PHYSICOCHEMICAL, FUNCTIONAL PROPERTIES OF EDIBLE INSECTS' CHITIN AND ANTIMICROBIAL PROPERTIES OF DERIVATIVES PRODUCED THROUGH IN VITRO CHITIN DIGESTION AND FERMENTATION

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Physicochemical, Functional Properties of Edible Insects' Chitin and Antimicrobial Properties of Derivatives Produced Through In Vitro Chitin Digestion and Fermentation

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this thesis to my wife, Grace Wambui, my daughter, Michelle Karuiru, and my entire extended family for their support and encouragement through the entire journey of my studies.

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ABBREVIATIONS AND ACRONYMS

AMPs	Antimicrobial Peptides
BSF	Black Soldier Fly
COS	Chito oligosaccharides
DDA	Degree of Deacetylation
DF	Dietary Fiber
EPs	Exopolysaccharides
FBC	Fat Binding Capacity
FTIR	Fourier Transform Infrared Spectroscopy
GCMS	Gas Chromatography Mass Spectrometry
GIT	Gastrointestinal Tract
HPLC	High Performance Liquid Chromatography
ILs	Interleukins
LABs	Lactic Acid Bacteria
SCFA	Short Chain Fatty Acids
SEM	Scanning Electron Microscope
WHC	Water Holding capacity

DEFINITION OF OPERATIONAL TERMS

In vitro digestion	It refers to the sequential or static digestion model used to assess chitin breakdown without the use of human or animal subjects.
Fermentation	It is a step that follows the in vitro digestion phase to investigate how the indigestible chitin is broken down by probiotic microorganisms.
Antioxidant properties	The measurable ability of a compound to prevent the oxidation of a target molecule. In this study, antioxidant activity was assessed using DPPH radical scavenging.
Antimicrobial properties	The measurable inhibitory effects of derivatives of in vitro digestion and fermentation against gram-positive and gram-negative strains, such as Escherichia coli and Staphylococcus aureus, expressed as inhibition zone diameter in mm
Derivatives	Products obtained after subjecting chitin to in vitro digestion and fermentation
Deacetylation	This is the chemical or enzymatic process of removing acetyl groups from the acetoamido group of the chitin polymer, thereby converting chitin into chitosan

ABSTRACT

Globally and in Kenya, there is a concerted effort to promote the consumption of edible insects. However, chitin poses a challenge to the utilization of edible insects as human food, since it is not digestible by the human gut's natural enzymes. Thus, the diverse biodiversity of edible insects offers a considerable chitin resource, prompting scientific investigations into its comprehensive benefits and potential applications. Therefore, this study aimed to characterize chitin extracted from House cricket (*Acheta domesticus*), field cricket (*Gryllus bimaculatus*) and black soldier fly larvae cocoons (*Hermetica illucens*) and to evaluate its potential health-promoting properties through in vitro digestion and fermentation studies. Chitin was chemically extracted and the functional groups were determined by Fourier Transform Infrared Spectroscopy (FTIR). The surface morphology of the chitin was examined using a Scanning Electron Microscope. Solubility, emulsion capacity, water holding capacity (WHC), oil binding capacity (FBC), degree of deacetylation (DDA) and purity of the extracted chitin were also determined. The extracted chitin was then in vitro digested by enzymes, followed by fermentation using ABY 10 and ABT 5 probiotic cultures. Derivatives of the *in vitro* digestion and fermentation process were then tested for antioxidant and antimicrobial properties. Further derivatives of *in vitro* digestion and fermentation were then determined as follows: fatty acid composition was determined using Gas Chromatography Mass Spectrometry (GCMS) while chitosan, chito-oligosaccharides, antimicrobial peptides and vitamins were determined using High Performance Liquid Chromatography (HPLC), Liquid Chromatography Mass Spectrometry (LCMS) and UltraViolet- Visible Spectroscopy (UV-VIS). Data analysis was done using STATA version 12. Data were subjected to one-way ANOVA to determine the differences in functional properties among the chitin samples. Further data were subjected to three-way ANOVA to determine the effects of the chitin sample, fermentation time and sample concentration on antioxidant activity, antimicrobial activity, fatty acid composition, chitosan, chito-oligosaccharides, antimicrobial peptides and vitamin content. The extracted chitin showed the characteristic functional groups i.e., O-H stretch, C=O stretch, N-H bend, CH2 ending and CH3 deformation, C-N stretch and C-O-C stretch. Chitin extracted from *Gryllus bimaculatus* recorded the highest values in oil absorption capacity (780.14%), emulsion capacity (65.67%) and emulsion stability (65.67%) ($p<0.001$). Chitin extracted from *Acheta domesticus* was more soluble in water than the commercial chitin. The highest level of deacetylation was reported in *Hermetia illucens* chitin (66.2%), while *Acheta domesticus* chitin had the least value (47.1%) ($p<0.001$). The microstructure images showed the presence of pores and fibers in all the chitin samples. The highest antioxidant activity in chitin samples fermented using ABY 10 was observed in derivatives of *Gryllus bimaculatus*, *Acheta domesticus* and *Hermetia illucens* chitin digested in vitro and fermented for 48 hours at a concentration of 5mg/ml (61.11%, 63.88% and 61.63%), while the least antioxidant activity was observed in the negative control ($p<0.05$). Derivatives obtained after in vitro digestion and fermentation of the chitin samples exhibited significantly different antimicrobial activity against *Escherichia coli, Vibrio cholerae*, *Bacteroides fragilis*, *Enterobacter agglomerans*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus*. The antimicrobial activity of the derivatives increased with an increase in fermentation time and sample concentration, with the highest activity

being observed after 48 hours of fermentation and at a concentration of 10 mg/ml. The predominant SCFA in all the samples was acetic acid (40.86% - 64.75%), followed by propionic acid (8.76% - 17.34%), then butyric acid (7.51% - 15.33%) as well as trace levels of 4-methylvaleric acid (1.01% - 3.88%), iso- valeric acid and valeric acid. The highest values of chitosan were obtained after fermentation of *Acheta domesticus* chitin using ABY 10 and ABT 5 for 48 hours (2.91 g/100g and 2.90 g/100g) and fermentation of *Hermetia illucens* indigestible chitin using ABY 10 for 48 hours (2.90 g/100g) ($p<0.05$). *Gryllus bimaculatus* indigestible chitin fermented using ABY 10 for 48 hours and *Acheta domesticus* indigestible chitin fermented using ABY 10 and ABT 5 for 48 hours had the highest chito-oligosaccharides yield (9.82 mg/g, 9.07 mg/g and 9.37 mg/g). *Gryllus bimaculatus* indigestible chitin fermented using ABY10 for 48 hours, *Acheta domesticus* and *Hermetia illucens* indigestible chitin fermented using ABY 10 for 48 hours yielded the highest defensin like AMP (19.40 mg/100g and 18.32 mg/100g). *Gryllus bimaculatus* indigestible chitin fermented using ABY 10 for 48 hours yielded the highest vitamin B12 content (5.74 mg/100g). In conclusion, the edible insect's chitin was similar to commercial chitin in regards to the FTIR spectra as well as surface morphology. ABY 10 proved to be more efficient in fermentation while *Hermetia illucens* indigestible chitin was more fermentable as compared to all the other samples. Regarding fermentation time 48 hours was found to be optimal in producing metabolites that had highest antimicrobial activity. The study findings collectively suggest that the development of chitin based food products could promote gut health.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Chitin is a polymer formed by N-acetylglucosamine units joined by β-(1-4)-N-acetyl-d-glucosamine linkages. The main structural distinction between cellulose and chitin is the presence of a hydroxyl substituent and an additional amine group on each monomer (Robinson & Robinson, 2018). Chitin is the second most common polymer on Earth, after cellulose (Kaur & Dhillon, 2013). Chitin can be found in insects, fungus, crustaceans, and certain types of algae. The majority of the 1012–1014 tons of chitin produced annually come from crustacean shells (Dhillon et al., 2013). The culinary and medical industries make extensive use of chitin and its derivatives, including chitosan (Santhosh et al., 2006). For instance, in Japan, chitin has been approved as a functional food (Aranaz et al., 2009). People who have consumed edible insects for long periods of time have been shown to possess the enzyme however, a majority lack this enzyme (Paoletti et al., 2007). Thus, a majority of people are not able to digest chitin. However beneficial bacteria in the GIT may be able to break down the chitin as documented in studies investigating the prebiotic properties of chitin (Kipkoech, 2019).

Through an extraction technique, chitin can be extracted from the exoskeleton of edible insects or the shells of crustaceans. Either chemicals or microorganisms can be used to extract the chitin. The chemical extraction of chitin is being employed at the commercial level, however, bioextraction of chitin is gaining more popularity since it is more environmentally friendly (Kaur & Dhillon, 2013). In general, chitin content in edible insects' ranges from 5 to 15%, with variation dependent on insect species and stage of development (Spranghers et al., 2017). For instance, studies have reported that the chitin content of grasshoppers ranges from 9.79% to 14.68% (Fombong et al., 2017). Chitin is a challenge associated with the consumption of edible insects since it is not digestible and there is therefore a need to explore how best chitin can be utilized since it accounts for a significant percentage of the insect (Paoletti et al., 2007).

Studies have documented the potential prebiotic properties of chitin (Kipkoech, 2019; Kipkoech et al., 2021). The prebiotic properties of chitin suggest it has the potential to support the growth and survival of probiotic bacteria, such as lactic acid bacteria. Probiotic bacteria normally produce bioactive compounds to aid them in colonizing the gut and surviving. Some of the bioactive components produced by probiotic bacteria include bacteriocins, short chain fatty acids, exopolysaccharides, amino acids and vitamins (Indira et al., 2019). Relatedly, organic acids such as lactic acid are also released as metabolites. The organic acids demonstrate their antimicrobial properties through reducing pH (Tenea & Yepez, 2016). Bacteriocins are antimicrobial peptides classified into four classes based on molecular weight. Examples of these bacteriocins include Enterocin, Nukacin, Nisin and Pediocin, among others. The short chain fatty acids, such butyrate, have antimicrobial properties as well as provide energy to the colon cells for normal functioning (Besten et al., 2013).

The fermentation of chitin has not been fully exploited to any great length. The most recent study assessed the effects of marination and fermentation on storage characteristics of Yellow mealworm (*Tenebrio molitor*) (Borremans et al., 2018). Probiotics, which can be supported by the fermentation of prebiotic fibers like chitin, play a crucial role in modulating the immune system by enhancing the activity of macrophages, natural killer cells and the production of immunoglobulins. A balanced gut microbiota, promoted by probiotic fermentation, strengthens intestinal barrier function and reduces the risk of pathogenic infections, thereby contributing to overall host immunity (Adnan & Pramaningtyas, 2020). In order to encourage the intake of edible insects and, consequently, the nutritional status, it is necessary to investigate chitin, a polysaccharide with prebiotic potential. Thus, the purpose of this work is to examine the antibacterial and functional characteristics of chemicals derived from the in vitro fermentation and digestion of crickets and BSF cocoons.

