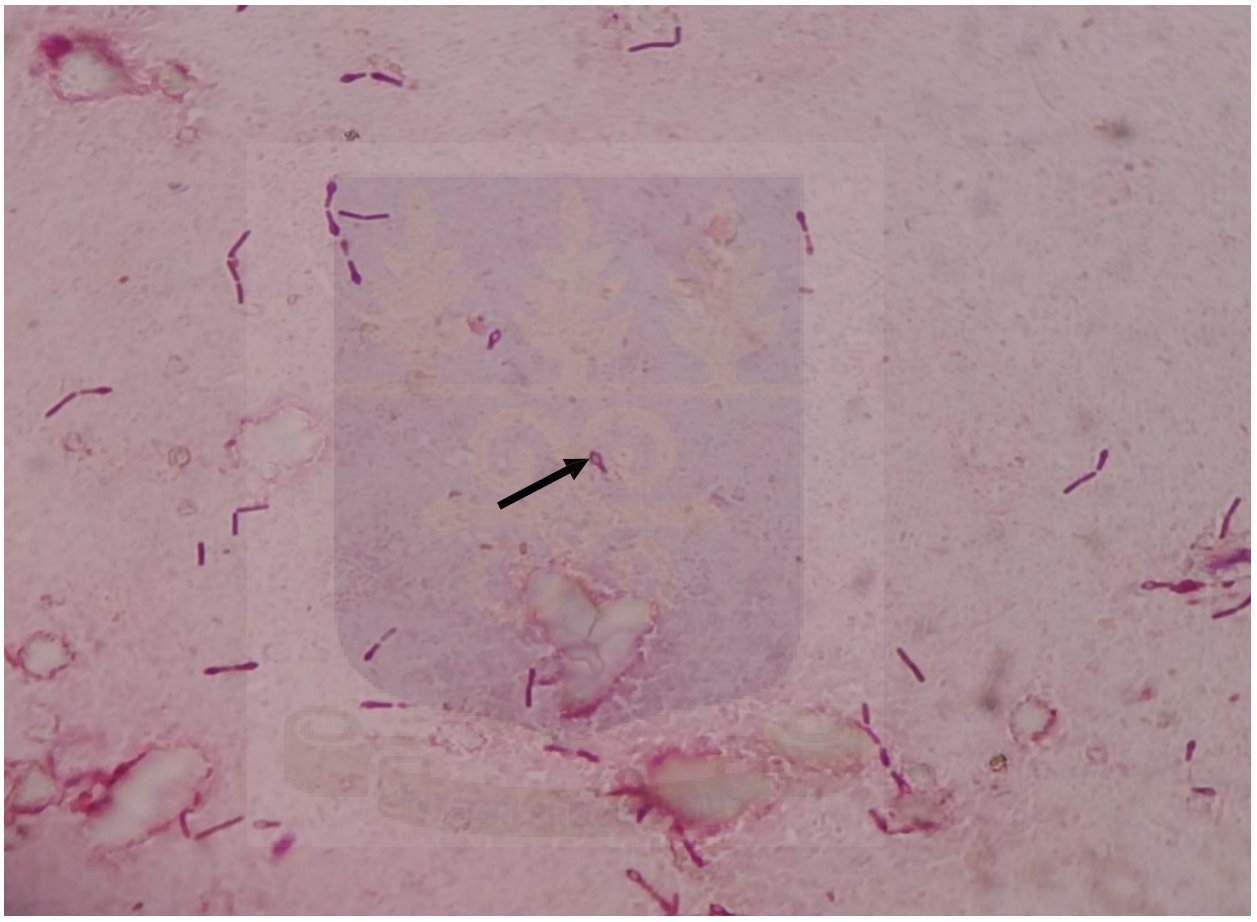


Figure 4.2: A Gram stained image of *Clostridium tetani* (Numerous in the field).

Bacillus spp, a Gram positive rod is responsible for numerous cases of food borne illnesses worldwide. The bacteria are shown in figure 4.3 as a long chain of large rods characteristic of *Bacillus spp*. They are commonly found in soils. The snails thus, were infected from soil with the agent.

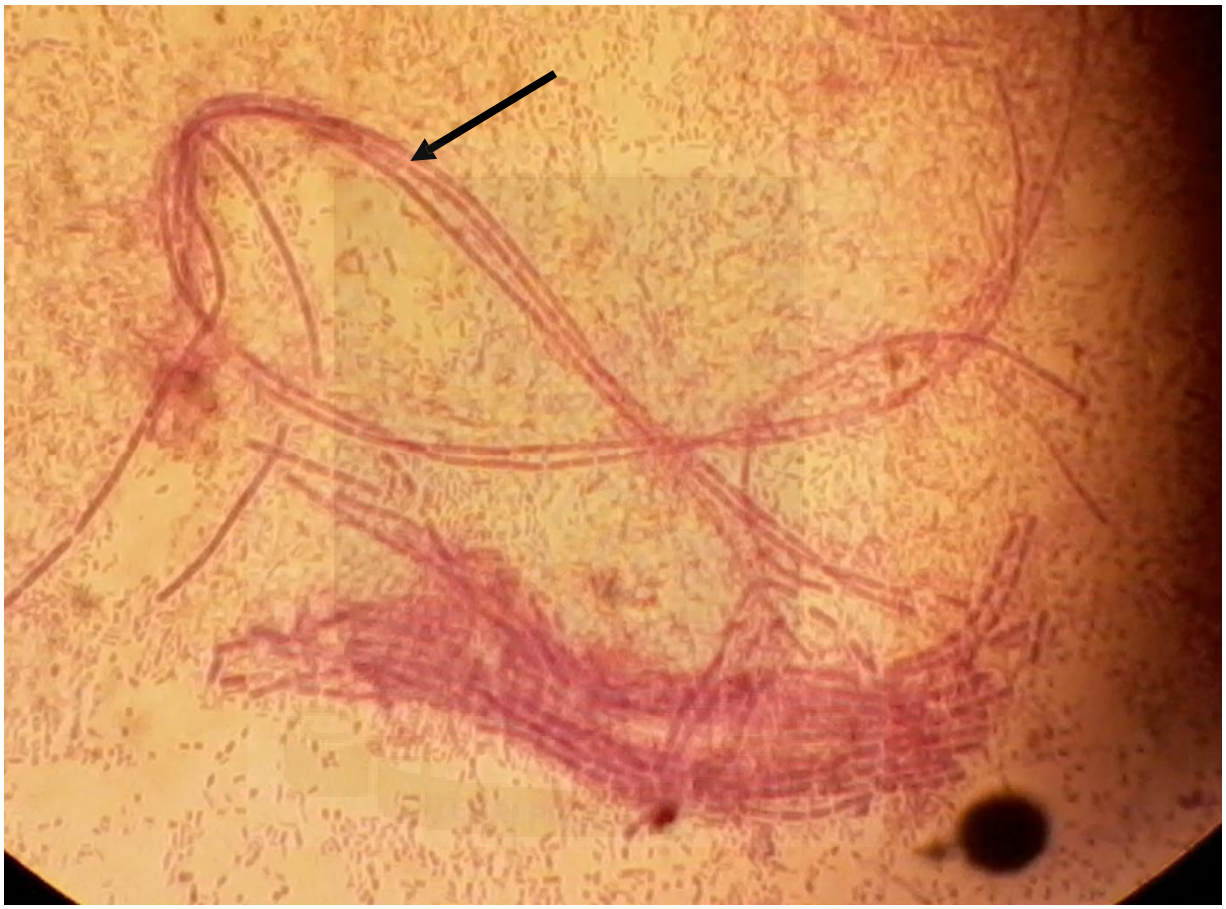


Figure 4.3: An image of *Bacillus spp* in a Gram stain

4.2.1.1 Analysis of bacterial loads between same specimen of *Achatina achatina* and *Achatina fulica.*

Table 4.2: Enterobacteriaceae count (EC) and Standard plate count (SPC) for same specimens of *Achatina achatina* and *Achatina fulica.*

Snail specimen	Snail species	Mean EC	P-value	Mean SPC	P-value
Faecal matter	*Achatina achatina* *Achatina fulica*	3.1x 10^5 1.8x 10^5	0.462	2.0x 10^5 9.1x 10^3	0.036
Hepatopancreas	*Achatina achatina* *Achatina fulica*	3.1x 10^5 7.9x 10^4	0.021	2.6x 10^5 4.4x 10^5	0.203
Haemolymph	*Achatina achatina* *Achatina fulica*	2.7x 10^5 9.1x 10^4	0.124	5.2x 10^5 1.4x 10^5	0.012
Slime	*Achatina achatina* *Achatina fulica*	5.6x 10^5 1.4x 10^5	0.002	5.6x 10^5 1.4x 10^5	0.002

Table 4.2 shows that at $p < 0.05$, there were significant difference between the enterobacteriaceae count for the hepatopancreas as well as that of the slime of *Achatina achatina* and *Achatina fulica*. The same table showed that, there were significant differences between the standard plate count for the faecal matter, haemolymph, as well as the slime of *Achatina achatina* and *Achatina fulica*.