1.2 Problem Statement

Globally and in Kenya, the consumption of edible insects is being promoted, characterized by the combined effort of local institutions and development partners

in farming and value addition of *Acheta domesticus*, *Gryllus bimaculutus,* black soldier fly, among other insects (Kinyuru, 2017). Studies have documented the use of crickets in the formulation of porridge flour for schoolchildren and baked products (Homann et al., 2017; Kipkoech, 2019). Other studies have documented the enrichment of wheat buns with termites (Kinyuru et al., 2009). However, chitin a polysaccharide obtained from marine sources, insects and fungi, is non-digestible by human gut enzymes. This indigestibility leads to specific consumption problems, including reduced nutrient bioavailability from insect-based foods and low consumer acceptance due to perceived poor digestibility. These problems are exacerbated by the fact that most people lack the chitinase enzyme that digests chitin a part from those who have consumed edible insects over a long time (Paoletti et al., 2007). Thus some researchers have recommended that chitin may need to be eliminated from insect-based foods (Jayanegara et al., 2017). However, chitin has been shown to have beneficial properties to the human body hence eliminating it from food products will deny the consumer the much needed benefits such as promotion of gut health by suppression of pathogenic microbes and promotion of beneficial bacteria. There is therefore needed to evaluate methods that may breakdown chitin in food products development hence produce products with superior health benefits. This will be in a bid to continuously support the efforts geared towards the utilization of insects as food locally and globally.

1.3 Justification

Leveraging the huge biodiversity of edible insects which accounts for about 95% of the animal kingdom, the study aims to investigate the underutilized functional potential of insect chitin (Feng et al., 2009). This work transforms insects from a protein substitute to a source of valuable functional components by concentrating on chitin, the second most prevalent biopolymer on Earth. Unlike insect proteins and lipids, which are readily digestible and have been extensively studied, chitin is a non-digestible dietary fiber with unique prebiotic, antimicrobial and immunomodulatory properties that remain largely unexplored, making it a more promising candidate for deriving bioactive compounds through gastrointestinal transformation. Further, by employing in vitro models to simulate human digestion and colonic fermentation, the

study explores how the Chitin is converted by GIT into bioactive compounds that have antioxidant and antibacterial properties. These bioactive substances improve the nutritional and health condition of insect-eating populations by suppressing harmful bacteria and promoting the growth of good microbes (Banerjee & Dhar, 2018; Kipkoech, 2019). Further, the documentation of the antimicrobial and antioxidant properties of compounds from the chitin fermentation process will synergize with the efforts that have been put into promoting the consumption of edible insects. Additionally, this study will be a point of reference for future studies on chitin value addition.

By establishing a clear link between insect consumption and improved metabolic health, this work provides the biochemical evidence necessary to overcome consumer neophobia toward the consumption of edible insects. Ultimately, these findings will facilitate the development of standardized insect-derived nutraceuticals that maximize the therapeutic potential of sustainable food systems.

1.4 Objectives

1.4.1 Main Objective

To characterize chitin extracted from various edible insects and evaluate its potential health-promoting properties through in vitro fermentation.

1.4.2 Specific Objective

1. To determine the structure and characteristics of chitin extracted from selected edible insects
2. To determine the antimicrobial and antioxidant activity of derivatives of in vitro digestion and fermentation of chitin.
3. To identify and quantify bioactive compounds obtained from in vitro digestion and fermentation of chitin.

1.5 Hypothesis

1. There is no significant difference in the structure and characteristics of chitin extracted from selected edible insects
2. Derivatives of in vitro digestion and fermentation of chitin have no antimicrobial and antioxidant activity.
3. There are no bioactive compounds obtained from in vitro digestion and fermentation of chitin.

CHAPTER TWO

LITERATURE REVIEW

2.1 Chitin

Chitin is a semi-crystalline polysaccharide and is the second most abundant biopolymer on earth. It is a long chain polymer made up of N-acetyle-D-glucosamine units linked up using β-(1→4)-linkages (Rinaudo, 2006). The polymer units are a derivative of glucose, which forms strong hydrogen bonds to create tough protective microfibrils (Muthukrishnan, 2012). This structural integrity makes chitin a crucial component of the cell walls of fungi, the exoskeleton of insects and arthropods (Elieh-Ali-Komi & Hamblin, 2017). Chitin is absent in vertebrates and plants, a characteristic that positions its metabolic pathways as promising targets for developing antifungal agents and selective pesticides (Muthukrishnan, 2012). The biocompatibility, biodegradability, and nontoxic nature have spurred extensive research into its applications in the medical sector in wound dressing as well as in food stabilization and water purification (Elieh-Ali-Komi & Hamblin, 2017).

2.2 Chitin Sources

2.2.1 Crustacean Seafood Waste

The conventional sources of chitin have been the marine ecosystem. i.e., from crustaceans' waste released by the seafood industry. About 45% of seafood waste from shrimps contains exoskeleton and cephalothoraxes. This waste contains about 20%-30% chitin, 30%-40% of valuable proteins, and 30%-50% calcium carbonate (Huq et al., 2022). This therefore implies that in order to obtain chitin, the waste has to be subjected to an extraction process, mainly a chemical process (Kumari et al., 2015). However, the exoskeleton of these marine organisms is highly mineralized, which makes the chitin extraction process quite rigorous (Huq et al., 2022). Further this chitin source is highly influenced by the seasonal availability of these marine organisms. Evidence further suggests that water pollution, as well as climate change has had a significant reduction effect in the production of these marine organisms

(Sumaila & Tai, 2020). This has further resulted to a considerable increase in the prices of chitin. Chitin sourced from seafood waste is associated with heavy and trace metal contamination, which has raised significant safety concerns in recent times (Marques et al., 2010; Zhao et al., 2016). Further there is a huge debate regarding the sustainability of crustacean farming (Gillett, 2008). These issues facing the number one source of chitin indicate the need for scientists to search for other sustainable sources of chitin (Hahn, Paul, et al., 2020a). Chitin is a multimillion dollar industry due to the documented wide application of chitin in the medical, pharmaceutical, food, agriculture, cosmetic and textile industries (Bakshia et al., 2019; Duan et al., 2019; Matica et al., 2019; Tao et al., 2019).

2.2.2 Insects

Globally, there has been a growing demand for more sustainable alternative sources of chitin. Insects have emerged as a promising and environmentally friendly source of chitin, with species such as crickets, grasshoppers, locusts and black soldier fly larvae gaining attention in recent years (Ma et al., 2022). The insect exoskeleton is particularly rich in chitin, making insects an attractive alternative to crustaceans for chitin extraction (Hahn, Paul, et al., 2020b). Recent studies have shown that the chitin content in insects can range from 5% to 20% of their dry weight, depending on the species and life stage (Spranghers et al., 2017). The advantage of insect-derived chitin lies not only in its abundance but also in the potential for more sustainable production methods, as insects can be reared on organic waste streams, contributing to a circular economy (Huis, 2013). Further edible insects have a huge biodiversity which has not been fully utilized (Dunkel, 2016). Insects seem to be a better source of chitin as compared to crustaceans since they have a high rate of fertility and reproduction. Additionally, edible insect farming infrastructure is being developed globally, which implies a consistent and sustainable source of chitin (van Huis, 2013). Evidence further suggests that the yield of chitosan a derivative of chitin from insects, is higher than that from crustaceans (Kaya, Baublys, et al., 2014).

2.2.3 Fungi

Chitin can also be sourced from microorganisms such as fungi, protists and algae. Fungi is the second most predominant source of chitin after crustaceans (Hahn, Paul, et al., 2020b). However, the utilization of these resources as a source of chitin has not been fully industrialized (Abdel-gawad et al., 2016). The mycelium of fungi such as *Mucor rouxii*, *Aspergillus niger*, *Rhizopus oryzae,* among others have been reported to contain chitin and chitosan in the cell walls (Huq et al., 2022). Further zygomyctes are a better source of chitin when compared with other classes of fungi. The synthesis of chitin in these microorganisms is done by the chitin synthase enzymes (Kappel & Mu, 2020). Majorly, the role of chitin in these microorganisms is to provide structural strength to the cell walls (Dhillon et al., 2013). The amount of chitin from fungal cell wall is dependent on the species, environmental factors, as well as age of the fungus (Elsoud & Kady, 2019). Chitin from fungi has gained significant global attention since it is not affected by season variability when compared to chitin obtained from crustaceans. Further the extraction process is rather inexpensive since it does not require the demineralization step (Dhillon et al., 2013). Additionally, the extraction of fungus chitin only requires minimal treatment with alkali and acids (F. Tao et al., 2019)

2.3 Chitin Extraction Methods

Chitin extraction methods are mainly two i.e. chemical and biological extractions. The traditional chemical method applies a sequential demineralization step using dilute HCL followed by a deproteinization step using NaOH to eliminate proteins and finally decolourization treatment (Kozma et al., 2022). However, this conventional approach is associated with high operational costs and environmental pollution (Yang et al., 2019). In response to this challenge, biological or green extraction methods is gaining popularity. The biological approach is based on microbial fermentation, which uses lactic acid bacteria to demineralize and deproteinize chitinous waste (Tan et al., 2020).

2.3.1 Chemical Extraction

The extraction process is a critical step in obtaining chitin from edible insects. The extraction procedure has a significant impact on the purity level, degree of acetylation, molecular weight, functional properties of chitin among others which further impacts on the application of chitin in various sectors. Commercially, the chemical extraction of chitin is being employed however the bio extraction of chitin is gaining huge interest (Kaur & Dhillon, 2013). Chemical extraction of chitin is usually a twostep process that involves demineralization using dilute hydrochloric acid (HCl) to remove calcium carbonate and deprotenization using an alkaline solution such as sodium hydroxide (NaOH) to eliminate proteins (Kozma et al., 2022). In most cases demineralization precedes deprotenization for the purpose of increasing surface area for deprotenization. However, the order of chitin extraction may be reversed in case the exoskeleton has high protein content (Kjartansson et al., 2006). Usually, demineralization is achieved through acidification while deprotenization is achieved by subjecting the chitin source to an alkali. The concentrations of acids and alkali used in the extraction process is usually dependent on the chitin source. Marine sources of chitin i.e. crustaceans require high acid concentration due to the high level of mineralization of these sources (Kou & Orcid, 2018; Kozma et al., 2022). The demineralization using strong acids have detrimental effects on the physico- chemical characteristics of chitin such as reduction in molecular weight, crystallinity index, degree of acetylation (DA) and viscosity (Gopi et al., 2020). The chemical extraction process is the most commonly used extraction method at the industrial level due to the low cost of reagents used and no need for specialized equipment (Yang et al., 2019). Evidence indicates that this chemical process releases acids and alkalis to the environment, which may cause environmental pollution and thus scientists are focusing on sourcing alternative methods of chitin extraction. Some of the proposed green extraction methods include the use of ionic liquids, deep eutectic solvents as well as the use of microorganisms (Bori et al., 2020; Boric et al., 2018; Morais et al., 2020).

2.3.2 Bio-Extraction

Bio extraction refers to the use of biological agents such as enzymes or microorganisms to selectively remove non-chitinous components from chitinous biomass under mild processing conditions offering an environmentally friendly alternative to chemical extraction (Sixto-Baraccal et al., 2023). Biological extraction methods of chitin are divided into enzymatic and fermentation methods (Kozma et al., 2022). Biological extraction methods normally yield chitin with high molecular weight and low degree of deacetylation, which exhibits superior rheological properties and mechanical integrity. Further bio extraction method does not fully demineralize and deprotenize the chitin which affects the purity of chitin (Jung et al., 2005; Kou & Orcid, 2018).

The enzymatic method normally uses proteinases that remove the proteins and isolate the chitin. This process normally yields chitin with low levels of deacetylation, and it also causes minimal damage to the chitin chain (Hongkulsup et al., 2016). Usually, the enzymatic method is optimum within a period of 2-8 hours (Mohan, Ganesan, Ezhilarasi, Kondamaraddey, et al., 2022). Evidence indicates that even with longer reaction times, the levels of deprotenization cannot exceed 90%. The enzymatic extraction method is significantly hampered by the high cost of proteinases and thus industrial enzymatic extraction may be very expensive (Hongkulsup et al., 2016; Kou & Orcid, 2018). On the other hand, the supernatant obtained from the enzymatic process normally have amino acids which makes this a nutritional resource and in the process, minimal waste is generated (Hamdi et al., 2017). It is worth noting that enzymatic extraction does not incorporate the demineralization step.