4.2.1.2 Analysis of bacterial load between different specimen of both snail species; *Achatina achatina* and *Achatina fulica.*

Table 4.3: Enterobacteriaceae count (EC) and Standard plate counts (SPC) between specimens of *Achatina achatina* and *Achatina fulica.*

Snail specimen	Mean EC	P-value	Mean SPC	P-value
Slime Haemolymph	3.5x 10^5 1.8x 10^5	0.067	3.3x 10^5 3.3x 10^5	0.998
Slime Hepatopancreas	3.5x 10^5 2.0x 10^5	0.073	3.3x 10^5 3.5x 10^5	0.862
Slime Faecal matter	3.5x 10^5 2.4x 10^5	0.331	3.3x 10^5 1.0x 10^5	0.031
Hepatopancreas Faecal matter	1.9x 10^5 2.4x 10^5	0.619	3.5x 10^5 1.0x 10^5	0.010
Hepatopancreas Haemolymph	2.0x 10^5 1.8x 10^5	0.867	3.5x 10^5 3.3x 10^5	0.857
Haemolymph Faecal matter	1.8x 10^5 2.4x 10^5	0.554	3.3x 10^5 1.0x 10^4	0.027

Table 4.3 shows that at $p < 0.05$, there were significant difference between the Standard plate count for the slime vs. faecal matter, hepatopancreas vs. faecal matter, as well as the haemolymph vs. faecal matter of *Achatina achatina* and *Achatina fulica*.

4.2.1.3 Analysis of bacterial load between specimen of *Achatina achatina* and *Achatina fulica* and source of snail.

Table 4.4: Enterobacteriaceae count (EC) and Standard plate counts (SPC) of snail specimen and Source of snail.

Snail specimen	Source	Mean SPC	P-value	Mean EC	P-value
Haemolymph	Abidjan	3.3x 10^5	0.472	1.5x 10^5	0.005
	Agbogbloshie	2.0x 10^5		1.1x 10^5	
	Ho	1.2x 10^5		3.4x 10^4	
	Nkurakan	1.5x 10^5		1.0x 10^4	
	Asenewa	3.8x 10^5		9.7x 10^5	
	Adawso-suhum	9.7x 10^5		5.4x 10^4	
	Kukurantumi	1.3x 10^5		3.7x 10^4	
	Kadjebi	9.2x 10^3		13x 10^5	
	Abetifi	1.0x 10^4		1.1x 10^5	
	Axim	1.3x 10^5		1.6x 10^6	
	Dodowa	8.0x 10^3		1.4x 10^4	

Table 4.4 shows that at $p < 0.05$, there was a significant difference between the enterobacteriaceae count for the haemolymph of *Achatina achatina* and *Achatina fulica* based on the source of purchase.

4.2.1.4 Analysis of bacterial load between specimen of *Achatina achatina* and source of snail.

Table 4.5: Enterobacteriaceae count (EC) and Standard plate counts (SPC) of *Achatina achatina* snail specimen and source of snail.

Snail specimen	Source	Mean SPC	P-value	Mean EC	P-value
Faecal matter	Abidjan	1.1x 10^5	0.006	3.7x10^5	0.998
	Agbogbloshie	NIL		NIL	
	Ho	NIL		NIL	
	Nkurakan	1.9x10^5		2.3x10^2	
	Asenewa	NIL		NIL	
	Adawso-suhum	1.8 x10^6		2.0x10^4	
	Kukurantumi	1.6x10^6		7.8x10^4	
	Kadjebi	12.0x10^6		7.2x10^4	
	Abetifi	2.3x10^3		6.1x10^4	
	Axim	3.1x10^4		2.9x10^4	
	Dodowa	NIL		3.1x10^3	

Table 4.5 shows that at $p < 0.05$, there was a significant difference between the standard plate count for the faecal matter of *Achatina achatina* based on the source of the snail.

4.2.1.5 Analysis of bacterial load between specimen of *Achatina fulica* and source of snail.

Table 4.6: SPC and EC of *Achatina fulica* specimen and source of snail.

Snail specimen	Source	Mean SPC	P-value	Mean EC	P-value
Haemolymph	Abidjan	1.8x10^5	1.000	1.9x10^4	0.003
	Agbogbloshie	2.5x10^4		6.2x10^4	
	Ho	1.8x10^5		3.2x10^3	
	Nkurakan	4.0x10^3		3.7x10^5	
	Asenewa	8.4x10^4		7.9x10^5	
	Adawso-suhum	3.8x10^4		5.0x10^5	
	Kukurantumi	6.6x10^5		3.0x10^5	
	Kadjebi	1.1x10^4		2.5x10^3	
	Abetifi	1.7x10^4		2.0x10^6	
	Axim	1.4x10^4		1.6x10^5	
	Dodowa	9.0x10^4		2.8x10^4	

Table 4.6 shows that at $p < 0.05$, there were significant differences between only the enterobacteriaceae counts of the haemolymph of *Achatina fulica* based on the source of the snail.

4.2.1.6 Analysis of bacterial load between specimen of *Achatina fulica* and source of snail.

Table 4.7: SPC and EC of *Achatina fulica* specimen and source of snail.

Snail specimen	Source	Mean SPC	P-value	Mean EC	P-value
Faecal matter	Abidjan	8.7x10^3	0.840	1.3x10^5	0.026
	Agbogbloshie	8.8x10^5		2.9x10^5	
	Ho	2.0x10^4		3.6x10^5	
	Nkurakan	1.9x10^3		2.1x10^4	
	Asenewa	1.4x10^5		8.7x10^4	
	Adawso-suhum	2.3x10^2		2.3x10^5	
	Kukurantumi	1.3x10^3		2.5x10^3	
	Kadjebi	NIL		NIL	
	Abetifi	NIL		NIL	
	Axim	6.2x10^3		1.9x10^6	
	Dodowa	NIL		NIL	

Table 4.7 shows that at $p < 0.05$, there were significant differences between the enterobacteriaceae counts of the faecal matter of *Achatina fulica* based on the source of the snail.

4.2.1.7 Analysis of bacterial load between specimen of *Achatina achatina* and *Achatina fulica* and the markets of snail purchase.

Table 4.8: SPC and EC of *Achatina achatina* and *Achatina fulica* and the markets of snail purchase.

Snail specimen	Market	Mean SPC	P-value	Mean EC	P-value
Slime	MAKOLA	9.0x10^5	0.010	4.5x10^5	0.421
	ODAWNAA	5.9x10^3		5.8x10^5	
	TEXPO	6.6x10^5		4.1x10^5	
	KORLE_BU	4.3x10^4		5.2x10^5	
	TEMA_STATION	3.3x10^4		1.6x10^4	
	ASIGAME	1.1x10^5		3.5x10^5	
	KEJETIA	1.1x10^5		3.7x10^4	
	MANDELA	8.2x10^3		5.6x10^5	
	ABURI	3.9x10^5		7.0x10^4	

Table 4.8 shows that at $p < 0.05$, there was a significant difference between only the standard plate count of the slime of *Achatina achatina* and *Achatina fulica* based on the market of snail purchase.

4.2.2 Results of Parasitological analysis

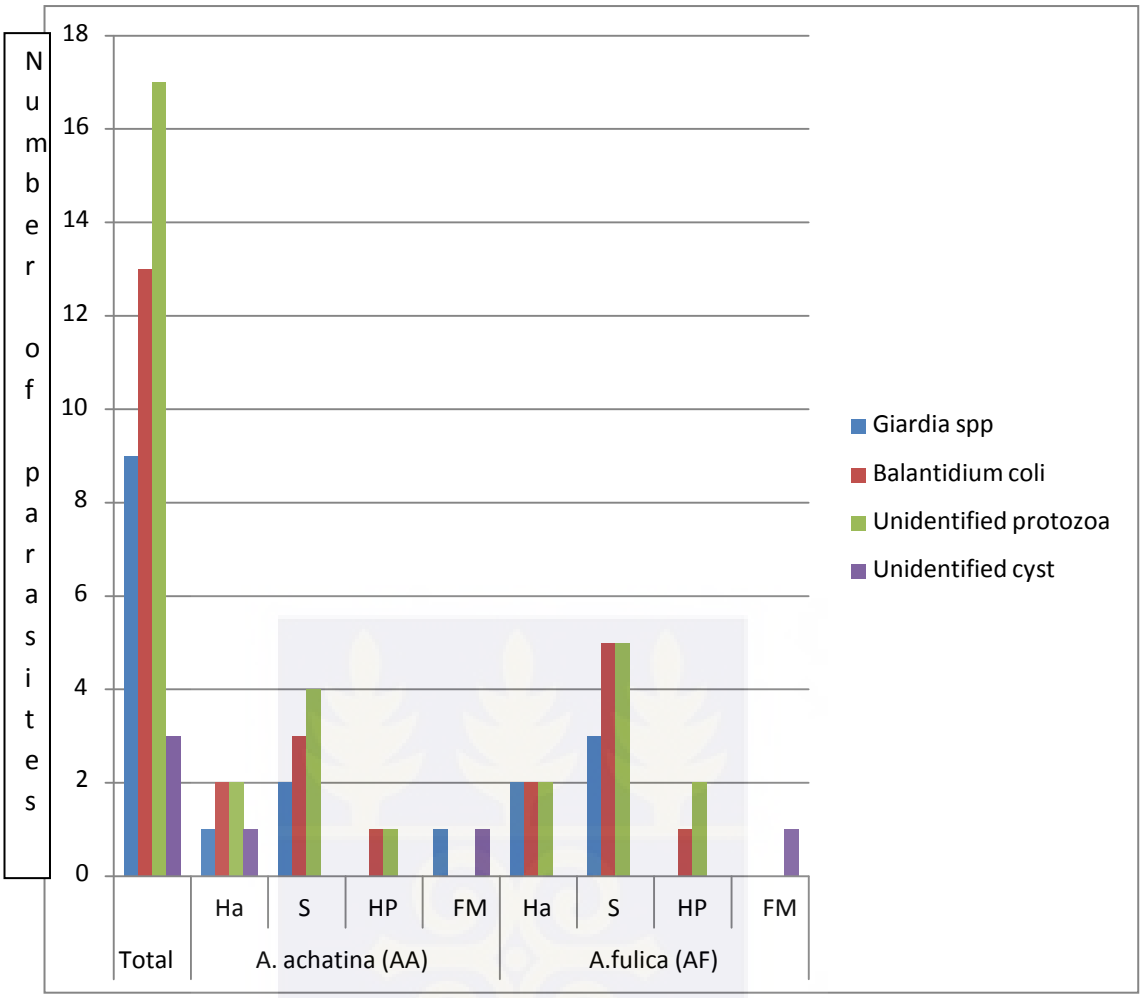
4.2.2.1 Enumeration of parasites

A total of 41 protozoan parasites and cysts were detected during the study from four parasites and cysts. The forty-one species included; *Giardia spp* (9), *Balantidium coli* (13), unidentified live protozoa (16) and unidentified cysts (3). The table below represents the distribution of the parasites to parasites in the various specimens of the two different snail species.

Table 4.9: Total samples infected with parasites isolated.

Parasitic agent	**Total parasites**	***A. achatina* (AA)**				***A. fulica* (AF)**			
		Ha	**S**	**HP**	**FM**	**Ha**	**S**	**HP**	**FM**
Giardia spp	9	1	2	0	1	2	3	0	0
Balantidium coli	13	1	3	1	0	2	5	1	0
Unidentified protozoa	16	2	4	1	0	2	5	2	0
Unidentified cyst	3	1	0	0	1	0	0	0	1

Note: Ha; Haemolymph, S; Slime, HP; Hepatopancreas, FM; Faecal matter.



Note: Ha; Haemolymph, S; Slime, HP; Hepatopancreas, FM; Faecal matter

Figure 4.4: Showing the distribution of total samples infected with parasites isolated.

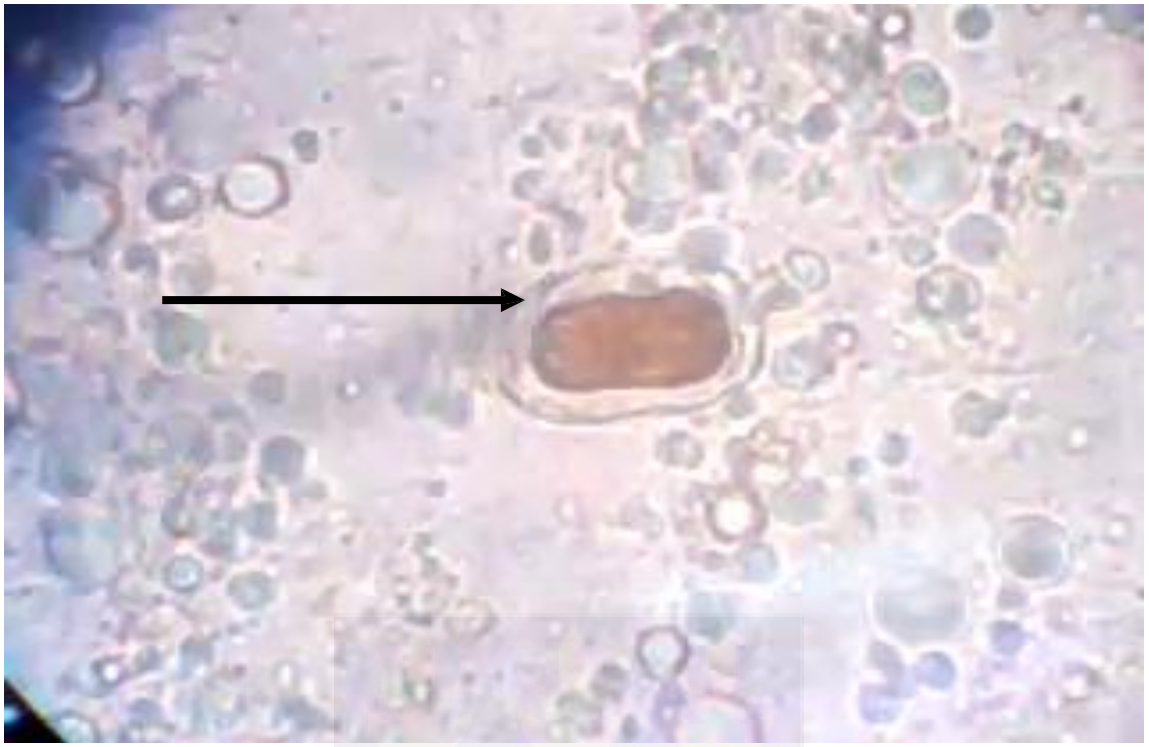


Figure 4.5: Showing an iodine stain of an unidentified cyst.

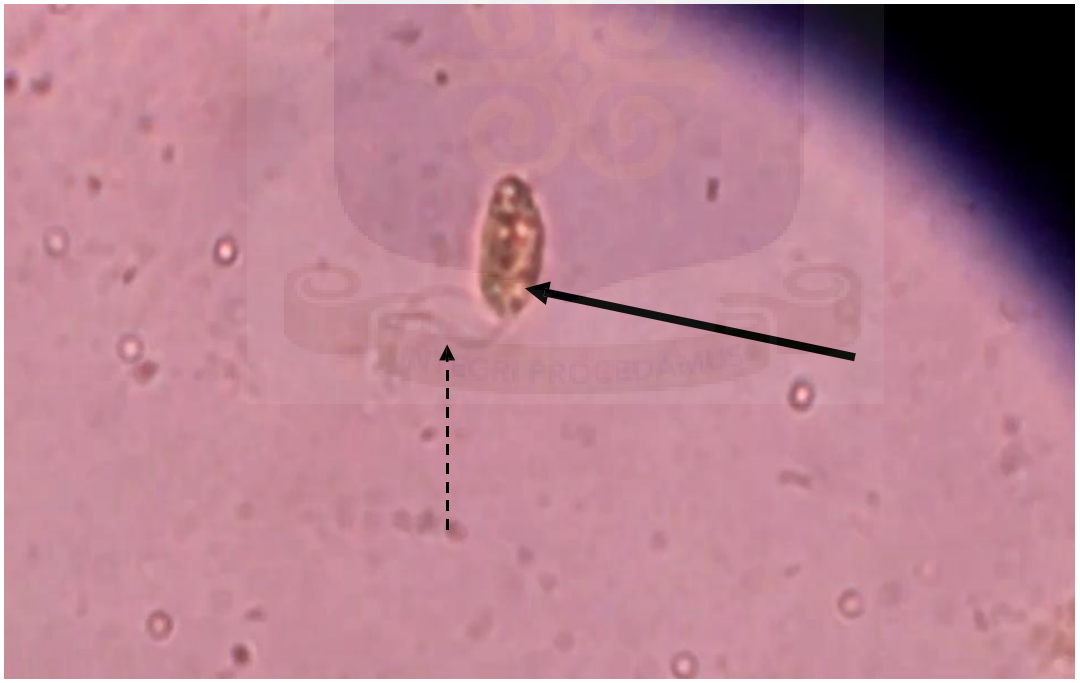


Figure 4.6: An iodine stain of an unidentified flagellate (The flagella is indicated by the broken arrow)