Fermentation extraction method normally employs bacterial cultures i.e. lactic acid and protease producing bacteria. The demineralization process is usually achieved by the lactic acid produced by the bacteria, while deprotenization is achieved by the protease enzyme produced by the protease producing bacteria (Mohan, Ganesan, Ezhilarasi, Kondamaraddey, et al., 2022; Morais et al., 2020). Some lactic acid producing bacteria that possesses some deprotenization capabilities can sufficiently extract chitin (Sedaghat et al., 2017). The fermentation process requires media to

enhance the growth of the bacteria. The media acts as the carbon source that is converted to acid (Cahyaningtyas et al., 2022). Most of the commercial culture media are very expensive and thus industrial fermentation may be costly. In order to lower the cost of fermentation, studies have experimented the use of waste peels as a carbon source. A study by Tan et al., (2020) reported that pulp from red grapes was a considerably good carbon source for chitin extraction, yielding chitin with low mineral and protein content. Additionally, the fermentation extraction of chitin generates a supernatant with valuable amino acids, making it a nutritional resource and consequently reducing waste generation by the extraction process (Cahyaningtyas et al., 2022).

2.4 Chitin Characteristics

2.4.1 Chitin Structure

Chitin is a polysaccharide structural component found in the exoskeleton of insects and other arthropods as well as from the cell wall of fungi. Chitin is made up of β-(1,4)-linked-2-acetamido-2-deoxy-D-glucopyranose units (Rudall, 1963). The crystalline forms of chitin are namely α, β and γ, α crystalline form is the most abundant of the three forms in nature. Furthermore, the α crystalline is the most stable among the three crystalline forms of chitin (Gardner & Blackwell, 1975). α-Chitin is characterized by antiparallel polymer chains that form extensive hydrogen-bonding networks, resulting in high mechanical strength, low solubility and superior thermal stability. In contrast, β-chitin features parallel polymer chains with weaker intermolecular interactions, leading to greater swelling capacity, higher reactivity and enhanced solubility, which makes it more suitable for certain biomedical applications such as drug delivery and wound dressings (Jang et al., 2004). Annually, about 1012 to 1014 tons of chitin are produced with the highest amounts being obtained from marine sources (Dhillon et al., 2013). Chitin is normally deacetylated to chitosan for commercial purposes. Chitin and chitosan has an economic value due to their versatile and biological properties, which makes them suitable for utilization in food, pharmaceutical, medical, agricultural and environmental sectors (Kaur & Dhillon, 2013). The insect exoskeleton is composed of chitin, minerals and proteins. The

minerals normally provide strength while the proteins give life to the exoskeleton. Chitin extraction therefore requires the removal of proteins and minerals (Kaur & Dhillon, 2013).

Insect species, growth stage, and gender all affect the surface shape of chitin. According to a study, the surface morphology of chitin extracted from black soldier fly larvae was complex with repeating units that resembled a honey comb, whereas the surface of chitin extracted from adult black soldier fly was smooth and composed of fibers distributed in a parallel pattern. Additionally, it was observed that the chitin of black army flies was porous (Wa et al., 2016). On the same note, the chitin that was isolated from adult and nymph grasshoppers has large nanopores and lengthy nanofibers.

The α crystal form of chitin displays bands at 1650 cm-1, 1620 cm-1 and 1550 cm-1 which correspond to C=O secondary amide stretch, N-H bend and C-N stretch, respectively (Wa et al., 2016). Bands may also appear at 1540 wave number and this indicates the level of chitin contamination with proteins. Other bands also occur at 3400 cm-1, 2920 cm-1, 1377 cm-1, 1309 cm-1 and 1152 cm-1 which relate to O-H stretching, asymmetric C-H stretching, CH3 asymmetric deformation, C-N vibration from amides and C-O-C asymmetric stretching (Gonil & Sajomsang, 2012). The crystallinity index of chitin from insects' ranges between 40%- 80% and it differs based on insect species, stage of development, sex and chitin extraction procedure (Kaya, Erdogan, et al., 2015). The crystallinity index of chitin influences the functional properties of chitin. For instance, low crystalline chitin has good sorptive properties. Additionally, chitin with a low crystalline index has better demineralization capacity (Aranaz et al., 2012).

2.4.2 Chemical Characteristics

The carbon, hydrogen and nitrogen content of chitin aids in computing the degree of acetylation of chitin. The amount of nitrogen in the chitin indicates the presence of leftover proteins. Additionally, a 100% degree of acetylation indicates the purity of chitin (Gonil & Sajomsang, 2012). Similarly, a degree of acetylation more than 100% indicates the presence of residual minerals in the chitin structure, which can be

explained by the demineralization process's efficiency. Both the insect's developmental stage and the extraction method employed have a significant impact on the purity of chitin. In contrast to chitin derived from adults, research has shown that chitin recovered from larvae and nymphs is purer (Wa et al., 2016).

2.4.3 Functional Properties

The functional properties of chitin and chitosan are highly influenced by the degree of deacetylation and the molecular weight (Trung et al., 2006). For instance, a higher degree of deacetylation (above 70-80%) results in a greater number of free amino groups, which enhances the functionality of the polymer, such as antimicrobial activity and metal ion chelation capacity. A lower degree of deacetylation (below 50-60%) yields a more hydrophobic, less soluble material with reduced bioactivity and lower affinity for biological interactions (Dutta et al., 2020). The concentration and position of the glucosamine influence charge distribution in chitin. The charge distribution in the chitin molecule also influences functional properties such as water-holding capacity, fat-absorption capacity and permeability of chitin membranes (Thein-Han & Stevens, 2004).

2.4.3.1 Solubility

Solubility is one of the factors that determines the quality of chitin and chitosan. Factors that influence chitin solubility include degree of deacetylation, temperature, the concentration of alkali used in deacetylation, and particle size, among others (Hossain & Iqbal, 2014). Chitin's crystalline structure with strong hydrogen and cohesive forces results to a highly aggregated three dimensional network which makes chitin insoluble in many conventional solvents. Chitin is usually insoluble in water, organic solvents, dilute acids and even alkalis (Kumari & Kishor, 2020). Additional data indicate that the solubility and solution characteristics of chitin are significantly influenced by the percentage of N-acetyl-d-glucosamine units in the chitin structure (Roy et al., 2017). Evidence suggests that the solubility of shrimp chitosan ranges from 48.3% to 97.65%, while fungal, insect and crab chitosan is approximately 98.9%, 60%-92% and 40%-85% respectively (Hahn, Paul, et al., 2020b; Hossain & Iqbal, 2014). The solubility of chitin and chitosan affects how they

are used as food processing ingredients. Compared to chitosan, which is easily soluble in diluted acidic solutions with a pH below 6, chitin is less soluble in organic solvents (Pillai et al., 2009). It has been shown that the water-insoluble chitin and chitosan exhibit antibacterial action against yeast and bacteria in food emulsions (Aranaz et al., 2012). This property is highly valuable to the food industry as it enables the use of chitin and chitosan as natural, non-toxic food preservatives that can extend the shelf life of emulsified products.

2.4.3.2 Water Holding Capacity

Studies have documented that low molecular weight chitin has a higher water holding capacity (Trung et al., 2006). The extraction process of chitin which involves demineralization and deproteinization, also significantly influences the water holding capacity of chitin (Hossain & Iqbal, 2014). Evidence indicates that the water holding capacity of chitin ranges between 381% to 673%, while that of chitosan ranges between 537.29% to 1150% (Kumari & Kishor, 2020; Rout, 2001; Trung et al., 2006). The differences in water holding capacity between chitin and chitosan are attributed to differences in crystallinity, number of salt forming groups and possibly residual proteins in the extract (Knorr, 1982). Water holding capacity is a key parameter in food processing as it influences sensory characteristics of food such as texture and mouth feel (Aremu et al., 2008). Chitin has therefore the potential of being utilized as an ingredient in the food industry.

2.4.3.3 Oil Binding Capacity

The fat binding capabilities of compounds are critical in food processing, especially in emulsion products, as well as in binding flavor compounds and vitamins. Evidence suggests that the fat binding capacity of chitin ranges between 316% to 320% (Kumari & Kishor, 2020). The sequences of steps involved in chitin extraction have been shown to affect the fat binding capacity of chitin. The fat binding capacity of chitin can be considerably affected by the differences in chitin particle sizes (Sampath, Ngasotter, Layana, Balange, et al., 2022). Evidence further indicates that chitin exhibits high fat binding capacity when demineralization is done prior to deproteinization and low fat binding capacity when deproteinization is done first

(Rout, 2001). Furthermore, pH has a significant influence on fat absorption capacity; chitin tends to absorb more oil at acidic pH (Elanchezhiyan et al., 2014). The fat binding capacity of chitin is of great advantage to health, for instance the binding of dietary fat and cholesterol by chitin and chitosan reduces the risks of chronic diseases such as coronary heart disease (CHD) (European Food Safety Authority, 2011; Mhurchu et al., 2004).

2.4.3.4 Emulsion Capacity

Emulsions are of significant interest due to their wide utilization in the food, pharmaceutical and cosmetic industries (Vignati et al., 2003). Chitin obtained from edible crickets has been documented to have good emulsification ability. Chitin has an emulsion capacity of 99%. Further evidence shows that chemical treatment of chitin, preferably with acids, results in an increase in emulsion capacities (Sampath, Ngasotter, Layana, Balange, et al., 2022). The chitin from edible insects is able to stabilize smaller oil droplets as compared to soy protein isolates (Hirsch, 2018). Furthermore, the creaming stability index of cricket chitin stabilized emulsions is slow and constant, which indicates good emulsion stability (Hirsch, 2018). The emulsion capacity of chitin is highly influenced by particle size; large particle size chitin has less emulsion capacity due to long adsorption time and inability to stabilize oil droplets with large interfacial curvature (Hirsch, 2018). Furthermore, chitin promotes the quality of food emulsions such as mayoinnase through its antimicrobial properties against bacteria and yeast in the liquid phase (Aranaz et al., 2012; Zhang et al., 2004).

2.5 Digestion of Chitin

Human genes that code for the expression of chitinase enzyme are conserved, they are expressed in diseased conditions (Paoletti et al., 2007). For instance, patients with Gaucher disease and acute malaria have been found to have elevated levels of chitotriosidase an enzyme that can hydrolyze chitin (Eijk et al., 2005; Malaguarnera et al., 2003). Further evidence indicates that chitotriosidase may also be involved in the innate immune response. Expression of Chia gene in the stomach, which is associated with chitin digestibility, is strongly influenced by feeding patterns (Tabata

et al., 2018). Further, individuals living in tropical areas who have been consuming insects for a long time have demonstrated high levels of acidic mammalian chitinase activity. The high activity of the chitinase enzyme is an adaptive response to feeding habits as well as increased resistance to parasitic infections, which are very common in the tropics (Paoletti et al., 2007). The chitinase enzymes will normally degrade chitin into N-acetyl-d-glucosamine units, which can be degraded further to other derivatives in the lower parts of the GIT (Ohno et al., 2016; Tabata, Kashimura, Wakita, Ohno, & Sakaguchi, 2017; Tabata, Kashimura, Wakita, Ohno, Sakaguchi, et al., 2017).

2.6 Fermentation

Chitin has been reported to have prebiotic properties. Based on a study, chitin was demonstrated to promote the growth of probiotic bacteria and consequently modify the gut microflora (Buruiana et al., 2017). Furthermore, in vitro studies have shown that chitin promotes the growth of *Lactobacillus fermentum*, *Lactobacillus acidophilus* and *Bifidobacteria adolescentis* (Edo et al., 2025). The beneficial bacteria are thus able to break down the chitin and release metabolites such as organic acids which suppress the growth of pathogenic bacteria (Kipkoech, 2019). Additionally, the deacetylated form of chitin has been reported to support the growth of probiotic microorganisms. The deacetylation of chitin is done by the use of chemicals and enzymes (Sixto-Baraccal et al., 2023). However, the enzymatic deacetylation of chitin is more advantageous in terms of utilization of chitosan in the food industry (Lee et al., 2002). Research has shown that chitosan exhibits bifidogenic qualities at 0.1% and 0.5% concentrations. Furthermore, it has been shown that chitosan, at a concentration of 0.1%, promotes the growth of Lactobacillus brevis and Lactobacillus casei (Lee et al., 2002). Furthermore, it has been shown that chitin and chitosan encourage the growth of more Lactobacillus species than fructo-oligosaccharide, one of the new prebiotics (Lee et al., 2002).

The probiotics suppress the growth of pathogenic bacteria and thus facilitate smooth functioning of various metabolic activities in the intestines through the generation of carbohydrates, vitamins, proteins and enzymes (Indira et al., 2019). The probiotic

bacteria benefit the host by increasing the resistance to colonization by other bacteria. Additionally, the bacteriocins produced by the probiotics fight the pathogenic bacteria and equally produce imunomodulators which regulate the host immune system (Jandhyala et al., 2015).

2.6.1 Derivatives of Chitin Fermentation

Fermentation of chitin and its hydrolysates i.e. N-acetylglucosamine and glucosamine has emerged as a sustainable biotechnological platform for producing a diverse range of bioactive compounds. Some of these compounds include chitosan, chitin oligosaccharides, short chain fatty acids, antimicrobial peptides and vitamins (Windels et al., 2025). These bioactive compounds have antioxidant, antimicrobial and immunomodulatory abilities, which has resulted in the utilization of chitin derivatives in medical, food, pharmaceutical and agricultural sectors (Parchen, 2025).

2.6.1.1 Chitosan

Chitosan and chitosan oligosaccharides are the most important deacetylated products of chitin. Chitosan is obtained from the deacetylation of chitin either chemically or biologically. The chemical process of chitosan synthesis involves the treatment of chitin with 40% to 60% potassium hydroxide or sodium hydroxide (No et al., 1989). The biological process of chitosan synthesis is achieved through enzymatic and fermentation processes (Kou et al., 2020). The enzymatic process uses enzymes such as carbohydrate esterases, which can deacetylate chitin. These enzymes are usually sourced from microorganisms (Lambertus et al., 2020). Chitosan is also produced through fermentation, which can either employ lactic acid producing bacterias such as *Streptococcus* strains as well as non-lactic acid producing bacteria (Doan et al., 2019; Kou et al., 2020). However lactic acid fermentation is the most widely used approach (Arbia et al., 2013). Usually, in the fermentation process, chitin acts as a carbon source to the bacteria and further, the bacteria produce enzymes that are able to degrade chitin to chitosan. Chitosan produced through fermentation has been documented to have higher Molecular weight (Kou et al., 2020). Evidently, then, chitosan has prebiotic potential and may thus enhance the growth of gut microflora. The assessment of the prebiotic potential of chitosan in vivo and in vitro is

imperative (Guan, 2022). Chitosan, just like chitin, has been documented to be nontoxic and may have biological properties such as antimicrobial capabilities (Rabea et al., 2003; Sinha et al., 2004). Chitin with a higher degree of deacetylation and lower molecular weight exhibits better biological effects as well as enhanced solubility (Kou et al., 2020; Younes & Rinaudo, 2015).

2.6.1.2 Chitosan Oligosaccharides

Chitosan oligosaccharides (COS) are usually derived from the degradation of chitosan (Thadathil & Velappan, 2014). COS have considerably better biological activity due to their high solubility in water as well as low toxicity (Muanprasat & Chatsudthipong, 2017). For instance, unlike chitosan, COS are more water soluble, easy absorption, and better cell membrane permeability, which makes COS a considerably valuable derivative of chitin degradation (Liaqat & Eltem, 2018). COS have been documented to have antimicrobial, antitumor, anti-inflammatory, anti-diabetic properties among other functional properties (Liaqat & Eltem, 2018; Yousef et al., 2012). These functional properties of COS make it suitable for utilization in the food and pharmaceutical sectors (Berger et al., 2004; Du et al., 2009). Thus, COS has gained significant attention among scientists globally. Evidence further suggests that COS have prebiotic potential and thus may have an impact on gut health (C. Zhang et al., 2018). For instance, dietary supplementation with COS was found to enhance the growth of *Bifidobacterium* spp and *Lactobacillus* spp while at the same time limiting the growth of pathogenic bacteria such as *Escherichia coli* (Yang et al., 2012). A few studies have assessed the effects of COS on gut microbiota in vitro, and thus there is a need to assess the impacts of COS on gut microflora as well as pathogenic bacteria in vitro (Zhang et al., 2018).

2.6.1.3 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are molecules that are secreted by diverse microbes, plants and animals (Wang, 2014; Wang et al., 2016). Usually, the AMPs are usually part of the first line of host defense across all species (Ostaff et al., 2013). The AMPS are further classified based on their amino acid composition and amphiphilic nature (Patel & Akhtar, 2017). Alpha helical AMPs include pleurocidin, cecropin,

magainin, mellitin and moricin, proline rich AMPs include apidaecin, drosocin and lebocin, while cysteine rich AMPs include defensin and drosomycin (Yi et al., 2015). AMPS impact their antimicrobial properties by interacting with microbial bilayer, thus forming lipid-AMP complexes which manipulate ion channels and consequently affect the microbial cell functionality (Sharma et al., 2016). Evidence further suggests that the AMPs prevent cell wall synthesis block nucleic acid synthesis and prevent protein synthesis and enzyme activity (L. Zhang & Gallo, 2016).

Bacteriocins are ribosomally synthesized AMPS produced by bacteria and recent research has demonstrated that during fermentation polymers such as chitin serve as effective prebiotic substrates that enhance the growth, metabolic activity and bacteriocin production of these beneficial microorganisms (Iyapparaj et al., 2013). Bacteriocins are low molecular weight compounds of about 20-60 amino acid residues that have the ability to kill other bacteria (Soumya et al., 2012). Both Gram-positive and Gram-negative bacteria produce bacteriocins. The beneficial bacteria in the gut, or rather the probiotics, produce bacteriocins that prevent pathogenic bacteria from colonizing the gut (Indira et al., 2019). Bacteriocins are classified into four classes; class I bacteriocins are called lantibiotics and are usually small peptides of <5 kDA. The most studied bacteriocins of class I are Enterocin W, Nukacin ISK, Nisin Q and Nisin Z (Sawa et al., 2012). Class II bacteriocins are more heat stable and they have a molecular weight of < 10 kDA. Some of the bacteriocins in this class include: - pediocin PA and Sakacin P, which have been documented to have antimicrobial activity against Listeria. Class III bacteriocins are large peptides with a molecular weight of about 430 Kda. Bacteriocins in this class include Acidofilicin A, Lactacins A, lactacins B and Helveticins J and W. The class IV bacteriocins are normally large complexes comprising of modified peptides with carbohydrate or lipid components (Tenea & Yepez, 2016).

2.6.1.4 Short Chain Fatty Acids

According to Leblanc et al. (2017), probiotic bacteria often produce short-chain fatty acids such as acetate, propionate, and butyrate by fermenting undigested polysaccharides, such as chitin. The final byproducts of fermentation are these short-

chain fatty acids. Because of their known antibacterial properties, short-chain fatty acids shield the gut from microbial infections (Ciarlo et al., 2016). The colon cells use short-chain fatty acids as an energy source, which guarantees the colonocytes' regular operation. Butyrate is one of the short-chain fatty acids that contributes between 60 and 70 percent of energy generation (Besten et al., 2013). Furthermore, butyrate has been shown to have therapeutic benefits for conditions like inflammatory bowel disease, colon cancer, heart disease, and diarrhea brought on by antibiotics (Tominaga et al., 2018).

2.6.1.5 Exopolysaccharides

Fermentation using probiotic bacteria produces enzymes such as glycosyltransferases and glycantransferases which normally convert sugar nucleotide precursors into exopolysaccharides (EPs). The exopolysaccharides have gained attention due to their positive impacts on health (Ates, 2015). Exopolysaccharides produced by lactic acid bacteria (LABs) have been documented to have antitumor, antioxidant activity, immunostimulatory potential, and ability to lower blood cholesterol levels (Tsai et al., 2014). Based on a study exopolysaccharides were documented to have an immune modulatory effect and antiulcer activity (Julendra et al., 2017). Additionally, other studies have documented the good emulsification capacity of the exopolysaccharides and thus are suitable for utilization in the food industry.

2.6.1.6 Amino Acids

The fermentation of chitin by probiotics results in the production of amino acids by the probiotic bacteria. The amino acids produced serve as precursors for the production of short chain fatty acids (Feng et al., 2018). The amino acids are fermented by probiotic bacteria to release phenols and indole. Furthermore, the amino acids aid in maintaining energy balance and preventing pathogenic infection of the gut (Tenea & Yepez, 2016). The amino acids normally undergo bioconversion to form organic acids, esters, alcohols and aldehydes that influence flavor and aroma in foods (Tenea & Yepez, 2016). The organic acids also affect pH, which prevents the growth of pathogenic bacteria.

2.6.1.7 Vitamins

During fermentation, probiotic bacteria are able to use carbon sources such as chitin to yield B-group vitamins. In an in vivo system, these vitamins are absorbed in the intestines and fat soluble vitamins which are absorbed with the aid of lipids (Tenea & Yepez, 2016). The B group vitamins are highly involved in the metabolism of macronutrients. Furthermore, riboflavin has been documented to aid in absorption of iron which helps in formation of red blood cells (Leblanc et al., 2017). Relatedly, vitamin K aids in enhancing the colonization of the gut by beneficial bacteria which further suppresses the pathogenic bacteria through competition (Cani, 2018).

2.6.1.8 Organic Acids

The antimicrobial effect of probiotics is highly related to the production of organic acids such as lactic acid, acetic acid, among others which influences the pH. The organic acids are generated from the bioconversion of carbon sources such as carbohydrates, chitin, proteins lipids among others during fermentation. Studies have documented high antimicrobial activity against Salmonella species at pH 3 which reduces with an increase in pH, as demonstrated with no antimicrobial activity at pH 7 (Tenea & Yepez, 2016). Similarly based on a study conducted in Kenya lactic acid bacteria were able to suppress the growth of *Salmonella typhi* through the production of organic acids which lowered the pH to about 3.6 (Kipkoech, 2019). Equally other studies have documented the limitation of nutrients and a low pH as key inhibitory factors to the survival and growth of pathogenic bacteria in the gut. Relatedly the reduction in pH promotes the growth of probiotic bacteria which outcompetes the pathogenic bacteria and as a result diminishes their rates of survival hence a suitable strategy by probiotic bacteria to suppress the growth of pathogenic bacteria (Blaut, 2002).