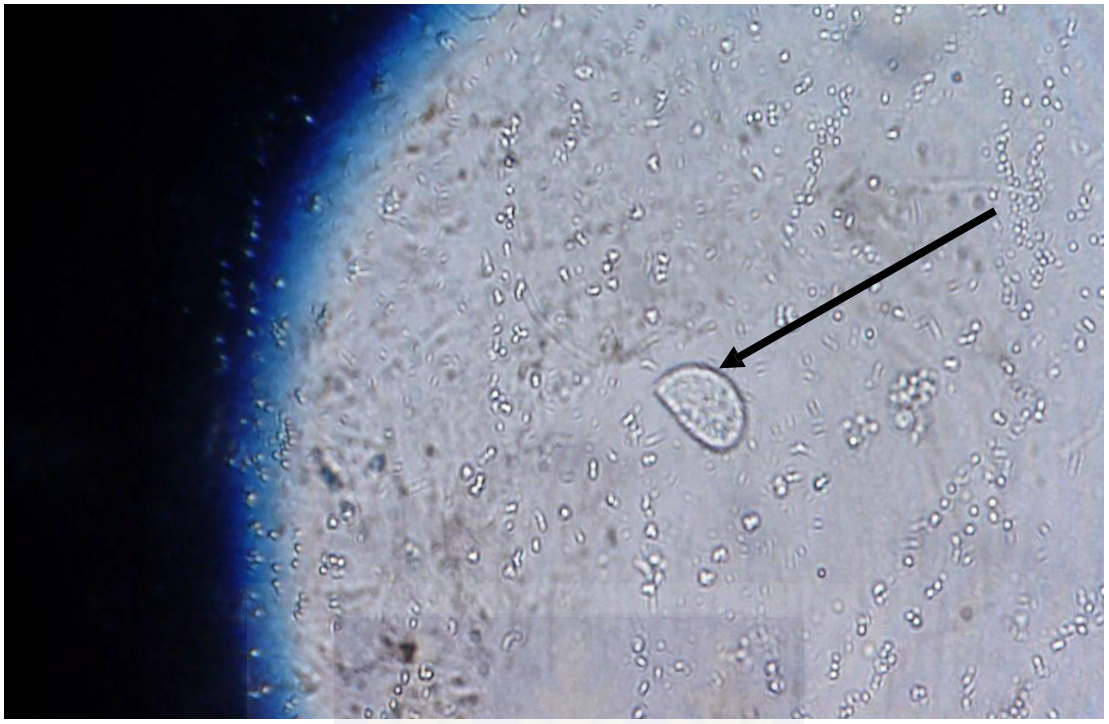


Figure 4.7: Showing an unidentified ciliate in a wet preparation

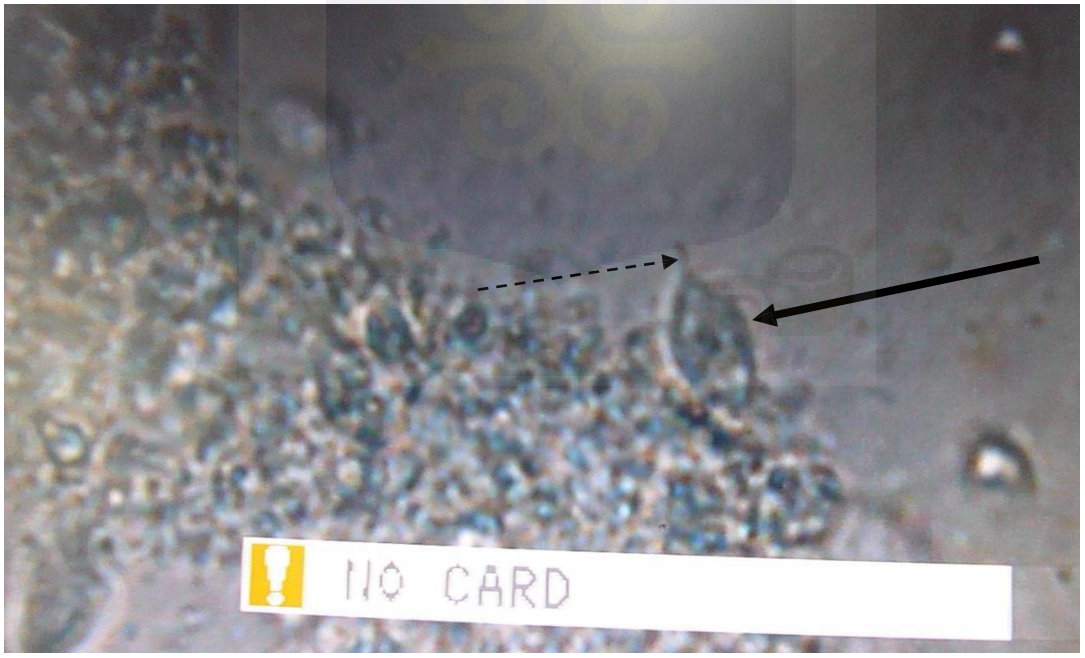


Figure 4.8: An unidentified flagellate in a wet preparation (Flagella shown by broken arrow)

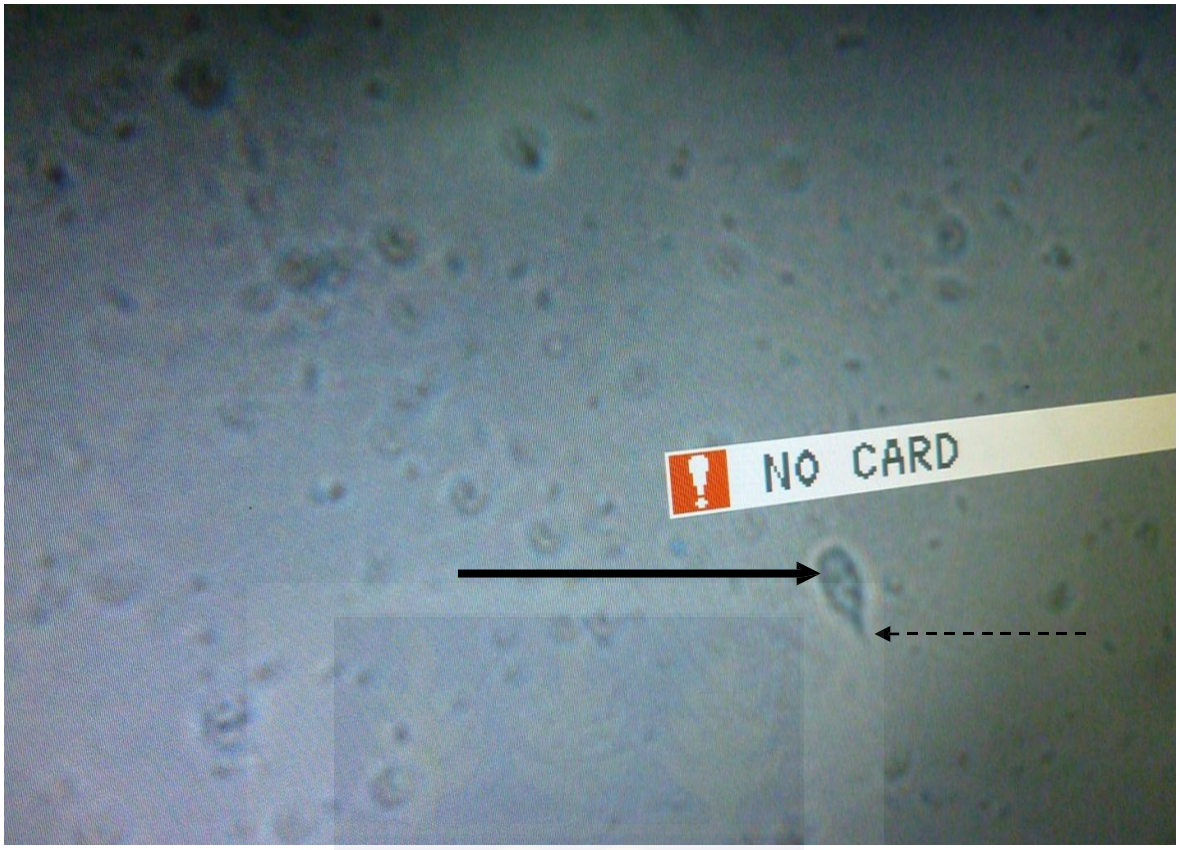


Figure 4.9: Showing an unidentified flagellate in a wet preparation

4.2.3 Mycological analysis

4.2.3.1 Enumeration of fungi

A total of 143 fungal agents from five different species were isolated during the research. The 32 species include; *Candida spp* (46), *Cryptococcus neoformans* (45), *Candida albicans* (28), *Aspergillus spp* (15) and *Rhizopus spp* (9). The table 4.10 below represents the distribution of the fungal agents in the various specimens of the two different snail species.

Table 4.10: Showing total fungal species per snail species and the corresponding specimen from which they were isolated

	Fungal agent	**Total fungi**	***A. achatina* (AA)**				***A. fulica* (AF)**			
			Ha	**S**	**HP**	**FM**	**Ha**	**S**	**HP**	**FM**
1	*Candida albicans*	28	5	4	6	1	3	7	2	0
2	*Candida spp*	46	12	9	0	1	5	9	8	2
3	*Cryptococcus neoformans*	45	8	11	7	0	5	8	3	3
4	*Aspergillus spp*	15	2	4	0	1	1	5	2	0
5	*Rhizopus spp*	9	1	3	0	0	0	4	1	0

Note: Ha; Haemolymph, S; Slime, HP; Hepatopancreas, FM; Faecal matter.

Candida spp as shown above, are yeast cells that cause disease in individuals with compromised immune systems. They are found in faeces of man and animals. They can

thus be found in soils contaminated with faeces containing the yeast cells. The characteristic oval shape of *Candida spp* budding is evident in the negatively stained image.

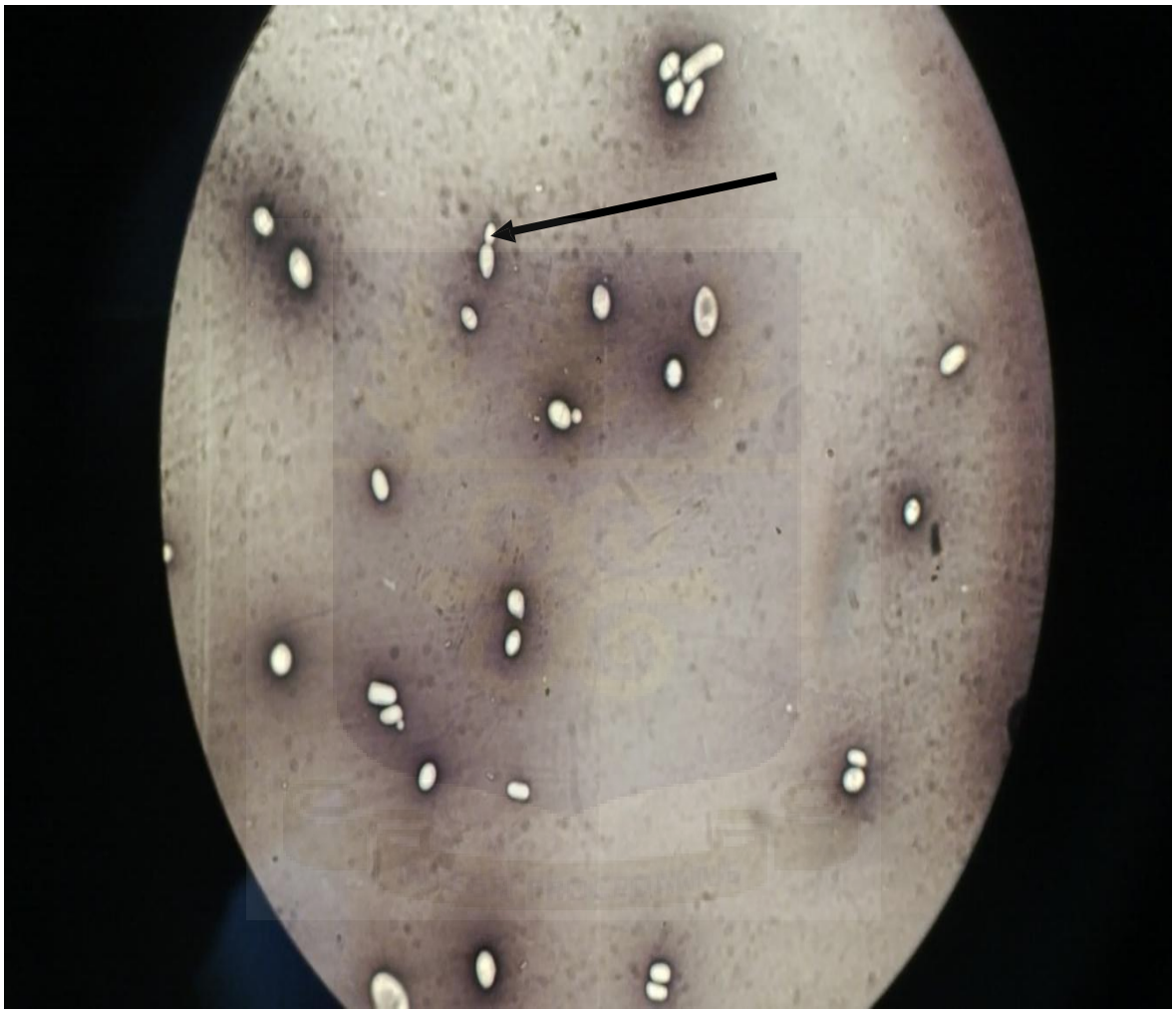


Figure 4.10: Showing a nigrosin stained image of *Candida spp*

The image below shows *Aspergillus spp* in a wet preparation. It shows non-septate hyphae with sporangia as observed in the image below. It is a soil borne fungal agent implicated in diseases of the respiratory system of man.

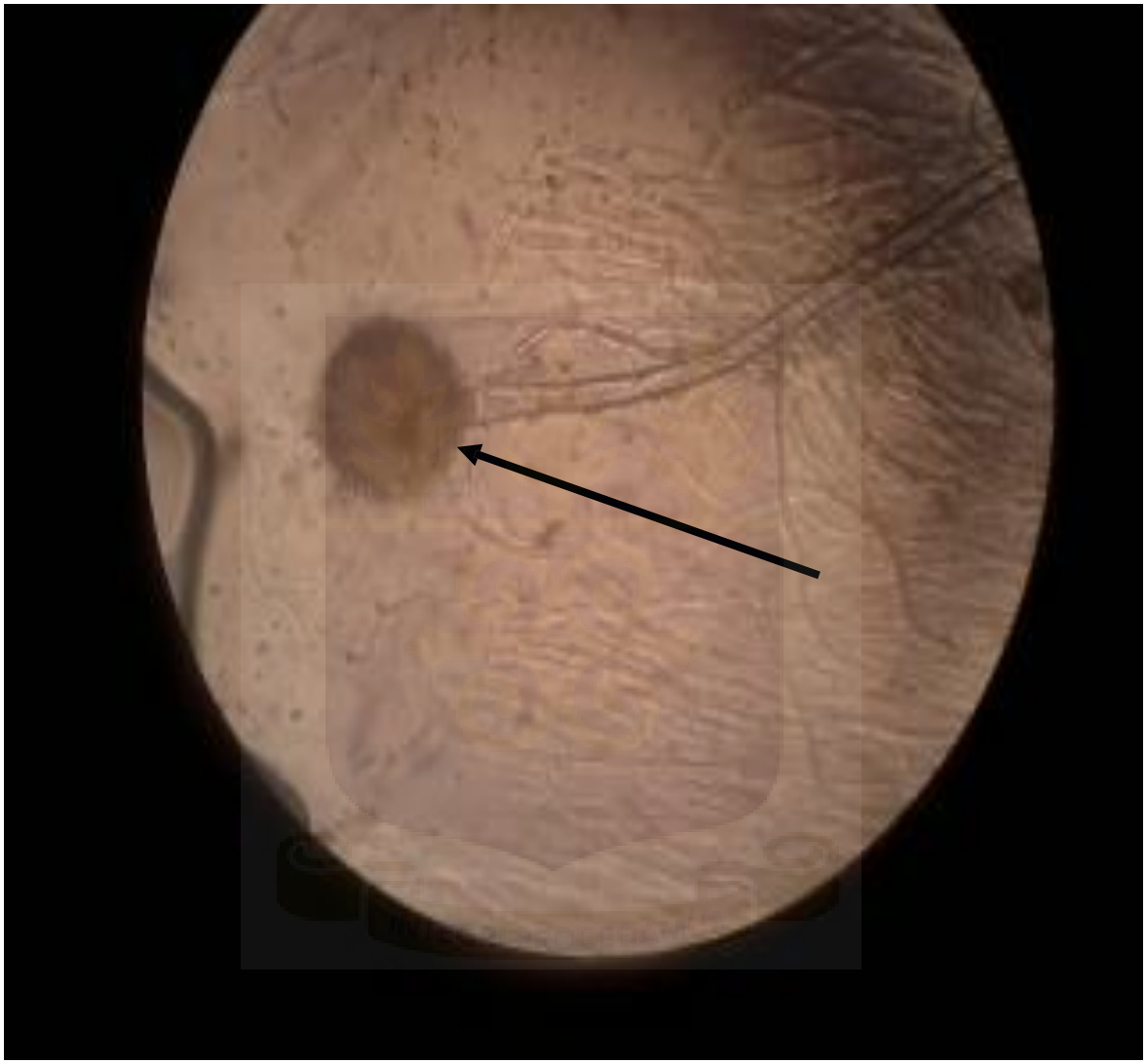


Figure 4.11: Showing an image of *Aspergillus spp* in a wet preparation

The image below shows the characteristic dark spores produced by *Aspergillus spp* on Sabouraud agar.

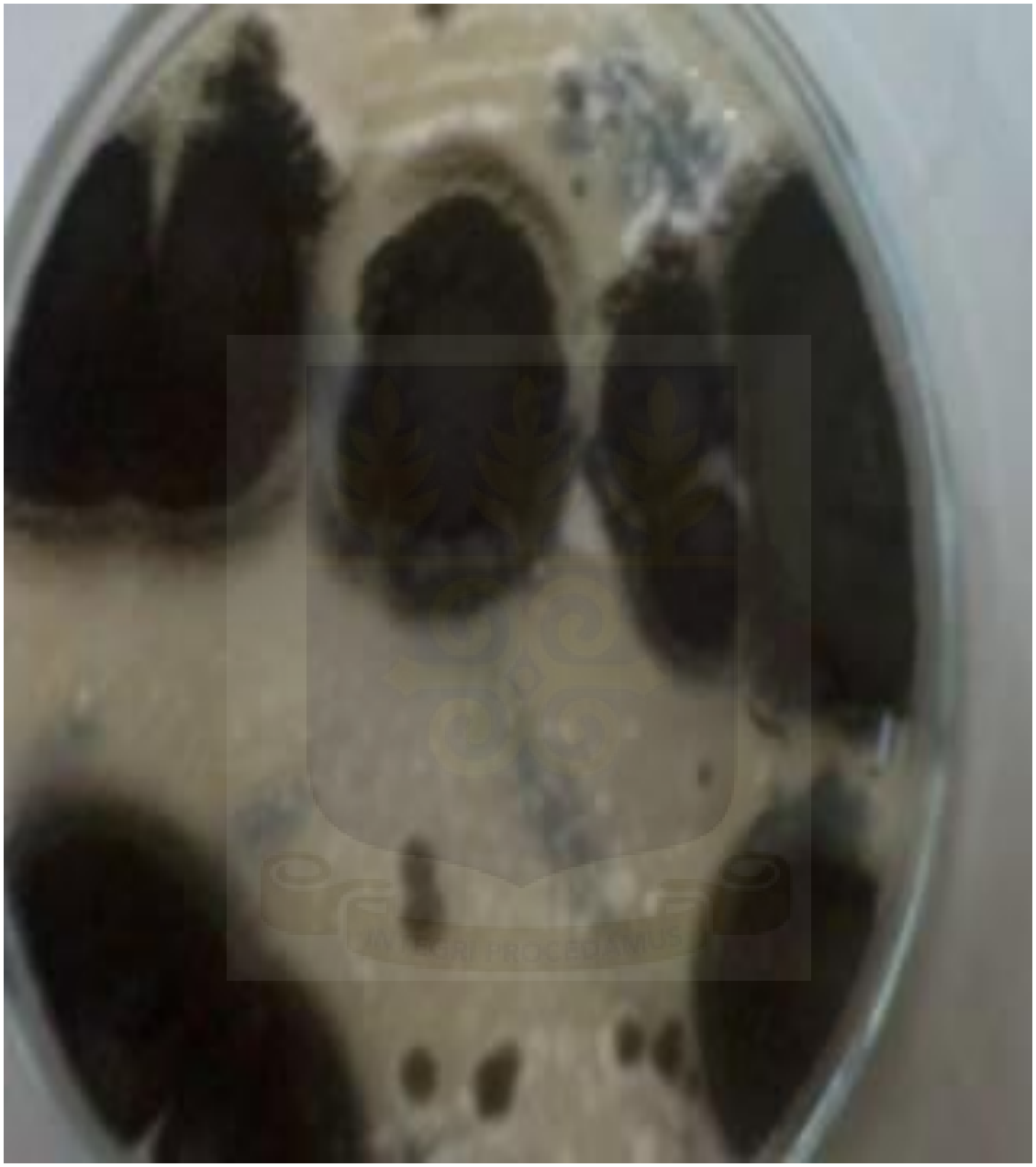


Figure 4.12: Showing an image of *Aspergillus spp* on Saboraud agar

Rhizopus spp, as shown in the wet preparation below shows the characteristic septation and sporangia. Snails contact this in contaminated soil.