2.7 Biological Properties of Bioactive Compounds

2.7.1 Antimicrobial Activity

According to Kalaivani et al. (2018), chitosan and chitin have been shown to have antibacterial action against a variety of microorganisms, including bacteria, yeast, fungi and algae. The interaction of the polymer's positively charged amino groups with the negatively charged microbial cell membranes is often what give chitosan and its derivatives, including chito oligosaccharides, their antibacterial properties (Ishraque et al., 2020). Research indicates that a polymer's antibacterial effectiveness increases with the number of positively charged amino groups it contains. Furthermore, the degree of acetylation and the molecular weight of the polymer both affect the antibacterial activity of chitosan and its derivatives. The antimicrobial properties of chitosan and chitin have resulted in its utilization in making biomaterials used in wound dressing (Mcclure, 2004). Further, chitin nano particles exhibited considerably high antimicrobial activity against pathogenic bacteria such as Listeria monocytogenes and Escherichia coli (Qin et al., 2016). The antimicrobial activity of chitosan has resulted in its wide application in the food industry as a preservative, in the cosmetic and medical industry (Ishraque et al., 2020).

Chitosan oligosaccharide (COS) a derivative of chitosan degradation, has been documented to exhibit biological properties including antimicrobial properties. Evidence further suggests that COS may have considerably higher antimicrobial activity than chitosan, due to its higher solubility and lower viscosity (Sousa et al., 2021). The antimicrobial property of COS has resulted in its utilization in nanotechnology with the aim of dealing with microbial resistance as well as tumor imaging and therapy (Bharathiraja et al., 2018; Mei et al., 2020). Evidence indicates that COS may exert its antimicrobial activity by perturbing ion transport in microorganisms (Xing et al., 2018; Yin et al., 2020).

The lipopolysaccharide produced by the probiotic bacteria normally forms a protective layer that enhances the colonization of the gut with beneficial bacteria and prevents colonization by pathogenic bacteria. Additionally, quorum-sensing molecules produced after activation of cells by probiotic bacteria activate antigen-

presenting cells which further activate naïve T cells. The T cells further activate B cells and promote the production of ig A which aids in eliminating pathogenic bacteria (Indira et al., 2019). Furthermore, the antimicrobial peptides i.e. bacteriocins produced by probiotic bacteria kill pathogenic bacteria in the gut. Studies have documented that bioactive compounds obtained from cricket chitin fermentation process significantly reduce the growth of *Salmonella typhi* (Kipkoech, 2019). However, further characterization of the bioactive compounds produced during chitin fermentation with lactic acid bacteria is needed. Relatedly lactic acid produced by LABs has been documented to strain the survival of Salmonella species through reducing pH levels (Tenea & Yepez, 2016). Additionally, the vitamins produced are essential, and for instance vitamin K aids the colonization of the gut with beneficial bacteria. The short chain fatty acid triggers the production of interleukins that negate inflammation. Butyrate aids the differentiation of T cells and also provides an anaerobic environment through its metabolism through β- oxidation process (Cani, 2018).

2.7.2 Antioxidant Activity

Chitosan oligosaccharides can be utilized as natural antioxidants due to their strong antioxidant activity (Laokuldilok et al., 2017; Yang et al., 2016). The antioxidant capacity of COS is influenced by its molecular weight, degree of deacetylation and the source material. In recent years, the food and medical sector has focused a lot of attention on the field of antioxidants since they help to deal with oxidative stress (Lan et al., 2019). Oxidative stress has detrimental effects on health for instance it results in a reduction in nutrient absorption, reduces metabolism, suppresses growth, as well as triggers other health disorders such as inflammatory diseases, cancer and even diabetes (Duan et al., 2014; Zhu et al., 2014). Chitosan a derivative of chitin degradation, has also been shown to have antioxidant activity however there is limited research that has explored these properties of chitosan (El-hack et al., 2020). The radical scavenging ability of chitosan is associated with the amino and two hydroxyl groups in the chitosan monomers (Castro et al., 2019; Wei et al., 2019).

2.7.3 Immunomodulation

The probiotics in the gut induce the production of immunomodulatory compounds. The probiotics interact with the gut and initiate a host immune response through production of immunomodulatory compounds such as cytokines, lymphocytes, interleukins among others (Indira et al., 2019). Furthermore, the probiotic bacteria control the expression of inflammatory genes, thus resulting to production of interleukins (IL) that regulate the production of immune cells (Plaza-Diaz et al., 2014). For instance, based on a study pathogenic microorganisms were inhibited from colonizing the host through production of igA antibodies in mucosal sites via IL-10 induction process (Kawashima et al., 2018).

2.8 Research Gaps

Many of the studies have only investigated the derivatives of chitin obtained through chemical processes, however very limited number of studies have explored the derivatives of chitin from a biological process, such as fermentation. Further very few studies have assessed the possible effects of chitin on gut health. Many studies have tested only the antimicrobial activity of chitosan or chitosan oligosaccharides; however, limited studies have assessed the antimicrobial activity of derivatives of chitin digestion and fermentation. Further, a few studies have assessed the fermentability of powders obtained from crickets and silkworm however a limited number of studies have subjected chitin to in-vitro digestion followed by in-vitro fermentation and tested the biological activity of the metabolites of these processes. This thus underscores the need to incorporate chitin fermenting probiotic microbes in food products which in turn ferment chitin in the gut, hence conferring gut health and other benefits.

CHAPTER THREE

FUNCTIONAL AND MICROSTRUCTURAL CHARACTERISTICS OF CHITIN EXTRACTED FROM EDIBLE INSECTS

3.1 Introduction

N-acetylglucosamine units joined by β-(1-4)-N-acetyl-d-glucosamine linkages form the polymer known as chitin. The presence of an extra amine group and a hydroxyl substituent on each monomer is the only structural difference between chitin and cellulose (Robinson & Robinson, 2018). According to numerous studies, this polymer, which is the second most abundant on Earth, has the potential to be used in a variety of fields in the future (Philibert et al., 2017). Chitin has recently gained attention due to its beneficial biological properties such as adsorption, emulsion capabilities, non-toxigenicity, non-antigenicity and the ability to chelate metal ions in the food sector (Shahidi & Abuzaytoun, 2005). Chitin can be utilized in water treatment to remove dyes and microbial agents (Zubair et al., 2020). It is worth noting that chitin has anti-inflammatory and anticancer effects (Azuma et al., 2015). The health benefits of chitin have led to an increase in demand for it and diversification in its utilization (Robinson & Robinson, 2018).

For a long time, chitin has been sourced from crustaceans, mainly from shrimps and lobsters. However, the utilization of chitin obtained from shrimps and lobsters has been hampered by the variability in quality caused by the vigorous extraction and deacetylation processes (Nwe et al., 2002; Tajdini et al., 2010). Furthermore, overfishing has significantly reduced the number of crustaceans and thus, dependency on crustaceans for chitin may in the future become unsustainable. Scientists are now focusing on alternative sources of chitin to expand the chitin supply base (Liu et al., 2012a).

Scientists have concluded that apart from being a source of macro and micronutrients, edible insects such as crickets are a promising and sustainable source of chitin (Crognale et al., 2022). In comparison to crustaceans, which are the conventional sources of chitin, edible insects are not affected by seasonality and they

can be easily mass reared owing to their short life cycle and their high reproductive rate (Triunfo et al., 2022). Furthermore, industrial insect rearing facilities are being established globally to meet the growing demand. Given the projected expansion of the insect sector in the near future, byproducts of insect farming such as *Hemetia illucens* pupae exuviae could constitute a significant supply of chitin for industrial application (Greven et al., 2019; Wang et al., 2020). Studies have shown that reared insects have significant amounts of chitin. For instance, house cricket has a chitin content of 4.3% - 7.1% (Ibitoye et al., 2019) while Black soldier fly (BSF) has a content of 8% - 23% (Soetemans et al., 2020) which is comparable to 20% obtained from shrimps which are the conventional sources (Benhabiles et al., 2012a; Finke, 2007). Furthermore, edible insects are a viable source of chitin since they have a huge biodiversity estimated to be at 95% of the animal kingdom (Liu et al., 2012a). Studies have also reported that chitin from edible insects has lower amounts of inorganic matter which eases the demineralization process (Hirsch et al., 2019). Given these facts, the suitability of edible insect chitin in industrial applications needs to be studied.

The successful utilization of chitin in the food, agricultural, pharmaceutical and other sectors is highly dependent on the physicochemical properties of chitin which normally vary based on the origin and the method used in extraction (Aranaz et al., 2012). However, the extraction methods and physicochemical qualities of edible insects have received little attention until very recently. Chitin stabilized emulsions are suitable for food processing (Tzoumaki et al., 2013). Studies have recommended further studies on the functional properties of chitin obtained from crickets as well as from other insects. Therefore, the present study aimed at investigating the functional properties of chitin obtained from selected edible insects.

3.2 Materials and Methods

3.2.1 Chitin Sourcing and Extraction

Approximately 250 grams of shrimp chitin (commercial chitin) was sourced from the market. Adult *Acheta domesticus*, *Gryllus bimaculatus* and *Hermetia illucens* pupal exuviae were harvested from JKUAT insect farm. The harvested insects were freeze

dried and ground using a stainless steel blender to obtain insect powder. Chitin was extracted according to a method described by Charoenvuttitham et al., 2006 and Toan, (2009). About 150 grams of ground insect powder and pupal exuviae was placed in 1000 mL of 0.5M boiling sodium hydroxide and the solution was boiled for 2 hours in a 3000 mL conical flask. The contents of the conical flask were then cooled for 30 min followed by centrifugation at 45 × g for 10 min. The supernatant was discarded and the residue was added into a conical flask containing 600 mL of 1% hydrochloric acid (Trung et al., 2006). The contents in the flask were then placed in a rotor shaker rotating at 40 rpm for 24 hours at room temperature. The contents of the conical flask were then centrifuged at 45 × g for 10 min. The supernatant was discarded and the residue was treated with 500 mL of 0.5M boiling sodium hydroxide for one hour in a conical flask (Puvvada et al., 2012). The contents of the conical flask were centrifuged and the supernatant was discarded. The residue was washed three times using boiling distilled water then dried at 70°C for 12 hours and stored in ziplock bags at room temperature.

3.2.2 Determination of Chitin Functional Groups

Chitin functional groups were determined according to a method by Sajomsang and Gonil, (2010). Approximately 0.1 to 1.0% insect chitin was mixed into 250mg fine pre-dried potassium bromide powder which is an alkali halide and then finely pulverized rapidly and put into a pellet press die. A manual hand press was then used for 3 min to form translucent pellets. The translucent pellet was then placed in sample compartment of the FTIR spectrometer, (Model Alpha; make Bruker, Germany) before allowing a beam of spectrum from the IR source to pass through. The molecular components and changes in molecular structure were identified by studying the infrared spectrum. The absorbance was evaluated between 800 and 3600 cm-1.