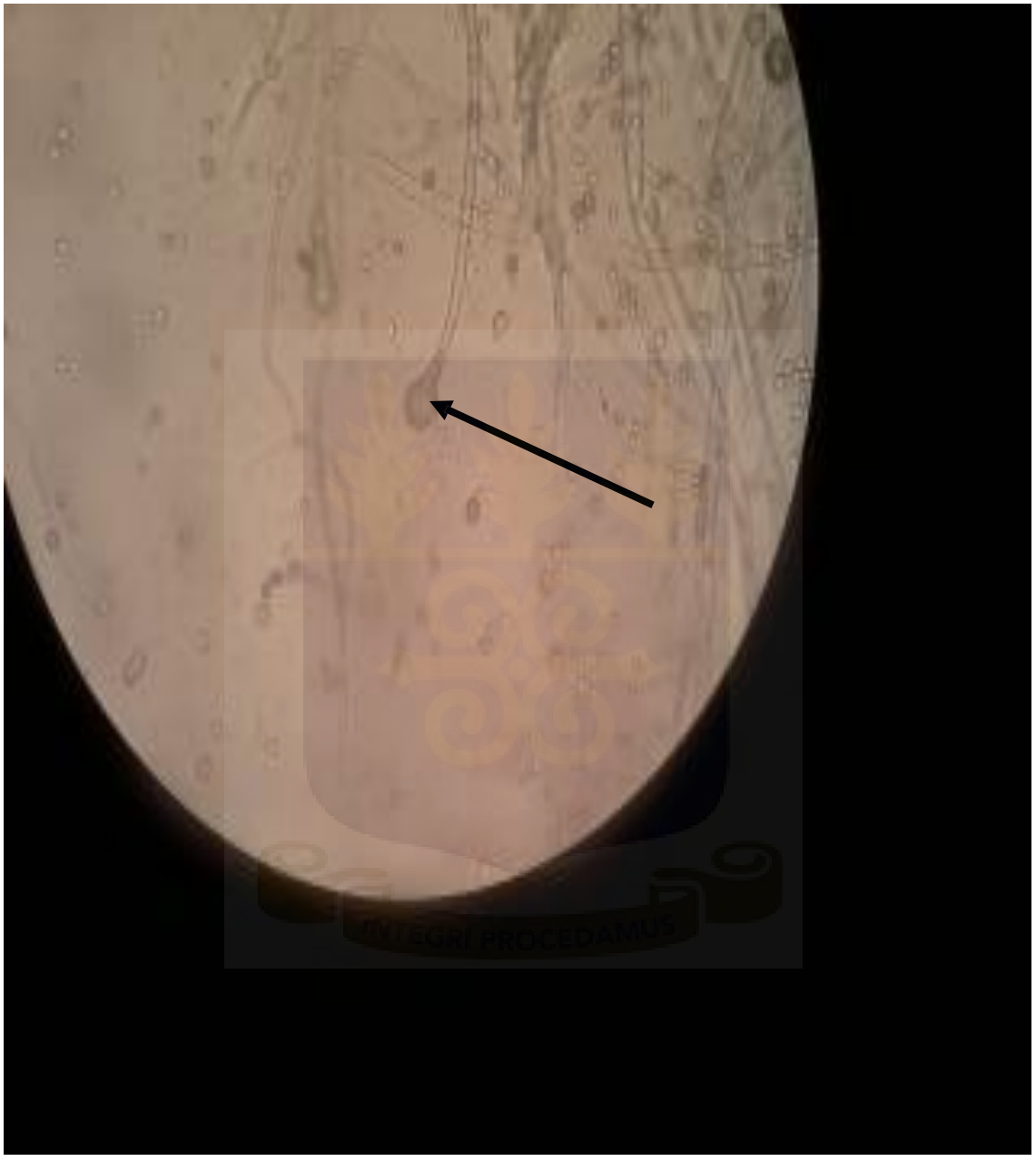


Figure 4.13: Showing an image of *Rhizopus spp* in a wet preparation

The image below shows a negative staining of *Cryptococcus neoformans.* The image shows its characteristic circular shape. The agent causes cryptococcosis in man; a respiratory disease.

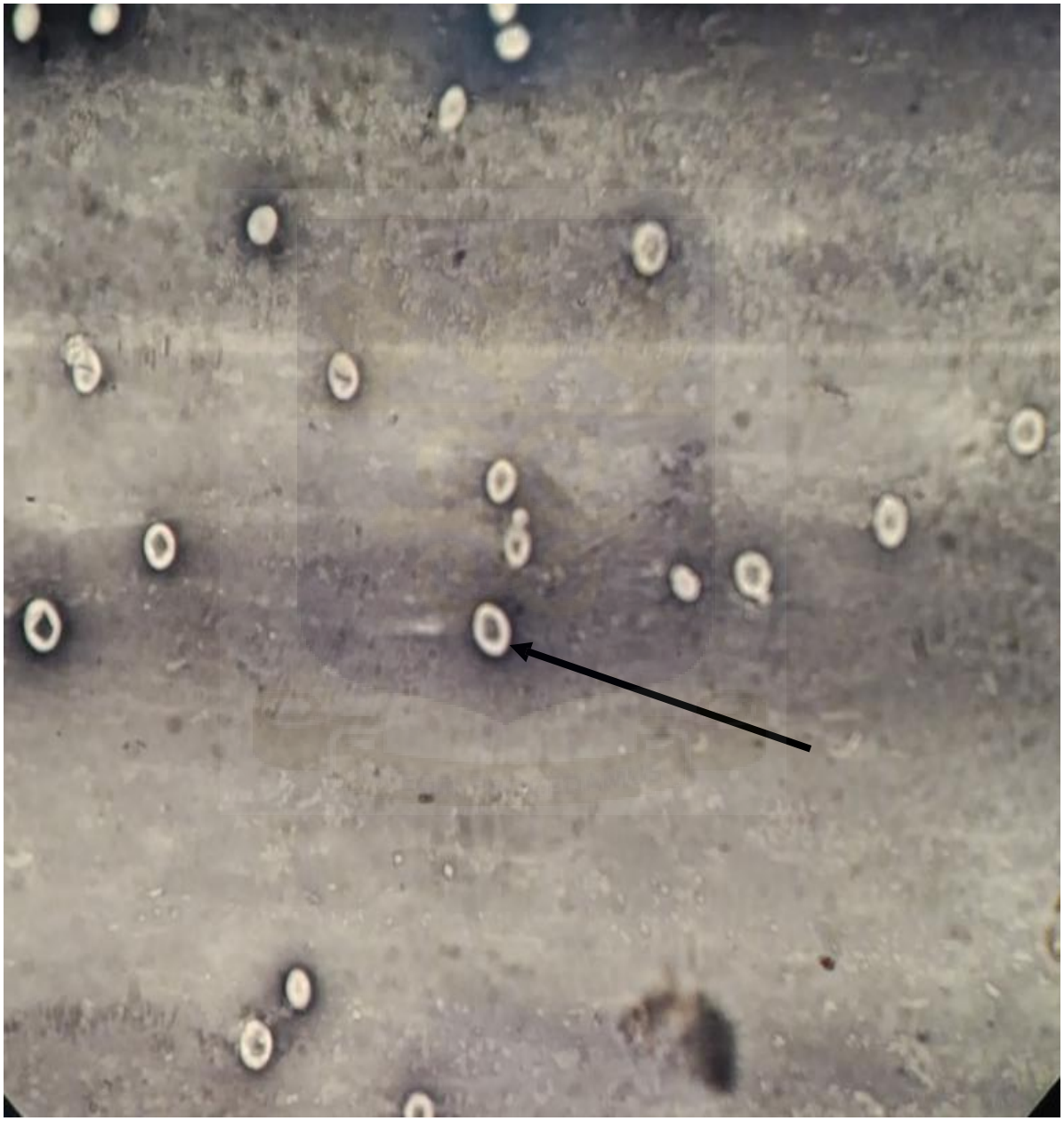


Figure 4.14: Showing a nigrosin stained image of *Cryptococcus neoformans.*

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Snail meat has served as a good source of proteins for man. The shell when ground to powder, has been used by many to improve skin regeneration in cases of burns. Its other emerging uses include the use of the live snails in beauty salons for facial therapy. Russia and Japan are amongst the countries exploiting the slime of the snail in beauty therapy (Jimenez, March 27; Japan Daily Press, July 2013). The act of drinking the raw snail haemolymph for medicinal values already elaborated upon is not a strange practice amongst some indigens in Ghana.

Contamination of foods through handling leads to foodborne illnesses worldwide. Routes of food contamination being mainly environmental include microbial, pesticide, heavy metals, etc (CDC, 2012). Microbial contamination could occur during transportation or vendor handling. Also, microbes present in specimens of the snail such as the faecal matter can contaminate other edible portions of the snail such as the meat during the processing stage. The majority of pathogens that cause food contamination and foodborne illnesses that are of public health concern worldwide are bacteria: however, viruses, parasites and fungi also contribute to the problem. Snails move by crawling on the ground and are, hence, in constant contact with the soil. They are also observed to burrow into soils whilst laying eggs as well as during periods of aestivation.

The inevitable contact of snails with soil makes them susceptible to infections with soil borne pathogens. Considering the use of snail for food coupled with the use of its unprocessed products for medicinal purposes poses a risk of infection acquisition by consumers. This research sought to assess the microbiological quality of edible snails from

selected markets in Ghana. In this study, microbes were isolated with the most predominant one being *Erwinia herbicola* and the least being *Enterobacter gergoviae*. During the study, more bacteria (85.7%) were isolated as compared to fungi (11.1%) and parasites (3.2%).