3.2.3 Determination of Chitin Solubility

One gram of the extracted insect chitin was dissolved in 10 mL of distilled water. The solution was then poured into centrifuge tubes and was kept in a shaker for 10 minutes. The solution was then centrifuged at 716 × g for 10 min using Beckman

CS-6 centrifuge. The chitin quantity in the supernatant was determined by drying the supernatant followed by gravimetric measurement. The solubility of chitin was then determined using the following equation:

$$\text{Chitin solubility (\%)} = \left(\frac{Weight\ of\ chitin\ in\ the\ supernatant}{Weight\ of\ the\ dissolved\ chitin}\right) \times 100 \quad (1)$$

3.2.4 Determination of Chitin Emulsion Capacity

Emulsion capacity was determined according to a method by Naczk et al. (1986). This was determined by mixing 1 gram of chitin with 100 mL of distilled water. The suspension was homogenized for 10 min. At the 5th min, corn oil was added continuously while stirring, and then the emulsion was centrifuged at 402 × g for 10 min. The volume of the emulsified layer was then measured.

$$\text{Emulsion capacity (EC) (\%)} = \left(\frac{Volume\ of\ emulsified\ layer}{Volume\ of\ the\ suspension}\right) \times 100 \quad (2)$$

3.2.5 Determination of Chitin Emulsion Stability

Emulsion stability was determined according to a method by Naczk et al. (1986) whereby 1 gram of chitin was mixed with 100 mL of distilled water. The suspension was homogenized for 10 min. At the 5th min corn oil was added continuously and stirred. The emulsion was heated at 85°C for 30 min then cooled back to room temperature. The emulsion was centrifuged at 402 × g for 10 min and the volume of the emulsified layer was measured.

Emulsion Stability (ES) (%) = (*volume of emulsion layer/ volume of suspension*) × 100. (3)

3.2.6 Determination of Chitin Water Holding Capacity

Water holding capacity (WHC) was determined according to AACC, (AACC, 2000). About 1 gram of the extracted chitin was put in a centrifuge tube and 3 mL of

distilled water was added. Centrifugation was done at 179 × g for 10 min (Beckman CS-6 centrifuge). After centrifugation the supernatant was decanted and its volume measured.

$$\text{Water holding capacity (\%)} = \left(\frac{Volume\ of\ water\ added - volume\ of\ supernatant}{weight\ of\ sample}\right) \times 100 \quad (4)$$

3.2.7 Determination of Chitin Fat Binding Capacity

Fat binding capacity (FBC) was determined using the procedure of Lin et al. (Humbert, 1974). A sample of 0.3 grams was mixed with corn oil (3 mL) in a pre-weighed 50 mL centrifuged tube for 1 min. After centrifugation at 179 × g for 30 min (Beckman CS-6 centrifuge), the supernatant was discarded and the tubes were re-weighed. The FBC was expressed using the following equation:

$$\text{FBC (\%)} = \left(\frac{W1 - W2}{W2}\right) \times 100 \quad (5)$$

Whereby W1 is the weight of sample and absorbed oil and W2 is the initial sample weight

3.2.8 Determination of Degree of Deacetylation (DDA)

The degree of deacetylation (DDA) of the chitin samples was determined using the weight percentage of Nitrogen obtained using Kjeldahl method. The following equation by Hussain et al., (2013) was used in computing the percentage DDA.

$$\text{DDA(\%)} = \frac{1400}{(364 * WN)} \times 100 \quad (6)$$

3.2.9 Determination of Chitin Purity

The purity of the extracted chitin content was determined using the following equation as described by Díaz-Rojas et al., (2006).

$$\text{Chitin}(\%) = \frac{(Nt.Cp+K-100)\times Cq}{(Cp-Cq)} \quad (7)$$

Where by Nt is total nitrogen, Cp is conversion coefficient of protein (6.25), K is the sum of ash, fat and moisture and cq is a conversion coefficient.

3.2.10 Microstructure Imaging

The microstructure imaging of the surface morphology of the chitin was examined using a Scanning Electron Microscope (JCM-7000 NeoScope BenchtopSEM (JEOL ltd, Tokyo Japan) according to a method described by Chatterjee et al., (2020). The extracted chitin samples were coated with carbon film and examined using the secondary electron mode with an accelerating voltage of 15 Kv to show microstructures at different magnifications.

3.2.11 Data Analysis

Data was reported in means and standard deviations. To determine the differences in functional properties among the chitin samples data were subjected to one-way analysis of variance (ANOVA) followed by mean separation by Bonferroni's method at $P \leq 0.05$. Data was analyzed using Stata version 12.

3.3 Results and Discussion

3.3.1 Functional Groups in Chitin

The FTIR spectra of chitin isolated from *Acheta domesticus*, *Gryllus bimaculatus* and *Hermetia illucens* showed similar bands with commercial chitin as shown in Figure 3.1. The identified bands were O-H stretch, C=O stretch, N-H bend, CH2 ending and CH3 deformation, C-N stretch and C-O-C stretch which based on literature, are characteristic bands of chitin. The similarity between the isolated chitin samples and the commercial chitin suggests that good quality chitin was obtained from the extraction process. However, chitin isolated from *Acheta domesticus* did not have bands representing CH2 and O=C=O stretch, while chitin isolated from

Hermetia illucens never showed bands for CH2. The O-H stretch, C=O stretch and N-H bend are the characteristic bands for chitin identification. All chitin samples showed characteristic vibration bands near 3405, 3408, 3394 and 3386 cm-1 which correspond to O-H stretch (Table 3.1). Similarly based on a study byIbitoye et al., (2018). O-H stretch bands in chitin isolated from house cricket, commercial (shrimp) and other commercial chitin were observed at 3433, 3431 and 3437 cm-1. Equally, chitin isolated from shrimps, crabs and prawns has shown bands that represent O-H stretch (Kaya, Erdogan, et al., 2014; Kumari et al., 2015; Paulino et al., 2006).

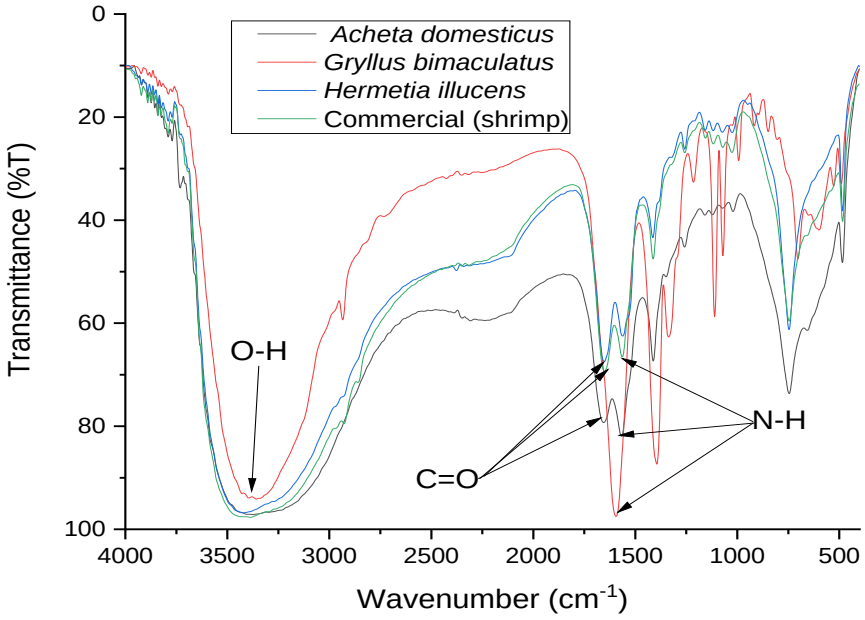

Figure 3.1: FTIR Spectra of Chitin Isolated from Commercial (Shrimps) *Acheta Domesticus, Gryllus Bimaculatus and Hermetia Illucens*

In the present study, C=O stretch was observed at 1655, 1649, 1643 and 1651 cm-1 in *Hermetia illucens, Gryllus bimaculatus, Acheta domesticus* and commercial chitin respectively (Table 3.1). The N-H bend vibration bands in *Hermetia illucens, Gryllus bimaculatus, Acheta domesticus* and commercial chitin were observed at 1566, 1594, 1563 and 1564 cm-1 respectively. Similarly, C=O stretch and N-H bending have been identified at 1650 cm-1 and 1548 cm-1 respectively in *Hermetia illucens chitin* (Purkayastha & Sarkar, 2020b). Equally occurrence of C=O stretch and N-H bend in

shrimp and Large black chafer bettle (*Holotrichia parallela)* chitin have been observed at 1654 and 1560 cm-1 respectively (Liu et al., 2012b). Evidence indicates that the characteristic vibration bands in the chitin samples in the current study were indicative of α- chitin (Purkayastha & Sarkar, 2020b). Similarly, other studies have reported that chitin isolated from cricket and BSF is α- chitin (Ibitoye et al., 2018; Soetemans et al., 2020).

Table 3.1: FTIR Bands (cm-1) of Chitin Isolated From Commercial (Shrimps) *Acheta Domesticus, Gryllus Bimaculatus and Hermetia Illucens*

Functional group and vibrating mode	**Commercial (Shrimp)**	***Acheta domesticus***	***Gryllus bimaculatus***	***Hermetia illucens***
O-H stretching	3405	3408	3394	3386
CH_3 symmetrical stretch and CH_2 assymetric stretch	2927		2934	
C=O stretch	1651	1643	1649	1655
N-H bend	1564	1563	1594	1566
CH_2 ending and CH_3 deformation	1412	1412	1394	1413
C-N stretch	1257	1256	1214	1259
C-O-C stretch	1071	1055	1070	1073

3.3.2 Functional Properties of Chitin

Texture and mouthfeel of chitin enriched baked and meat products have been associated with the FBC of chitin (Grumezescu & Maria, 2018). In the current study *Gryllus bimaculatus* chitin recorded the highest FBC (780.14%), followed by commercial chitin (630.01%) while *Acheta domesticus* chitin had the least value (457.87%), as shown in table 3.2. Interestingly, all the insect chitin samples had desirable FBC since they could absorb at least four times more fat in relation to their weight. Evidently, *Gryllus bimaculatus* chitin could be a potential alternative to commercial chitin in food industrial applications, given its significantly high FBC.

The FBC of *Acheta domesticus* and *Hermetia illucens* chitin were comparable with the range of 316-563% reported by Cho et al. (Cho et al., 1998). The FBC of commercial chitin in the present study was significantly higher than values reported by other studies (Knorr, 1982; Marei et al., 2016; Sampath, Ngasotter, Layana, Balenge, et al., 2022). The variations in FBC among chitin samples could be attributed to differences in molecular weight and density (Panith et al., 2016). Evidence indicates that the FBC of chitin is significantly affected by particle size, with flake chitosan having less FBC as compared to powdered chitin (Liu et al., 2008). It is worth noting that chitin may have beneficial impacts on human health due to its ability to bind fat and cholesterol, hence reducing the amounts absorbed into the body (Huang & Tsai, 2020).

In the present study, commercial chitin (853.4%) had the highest water holding capacity (WHC) followed by *Gryllus bimaculatus* chitin (635.80%) while *Hermetia illucens* chitin (332.28%) had the least value (Table 2). Similarly, other studies have reported high WHC (748%) in shrimp chitin (Nessa et al., 2010). Other studies have documented that the WHC of commercial chitin ranges from 318 to 805% (Cho et al., 1998; Sampath, Ngasotter, Layana, Balenge, et al., 2022). The WHC of *Acheta domesticus*, *Gryllus bimaculatus*, and *Hermetia illucens* chitin samples was comparable to values of commercial chitin reported by Cho et al. (Cho et al., 1998). This therefore indicates the potential of edible insect chitin application in the food industry. The WHC of chitin is influenced by the crystallinity, number of salt forming groups and differences in protein content (Knorr, 1982). Additionally, low values of WHC of chitin have been attributed to a lower degree of deacetylation (Nessa et al., 2010).

Table 3.2: Functional Properties of Chitin Isolated from Commercial (Shrimp), *Acheta Domesticus, Gryllus bimaculatus and Hermetia Illucens*

Chitin source	FBC (%)	WHC (%)	Solubility (%)	Emulsion capacity (%)	Emulsion stability (%)
Shrimp (commercial)	630.01±13.59^{c}	853.4±8.71^{d}	10.59±0.80^{b}	23.67 ± 2.52^{b}	21.67+1.53^{b}
Acheta domesticus	457.87±2.27^{a}	447.45±0.78^{b}	22.71±0.54^{d}	36.49±1.78^{c}	33.21±1.32^{c}
Gryllus bimaculatus	780.14±10.69^{d}	635.80±1.54^{c}	6.14 ±0.18^{a}	65.67±3.06^{d}	65.67±4.73^{d}
Hermetia illucens	478.53 ± 14^{b}	332.28±0.55^{a}	16.35±0.90^{c}	15.83±0.72^{a}	12.08+1.91^{a}
P value	<0.001	<0.001	<0.001	<0.001	<0.001

Values are means ± SD. Means with different superscript letters along the columns are significantly different at $p<0.05$.

Acheta domesticus chitin had significantly higher solubility in water as compared to the other chitin samples. The solubility of the chitin samples was within the range reported in commercial chitin samples (3-16%) (Sampath, Ngasotter, Layana, Balenge, et al., 2022). Generally, chitin is expected to be less soluble in water than chitosan due to its semicrystalline structure and the presence of strong intra and intermolecular hydrogen bonding network (Ru et al., 2019). Furthermore, its solubility is influenced by particle size, degree of deacetylation and biological origin (Hossain & Iqbal, 2014; Mahdy Samar et al., 2013). Chitin with a degree of acetylation of 0.45 to 0.55 is likely to be water soluble, probably due to the random distribution of the N- acetylglucosamine and glucosamine residues along the main chain (Kurita, 2006; Rinaudo, 2006). Chitosan, a derivative of chitin is usually more soluble in water, acid and alkaline solutions and is thus more preferred over chitin for a wide range of applications (Gartner et al., 2010).

There was a statistically significant difference in emulsion capacities among the chitin samples whereby *Gryllus bimaculatus* chitin exhibited higher values while *Hermetia illucens* had the least values (Table 2). It is worth noting that *Gryllus bimaculatus* and *Acheta domesticus* chitin had superior emulsion capacity as

compared to commercial chitin. Furthermore, *Gryllus bimaculatus* and *Acheta domesticus chitin had* significantly higher emulsion capacity than (6.96%) observed in crickets (Hirsch, 2018). Moreover, the chitin samples had significantly lower emulsion capacity as compared to shrimp chitin (90%) (Knorr, 1982; Sampath, Ngasotter, Layana, Balenge, et al., 2022). Additionally, the emulsion capacity of the chitin samples was significantly lower than that of conventional emulsifiers such as caseinate (250-700%) (Mohanty et al., 1988). The significantly low emulsion capacity of chitin samples could be potentially explained by the large chitin particles that were unable to participate in emulsification (Hirsch et al., 2019).

There was a statistically significant difference in the emulsion stability among the chitin samples ($p<0.001$). The *Gryllus bimaculatus* chitin showed significantly higher emulsion stability than the commercial chitin. Cricket chitin forms stable emulsions, indicated by a slight and steady decrease in creaming stability index over time (Hirsch, 2018). Chitin is reported to stabilize emulsions through pickering stabilization whereby a solid non dissolved particle aids in stabilization by absorbing onto the interface of the immiscible liquids hence preventing coalescence of the dispersed droplets (Pickering, 1907). Chitin is a promising pickering stabilizer due to its ability to stabilize much smaller oil droplets (3.33µm) as compared to traditional surfactants such as soy protein isolates which stabilize oil droplets of about 60- 70 µm (Liu & Tang, 2013). Additionally, it has been reported that chitin is a suitable emulsion stabilizer that works well when combined with other emulsifiers and antioxidants (Harkin et al., 2019). Thus, the considerably good functional properties of chitin make it a viable functional ingredient for stabilizing emulsions, improving texture, and serving as a fat replacer in food products.

3.3.3 Degree of Deacetylation and Purity of Chitin

In the present study, *Hermetia illucens* chitin recorded the highest Degree of Deacetylation (DDA) values while *Acheta domesticus* chitin had the least value (Table 3.3). The high DDA in *Hermetia illucens* chitin could be explained by the fact that chitin was extracted from cocoons rather than the whole insect meal and thus, the potential for extensive deacetylation. The DDA values of commercial chitin were

comparable with 48.16% reported in shrimp chitin (Zhang et al., 2017). Similarly, other studies have reported a DDA range of 16 to 47% in chitin obtained from crabs (Xu et al., 2019). The differences observed in DDA values could be attributed to species differences. The variation in DDA values among chitin samples could potentially be attributed to modifications of the chitin extraction procedure. Modification of the chemical extraction process through increasing the NaOH concentration up to about 40% and increasing the extraction time may result in higher DDA values (Xu et al., 2019). However, in the present study, the extraction procedure was not modified.

Table 3.3: Degree of Deacetylation (DDA) and Purity of Chitin Extracted from Shrimp (Commercial), *Acheta Domesticus, Gryllus Bimaculatus and Hermetia Illucens*

Chitin	**DDA (%)**	**Purity (%)**
Shrimp (commercial)	50.50 ± 1.83^{a}	98.44 ± 1.17^{c}
Acheta domesticus	47.31 ± 2.24^{a}	76.31 ± 3.02^{a}
Gryllus bimaculatus	57.48 ± 1.15^{b}	95.41 ± 1.10^{c}
Hermetica illucens	66.21 ± 1.51^{c}	86.33 ± 1.71^{b}
P value	<0.001	<0.001

Values are means ± SD. Means with different superscript letters along the columns are significantly different at $p<0.05$.

It is worth noting that DDA is an indication of the number of surface amino groups (Wu et al., 2008). Studies have documented a positive correlation between DDA and emulsion capacity and stability. Evidence shows that the antibacterial properties of chitin are enhanced by an increase in DDA and a decrease in pH whereby the pH influences the degree of ionization of the amino group which consequently confers the antibacterial properties. Basically, the positively charged amino groups may interact with the negatively charged microbial cell membranes which further leads to loss of intracellular electrolytes such as potassium and other low molecular weight proteinous compounds, thus hindering the growth of pathogenic microorganisms

such as *Escherichia coli* (Xu et al., 2019). Studies have further associated the DDA of chitin with functional properties. For instance, chitin with a DDA of about 75% was reported to have significantly high water holding capacity (Silva et al., 2021). In the present study, *Hermetia illucens* chitin, which had a considerably high DDA, showed a significantly high fat-binding capacity. This positive correlation between a higher degree of deacetylation (DDA) and enhanced fat binding capacity (FBC) can be attributed to the increased exposure of amino groups (-NH_2) along the chitosan polymer chain, which creates more hydrophobic binding sites as well as porosity of chitin (Purkayastha & Sarkar, 2020a).

Commercial chitin and *Gryllus bimaculatus* chitin had the highest purity levels followed by *Hermetia illucens* while *Acheta domesticus* had the least levels of purity (Table 3). Evidence indicates that the purity of commercial chitin reported in the present study was comparable to 96.1% reported in shrimp chitin (Zhang et al., 2022). Equally, other studies have reported high levels of purity in commercial chitin (91.3%) (Zhu et al., 2005a). Interestingly, the purity of *Hermetia illucens* chitin reported in the present study was slightly lower than 96.8% reported in BSF (Soetemans et al., 2020). Studies propose that the purity of chitin ranges from 85 to 97%. Other studies suggest that the chitin purity is influenced by insect species, stage of maturity and the purification method (Hahn, Roth, et al., 2020; Khayrova et al., 2020). Chitin extracted from *Acheta domesticus* had the least purity levels which could be attributed to partial conversion of chitin to chitosan during the extraction process (Psarianos et al., 2022). It is also worth noting that the purity of the chitin samples was determined using a model and thus, we recommend the use of solid-state nuclear magnetic resonance spectroscopy to validate the model.

3.3.4 Microstructure Images of Chitin

In the present study, the surface morphology of *Gryllus bimaculatus* (Figure 3.4) and *Hermetia illucens* (Figure 3.3) chitin was characterized by pores, microfibrils and repeating units of square and hexagonal boxes. However, *Acheta domesticus* (Figure 3.2) and commercial chitin (Figure 3.5) differed in surface morphology with *Gryllus bimaculatus* and *Hermetia illucens* in that they lacked the repeating units of

hexagonal boxes. This observation suggests that differences in the surface morphology of chitin could be attributed to species differences. The microfibrillar structures observed are primarily composed of bundles of chitin chains stabilized by extensive inter and intra-molecular hydrogen bonding, which provide the polymer with its high tensile strength and crystalline rigidity (Hamed et al., 2015). The repeating hexagonal and square units represent highly organized calcified chitin-protein matrices and are thus high areas of crystallinity which correlates to poor solubility (Mohan et al., 2018). It is also worth noting that the pores observed in all the chitin samples were of different diameters and shapes, whereby some were oval and others circular. Chitin surface morphological differences have been shown to be dependent on the level of magnification and the area being observed (Ibitoye et al., 2018). Evidence suggests that chitin may have a mix of porous and microfibrillar structure, just a microfibrillar structure and a non-porous non-microfibrillar structure (Kaya, Baran, et al., 2014). Similarly, the surface morphology of *Hermetia illucens* and *Acheta domesticus* chitin was similar to what was reported by other studies (Ibitoye et al., 2018; Purkayastha & Sarkar, 2020b).

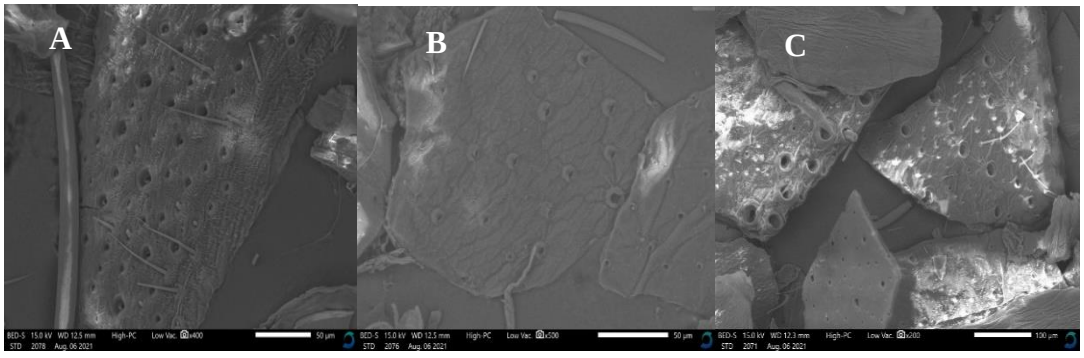

Figure 3.2: Figures (A, B and C) Scanning Electron Micrographs of *Acheta Domesticus* Chitin

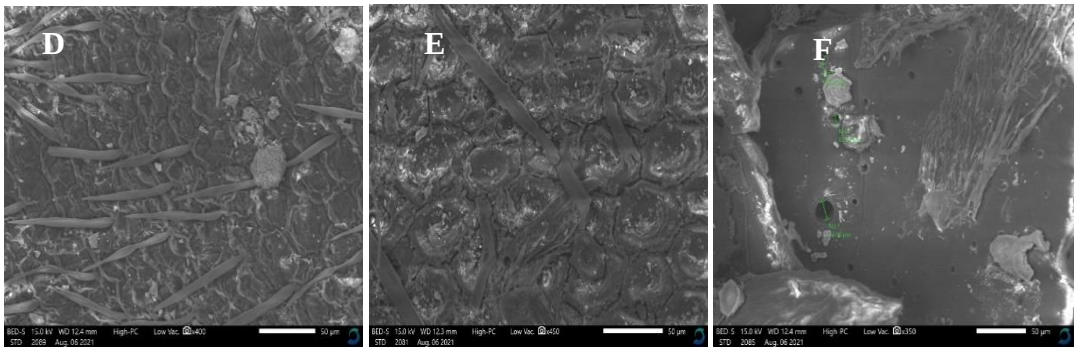

Figure 3.3: Figures (D, E and F) Scanning Electron Micrographs of *Hermetia Illucens* Chitin