The results from the questionnaire also showed the possible reason why the demand for snails is currently not met by inland sources. The reasons were attributed to reduced rainfall and two main human activities: mining and the use of chemical sprays in agriculture. Six (54.6%) of the vendors attributed the reduction of snail availability to chemical spraying on farmlands, 4 (36.4%) attributed the observation to mining activities, and 1(9.1%) attributed the observation to reduced rainfall.

In addition to the vendors knowledge on the use of snail products/ specimen (haemolymph, slime, hepatopancreas and faecal matter) for medicinal value, dermato therapeutic purpose amongst other uses, the specimen were mentioned for use against epilepsy, stroke, anaemia, asthma, hypertension and burns. It is also used for cleansing the eyes, as well as energy source, calcium source, improving intelligence, promotes smooth skin and adds taste to soups. Concerning the use of the haemolymph, 11vendors consented to having knowledge of its use as an energy source and taste value in soup, 10 vendors consented to having knowledge of its use for hypertentension, 10 for asthma, 9 for anaemia and as a calcium source, 5 for burns and use by pregnant women (drunk in the raw state), 4 for stroke, 3 for treating diabetes. Each of the remaining vendors also consented to knowledge of its use for epilepsy, intelligence, eye cleansing and for smooth skin.

With regards to the use of the slime, 4 (36.4%) vendors consented to having knowledge of its use for treating burns whilst 1 (9.1%) admitted that use of the slime makes the skin smooth. No vendor admitted to knowing the use of the slime for the purposes mentioned for the haemolymph. No medicinal value was attributed to the faecal matter of the snails.

5.1 Bacteriological analysis

Of the total different bacteria isolated, 24 (75%) percent were Gram negatives and 8 (25%) were Gram positives. The Gram negatives included *Erwinia herbicola*, *Klebsiella pneumoniae, Citrobacter freundii, Citrobacter spp, Yersinia enterocolitica*, *Citrobacter koseri*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Morganella morganii*, *Yersinia pseudotuberculosis, Shigella spp, Klebsiella oxytoca, Enterobacter spp*, *Escherichia coli*, *Providencia stuartii*, *Salmonella spp*, *Pseudomonas spp*, *Klebsiella ozonae*, *Providencia rettgeri*, *Proteus spp*, *Yersinia spp*, *Hafnia alvei*, *Klebsiella rhinoscleromatis* and *Enterobacter gergoviae*. Gram positives on the other hand included *Clostridium spp*, *Clostridium tetani, Bacillus spp, Bacillus cereus, Streptococcus pneumoniae, Streptococcus spp, Staphylococcus spp, and Staphylococcus aureus*.

The occasional or frequent drinking of the haemolymph for medicinal value poses a risk of microbial infection of the gastrointestinal tract with many of the above isolated bacteria. These bacteria are implicated in disease states such as diarrhoea, typhoid fever, paratyphoid fever, peritonitis, septic arthritis, meningitis, pneumonia, endolphthalmitis, chorioamnitis, endocarditis, otitis media, as well as bacteraemia with fatal consequences (Ryan, 2004). This is especially risky in individuals with ulcers in the gastrointestinal tract. The inoculation of the haemolymph unto foot with rot (necrotizing tissue) as a remedy poses the risk of infection for its users with disease agents such as *Streptococcus spp, Clostridium tetani, and Staphylococcus aureus* amongst others, which are implicated in disease states such as ecthyma, central nervous system infections, necrotizing fasciitis and tetanus amongst other diseases. Foots with rot are likely to provide ambient conditions for *Clostridium tetani* replication and subsequent neurotoxic effect. Furthermore, the inoculation of the haemolymph unto the eye to cleanse it poses the

risk of infection with disease agents such as *Streptococcus spp* which is implicated in bacterial conjunctivitis (mydras eye).

The sick and the poor are most vulnerable to resorting to the above outlined options. The risk of infection with any of these microbial agents is even higher in individuals with a compromised immune system and those with sub-optimal immune systems. Individuals who handle or frequently come into contact with snail or snail specimens and have cuts stand a high risk of infection as well.

From the study, a statistically significant difference was observed (Table 4.2) between the enterobacteriaceae count for the hepatopancreas (p-value of 0.02) as well as the enterobacteriaceae count of the slime (p-value of 0.00) of *Achatina achatina* and *Achatina fulica*. This result indicated that *Achatina achatina* had consistently higher enterobacteriaceae counts than *Achatina fulica*. The observation could perhaps be from the higher demand for *Achatina achatina* compared to *Achatina fulica*. This could be due to the frequent handling by vendors hence contamination of the snails by vendors through display, handling and packaging together in a sack, crawling of snails on soils, as well as the picking of microbes by snails when they fall onto the grounds and the washing of snails with the same water before sale to buyers. This reflects the little awareness of vendors on cross-contamination and its likely implication. This was evident from the questionnaire as vendors believed cooking killed all pathogens hence neglecting the need to reduce or avoid contact of snails with dirt and faecal matter of other snails. The water used to wash the snails was observed to be unclean and this causes further contamination of the snails thereby posing more health hazards to consumers.

Table 4.2 again showed a statistically significant difference between the standard plate counts for the slime (p-value of 0.00), haemolymph (p-value of 0.01) and the faecal matter (p-value of 0.04) of *Achatina achatina* and *Achatina fulica*. This indicates the possible contamination of snails by the environment. The untidy or dirty environments might be a source of the contamination via airborne transmission from nearby public places of convenience, heaped rubbish, houseflies, as well as the occasional fall of snails unto market soils. Vendor environments were dirty as mentioned already. The mean counts for *Achatina achatina* specimens were higher than *Achatina fulica* in both cases of enterobacteriaceae count and standard plate count. The questionnaire showed that, of the two snail species, the most sold species was *Achatina achatina*. Thus, significantly higher counts for *Achatina achatina* could be due to the frequent handling of *Achatina achatina* than *Achatina fulica* based on the higher demand for it.

Analysis conducted to deduce which specimen was more contaminated (Table 4.3), showed a statistically significant difference between the standard plate count for the comparison of the hepatopancreas and faecal matter (p-value of 0.01), haemolymph and faecal matter (p-value of 0.03) and the slime and faecal matter (p-value of 0.03). The results showed that all specimens (slime, haemolymph and hepatopancreas) had statistically significant mean standard plate counts which were higher than the mean standard plate count of the faecal matter. This observation may be due to the presence of toxins, unfavourable pH, change in oxygen content or the presence of an inhibitory compound in the faecal matter that did not support survival of many bacteria. The observation may also be due to the fact that only 50 (instead of 75 each) faecal matter for each snail species were obtained during the study.

With regards to the source of snail and snail specimen contamination (table 4.4), statistically significant differences were observed between the enterobacteriaceae count for the haemolymph (p-value of 0.01) of both snail species, as well as the standard plate count for the faecal matter (p-value of 0.02) of both snail species.

This is an indication of possible contamination by vendors from these sources through handling, packaging or transportation to markets for sale for the observed enterobacteriaceae counts. The standard plate count per source of snail on the other hand, is an indication of the possible contamination from the environments (soil, water, rubbish, etc) of the sources of the snails. The same analysis (Tables 4.5, 4.6 and 4.7) on the source of snail but using individual species revealed that *Achatina achatina* showed a statistically significant difference only between the standard plate count for the faecal matter (p-value of 0.01) and the source of the snail. *Achatina fulica* on the other hand showed a statistically significant difference only between the enterobacteriaceae count for the haemolymph (p-value of 0.00), as well as the faecal matter (p-value of 0.03).

Regarding the market of snail purchase and snail specimen contamination (Table 4.8), a statistically significant difference was observed between the standard plate counts for the slime (p-value of 0.01) of both snail species. This difference between standard plate counts per market of snail purchase is an indication of the possible contamination from the environments (soil, water, rubbish, etc) of the markets.

5.2 Parasitological analysis

In this study, cysts and trophozoites of flagellates and ciliates were found. Of the total number of 41 samples positive for parasites (Table 4.11), 13 samples showed *Balantidium coli*, 11 showed unidentified flagellates, 9 showed *Giardia spp*, 5 showed ciliates and 3

showed unidentified cysts. The parasites *Giardia spp* and *Balantidium coli* identified are of faecal origin and is an indication of the unhygienic habitats of the snail. Infections with *Giardia spp* present clinical findings such as diarrhoea, flatulence, duodenal inflammation, protein malabsorption, nausea, anorexia and abdominal cramps without fever. Most infections with *Balantidium coli* are asymptomatic. *Balantidium coli* has the ability to secrete hyaluronidase which probably helps the organism to invade the mucosa. Severe balantidiasis can lead to perforation of the colon, hepatic abscess and appendicitis.

5.3 Mycological analysis

The study revealed the presence of *Candida albicans, Candida spp* (Figure 4.9), *Cryptococcus neoformans, Aspergillus spp* and *Rhizopus spp*. Candida albicans was observed in all specimens of *A. achatina* except *A. fulica* faecal matter. Diseases of *C. albicans generally* occur in individuals with an immunocompromised state. Thus, *C. albicans* becomes an infectious agent when there is a change in body environment allowing it to grow out of control. In extreme and rare situations when one's body cannot resist this infectious agent, an infection of the bloodstream can occur affecting the heart, kidney, heart, lungs (a bloody sputum discharge), eye (pain and blurred vision), brain (seizures) and other organs. Considering the application of the snail products, users with immunocompromised, states as well as those with broken skin and ulcers are most vulnerable (Health Central, 2013).

Cryptococcus neoformans (Figure 4.13), a soil borne fungus causes cryptococcosis. The agent affects the lungs and may become disseminated throughout the body. The infection is seen in people with weakened immune systems (Medline plus, 2013). When disseminated, it has effects on the brain and nerves. Aspergillosis caused by fungus of the

genus Aspergillus (*Aspergillus spp* as displayed by Figure 4.10) similarly affects the respiratory system (CDC, 2013). Aspergillus skin infections have been reported as occurring from contamination of biomedical devices (CDC, 2013). The infection via haemolymph or slime application to skin could serve a possible route for infection especially in individuals with an immunocompromised state as well as those with broken skin.

Rhizopus spp are generally non-pathogenic although some species are said to be opportunistic agents of human zygomycosis which can cause fatalities. *Rhizopus* may cause rhinocerebral infections presenting as facial pain, eye pain or bulging eyes. They also cause pulmonary infections which may present as fever, cough, breathing difficulty, as well as gastrointestinal and skin infections which present as abscess inside the abdomen and necrotic skin lesions respectively. Complications of Rhizopus infections result in carotid artery thrombosis (rhinocerebral infection). *Rhizopus* is also a soilborne agent, with some of the infectious ones toxin producing (Rightdiagnosis, 2013).

The isolation of these fungal agents during the study is understandable considering that the snails are in constant in contact with the soil. Furthermore, most of the isolated fungi can be obtained from bird droppings. The markets are not with adequate spaces and are congested, the drainage system is poor, and vendors are in close proximity to refuse/rubbish and toilets. There are rarely, if any, few markets that are designated for the sale of particular products (e.g, a market for vegetables only, live organisms only, etc). As a result, birds are sold where vegetables, snails, etc are also sold. There are no strict management systems in place to monitor how bird droppings for example are disposed off to ensure a re-infection of susceptible market products does not occur. Hence snails,

considering the manner in which they are displayed at markets are likely to fall off onto the untidy floors where they contact the microbes under discussion. The occasional or frequent drinking of the haemolymph as well as skin application of the slime for medicinal value poses a risk of infection with any of the above isolated fungi.

CONCLUSION

From the objectives of this study, microbial isolates found included non-pathogenic (majority of which were opportunistic) and pathogenic microbial agents. Hence, the snails on the Ghanaian market are not microbiologically safe for consumption. Also, making reference to microbiological food safety, foods requiring further cooking should not contain $5x10^5$ bacterial cells per gram of Standard plate count of the food. Counts of *Bacillus cereus* ($5x10^3$), *Clostridium perfringens* ($5x10^2$), etc, and higher than designated values, indicate the foods as unacceptable. During the study, bacteria load count ranges (10^2- 10^6) obtained fall within and above the levels set for most foods hence by that comparison can be inferred to be microbiologically unacceptable or not of quality microbiologically.

Also different bacteria, parasites and fungi both pathogenic and opportunistic have been isolated with all possessing different degrees of virulence. The absence of a standard for the snail nor such similar specimens worked on makes it impossible to conclude on whether or not the bacterial counts are within acceptable limits or not. For example by Hazard Analysis Critical Control Points (HACCP) standards, as low as 10 cells of *Shigella spp* can cause illness following the ingestion of very low numbers of viable bacteria depending on host susceptibility (Kothary, 2001). However all counts exceeded 10 though too few to count. The risk therefore with the administration of the unprocessed

specimens orally or otherwise is of immense public health concern bearing in mind the potentially pathogenic microbes isolated.

RECOMMENDATION

Based on the findings of this research, the following are thus recommended;

1. Every household should ensure adequate cooking time and temperature is attained before consumption of snails since the meat can easily be contaminated with the other specimens of the snail during the traditional extraction and processing.
2. City planning authorities like the Accra Metropolitan Assembly (AMA), Kumasi Metropolitan Assembly (KMA), Tema Metropolitan Assembly (TMA), etc must re-assess the plan of markets, especially the local markets in terms of water drainage. This will help reduce the accumulation of waste materials within the market premises.
3. A market sanitation team can be set up to monitor and ensure market sanitary practices
4. The Ghana Forestry Commission should strengthen their policies on afforestation. Afforestation strategies should be implemented or the clamp down on deforestation activities should be intensified to save the ecosystems. This is essential as our forests play a pivotal role in the rainfall pattern. Snails love forests hence; it will go a long way to attempt to restore increased snail population of snails in our forests.
5. Raw snail specimen should be treated with the universal law of safety (all specimen should be handled as potentially pathogenic).
6. A General Hygienic Practice (GHP) plan should be designed for snail vendors to reduce the various points of microbial contamination or cross contamination of the live snails. This will be critical with respect to the manner of packaging for

transportation, manner in which they are openly displayed, as well as the state of the untidy floors unto which they are frequent observed to fall.

7. A Hazard Analysis Critical Control Point (HACCP) plan should be designed and implemented for usage by commercial snail vendors.
8. Health policy makers should advise the general public on the inappropriate use of snail or snail specimens for supposed medicinal benefits.

FURTHER STUDIES

1. Full toxicology studies should be carried out on the edible land snails to outline the toxins present. This is important considering the spore-forming bacteria species such as *Bacillus cereus, Bacillus spp, Clostridium tetani, Clostridium spp,* and *Staphylococcus aureus* amongst others isolated during the study. Some of these spore-forming bacteria unidentified to the species level could be of the *perfringens, botulinum, subtilis*, etc species known to produce very fatal toxins.
2. Studies should be carried out to come up with a bacteria load standard for the snail.
3. A full chemical study was not done during the study. Nonetheless, the chemical analysis conducted revealed a very strong correlation between the pH and bacteria loads in snail specimens studied. Further studies, ought to be done to determine the possible effects of the chemistry on these specimens to be able to do a wholesome *Achatina achatina* and *Achatina fulica* comparative analysis or studies.
4. Further studies should be done on the metal (eg. Iron (Fe), copper (Cu), etc) composition of the haemolymph to determine if frequent consumption does not

pre-dispose one to metal overload disease states such as haemosiderosis and copperiedus.

5. Further studies should be carried out to determine the viral agents that may be present in the specimens.
6. Analysis should be carried out on the specimens using molecular technique as the technique is more reliable and can help obtain a profile of the microbes present in the snail. This will enable more reliable conclusions to be drawn.
7. Investigations into the medicinal values of the snail products are required. If findings are positive, it will enable pharmaceutical industries to develop products that will serve as a cure for many human ailments.

LIMITATIONS OF THE STUDY

1. Financial constraint; due to this limitation virological analysis could not be carried out.
2. Equipment inaccessibility; the absence of an electron microscope did not allow view of viral morphology as basis for inference of family of viruses present.
3. Duration; the study period was less than a year hence many studies could not be carried out.

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APPENDIX A

STAINS

A. Gram stain

This staining technique was used to differentiate Gram positive (GP) organisms from Gram negative (GN) organisms based on their different cell wall constituents. The staining reaction reveals GP as violet/purple stained cells because of the presence of a thick peptidoglycan layer in their cell wall. Gram negative organisms on the other hand show as red/pink stained cells due to the presence of a relatively thin peptidoglycan layer in the cell wall compared to Gram positive organisms.

B. Catalase test

This test is done to differentiate organisms possessing the enzyme; catalase from those that do not. Catalase converts hydrogen peroxide to water and oxygen. Oxygen use often results in the generation of two toxic molecules namely hydrogen peroxide and the free radical superoxide. Bacteria that contain catalase are usually aerobic or facultative anaerobes. A positive reaction is indicated by a continuous bubble formation when the catalase is introduced in the bacteria colonies.

C. Coagulase test

This test is used to detect the presence of the enzyme coagulase in a given bacteria isolate. The production of coagulase accelerates the formation of fibrin clot from its precursor fibrinogen. This clot may protect the bacteria from phagocytosis by walling off the

infected area and by coating the organism with a layer of fibrin. The test uses rabbit plasma that has been inoculated with staphylococcal colony. The test can be done by the slide test or the tube test method. The test is said to be positive for *Staphylococcus aureus* (when clumping is observed) and negative if no clumping is observed (confirms any other species of Staphylococcus except *Staphylococcus aureus*).

D. Spore stain

A spore is a highly resistant structure formed in response to adverse conditions by two genera of medically important Gram positive rods: Bacillus (includes the agents of anthrax) and the genus Clostridium (includes the agents of tetanus and botulism). Spore formation (sporulation) occurs when nutrients such as sources of carbon and nitrogen are depleted. The spore forms inside the cell and contains bacterial DNA, a small amount of cytoplasm, cell membrane, peptidoglycan, very little water, and most importantly a thick keratin-like coat (responsible for the remarkable resistance of the spore to heat, dehydration, radiation, and chemicals). This resistance may be mediated by dipicolinic acid, a calcium ion chelator found only in spores.

E. Ziehl-Neelson stain

This staining is done to detect the presence of acid fast bacilli. The dry weight of mycobacterium consists of as much as 40% lipid (this accounts for the unusual growth and staining characteristics). The presence of mycolic acid (a large α-branched, β-hydroxy fatty acid), wax D, and cord factor amongst others give the bacteria its peculiar features.

APPENDIX B

MICROBIOLOGICAL QUALITY OF EDIBLE LAND SNAILS FROM SELECTED MAJOR MARKETS IN GHANA.

QUESTIONNAIRE

1. Code of vendor……
2. Gender…….
3. State of environment…….. [Dirty/ Neat]
4. Reason for "3" above……
5. Educational status…….. [Primary/ JHS/SHS/Tertiary]
6. Age…….
7. Region of Ghana…….
8. Name of Market……..
9. Source of snail…….
10. *Achatina achatina* and *Achatina fulica* vendor……[Y/N]
11. Which snail species is most sold……..…….
12. Have you knowledge of any allergy or reaction upon snail consumption?

    [Y/N]…….

13. Uses of haemolymph……

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14. Uses of slime……

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15. Uses of hepatopancreas……

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16. Uses of faecal matter…….

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17. Reason for reduction in snail availability in Ghana.....

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