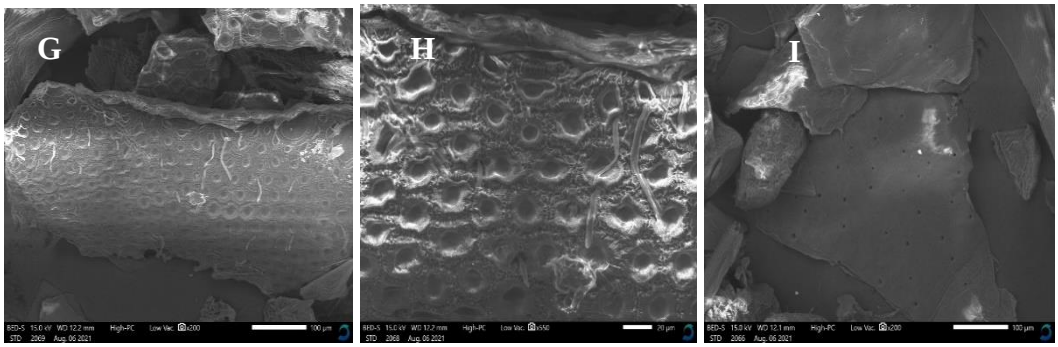

Figure 3.4: Figures (G, H and I) Scanning Electron Micrographs of *Gryllus Bimaculatus* Chitin

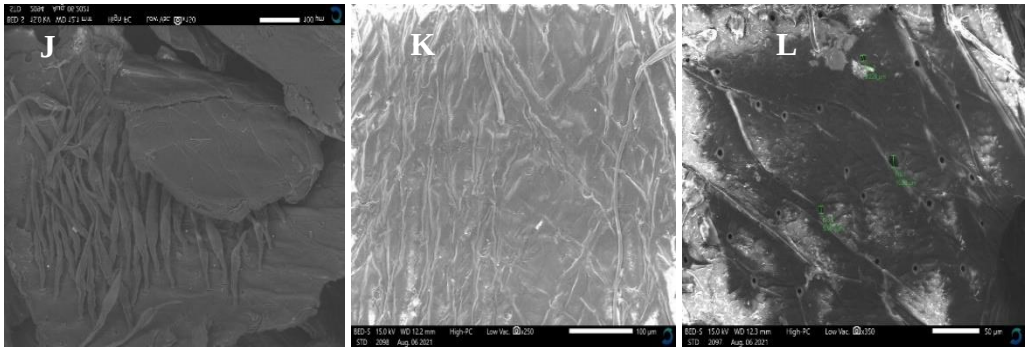

Figure 3.5: Figures (J, K and L) Scanning Electron Micrographs of *Commercial* Chitin

The porosity of chitin has been associated with the absorbent properties of chitin. Studies have documented that the porosity of chitin and the pattern of pores enhance its water absorption capacity (Chakravarty et al., 2018; Huang & Tsai, 2020). Therefore, the considerably high water absorption capacity of the chitin samples as shown in table 2 could be attributed to its porosity. Chitin fibers have been documented to have antimicrobial properties and have thus been utilized in making biodegradable bandages and used in wound dressing in tissue engineering. Chitin fibers increase the surface area of chitin, hence making it ideal for dye removal and wastewater treatment (Wang & Chen, 2014). Fibrous chitin has also been utilized in the cosmetic (Bhardwaj & Kundu, 2010), textile (Hirano, 2001) and pharmaceutical industry (Abdelgawad et al., 2014; Sibaja et al., 2015). Therefore, the similarity in surface morphology between the commercial chitin and insect chitin shows that insect chitin is a potential alternative that should be exploited.

3.4 Conclusion

In conclusion, the chitin samples in the present study were similar to commercial chitin as indicated by the FTIR spectra. All the chitin samples were more soluble in water when compared to the commercial chitin. The *Gryllus bimaculatus* chitin had significantly higher fat binding capacity, emulsion capacity and emulsion stability as compared to the commercial chitin while the commercial chitin showed superiority in regards to water absorption capacity. Interestingly, all the chitin samples could absorb four times more water and two times more fat, than their weight which is indicative of the potential for utilization in baking and the meat industry. The commercial chitin and BSF had the highest DDA while commercial chitin and *Gryllus bimaculatus* had the highest values of purity, followed by BSF. The SEM images showed similar surface morphology in all the chitin samples, which were characterized by the presence of microfibers and pores. The similarity between commercial chitin and insect chitin implies the need to utilize insect chitin in the food, pharmaceutical, medical and textile industries among other applications.

CHAPTER FOUR

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF DERIVATIVES OF IN VITRO DIGESTION AND FERMENTATION OF EDIBLE INSECT'S CHITIN

4.1 Introduction

Globally, the increase in cases of microbial resistance has prompted scientists to focus on natural molecules that may have antimicrobial and antioxidant properties. Scientists have thus been evaluating various biopolymers that may have antibacterial properties and this includes chitin. Chitin is the second most abundant biopolymer on earth and has gained a lot of attention because of its antioxidant and antimicrobial potential (Guarnieri et al., 2022). These properties of chitin have enhanced its extensive use in medical, pharmaceutical, textile and food sectors. Chitin has been sourced from crustaceans, a source that is significantly threatened by overfishing, environmental pollution and climate change (Sumaila & Tai, 2020). Therefore, scientists are investigating the potential of edible insects as alternative sources of chitin.

Evidence suggests that edible insects have prebiotic potential. For instance, evidence shows that cricket chitin enhanced the growth of *Bifidobacterium animalis* and *Lactobacillus rhamnosus* GG (Stull et al., 2018). Further edible insects' chitin and its deacetylated derivatives, chitosan exhibit significant antioxidant and antimicrobial activity against Escherichia coli, Staphyloccoccus aureus and Salmonella species. Additionally, a study by Fernandes et al., (2008) documented that chitin promotes the proliferation of gut microbiota such as *Facecalibacterium*, *Ruminococcaceae* and L*achnospiraceae* genera during colonic fermentation. Critically, the bioactivity of insects' chitin is not static and thus changes as it traverses the GIT where fermentation generates low molecular weight derivatives such as chitooligosaccharides, which show enhanced solubility and biological activity (Hahn, et al., 2020).

However, despite these significant reports, a major gap persists in the understanding of how the sequential processes of in vitro digestion followed by fermentation affect the bioactivity of insect chitin derivatives (Alyani et al., 2020). Current literature has focused on the antimicrobial activity of chitosan that has been chemically obtained by degrading chitin obtained from crustaceans and edible insects (Kaya et al., 2015; Kipkoech, Kinyuru, Imathiu, & Meyer-rochow, 2021). Other studies have assessed the antimicrobial and antioxidant activity of commercial Chito-oligosaccharides (COS) and chitosan nanoparticles (Divya, Vijayan, George, & Jisha, 2017; Ngo, Lee, Kim, & Kim, 2009). Other studies have also tested the effect of consuming whole crickets on the gut microbiota (Mendoza-Salazar et al., 2021; Stull et al., 2018). Studies comparing whole insect powders and isolated chitin have shown that chitin may be partially responsible for the observed increase in gut microbial diversity, yet the specific contribution of derivatives generated through digestion and fermentation to antimicrobial and antioxidant activity remains unresolved. Further, the demonstration that chitosan shows lipid peroxidation inhibition ability alongside conventional radical scavenging activity shows the need for a comprehensive profiling of bioactivity of fermentate obtained after in vitro digestion and fermentation of chitin (Hisham et al., 2024).

4.2 Methods

4.2.1 Chitin Extraction

Acheta domesticus, *Gryllus bimaculatus*, and *Hermetia illucens* cocoons that were collected from the insect farm at Jomo Kenyatta University of Agriculture and Technology were dried and ground. The method described by Charoenvuttitham et al., 2006 and Toan, (2009) was adopted where 150 grams of ground *Acheta domesticus* and *Gryllus bimaculatus* powder and 150 grams of ground *Hermetia illucens* cocoons was added to 1000ml of boiling 0.5M sodium hydroxide in a 3000 mL conical flask and then boiled for 2 hours. The content of the conical flask was cooled for 30 min and then centrifuged at 45×g for 10 min. The supernatant was discarded and the residue was added into a conical flask containing 600mL of 1% hydrochloric acid followed by shaking in a rotor shaker rotating at 40 rpm for 24

hours at room temperature. The contents of the conical flask were then centrifuged at 45xg for 10 minutes. The supernatant was discarded and the residue was added to 500ml of 0.5M sodium hydroxide in a conical flask and boiled for one hour. The contents of the conical flask were subjected to centrifugation at 45xg for 10 min. The supernatant was then discarded while the residue was washed three times with boiling distilled water then dried at 70°C for 12 hours and stored in zip lock bags at room temperature.

4.2.2 In Vitro Digestion

The chitin samples were in vitro digested i.e. oral, gastric and intestinal digestion according to a method by Minekus et al., (2014) with a few modifications.

4.2.2.1 Oral Digestion

Simulated salivary fluid was made by dissolving 1.055g of alpha-amylase into a solution of 137mM NaCl, 2.7mM KCl, 10mM NaH_2PO_4 and 1.8mM KH_2PO_4. To 1 gram of the chitin samples 1.5 ml of freshly prepared simulated saliva was added. The solution was incubated for 10 min at 120 strokes per minute in a shaking water bath set at 37°C. After incubation, the pH of the solution was adjusted to 2.0 using 0.1N HCl.

4.2.2.2 Gastric Digestion

Simulated gastric fluid was made by dissolving 0.2g of pepsin into 5 ml 0.1 N HCl. About 2.5 mL of the simulated gastric fluid was added to the solution obtained after oral digestion. The solution was then incubated in a shaking water bath at 37°C for 2 hours at 120 strokes per minute. The solution was then placed on ice for 10 min. The pH of the solution was then raised to 7 using 1 M $NaHCO_3$.

4.2.2.3 Intestinal Digestion

Simulated intestinal fluid was made by dissolving 0.05 g of pancreatin, 0.025 g of lipase and 0.3 g of bile extract in 25 mL 0.1 M $NaHCO_3$. To the solution obtained after gastric digestion 12.5 mL of the pancreatin, bile extract, and lipase solution was

added. The solution was then incubated in a shaking water bath at 37°C for 2 hours at 120 strokes per min. The solution was then placed on ice for 10 minutes. The pH of the solution was then adjusted to 7.2 by adding 0.5 M NaOH.

A 10 mL portion of each sample (previously digested) was placed in dialysis tubing using pipettes. The outside compartment of the dialysis tubing of each sample was loaded with a 25 mL solution of 0.9% NaCl with albumin. The samples were then dialyzed at room temperature for 24 hours. The solution outside and inside the dialysis tubing were emptied into separate test tubes and measured gravimetrically. The solution that was inside the dialysis tubing, which was the indigested chitin was then freeze-dried and stored in zip-lock bags awaiting the fermentation process.

4.2.3 Fermentation

Fermentation of the indigestible chitin was done according to a method by Martín-Carrón and Goñi, (1998) with a few modifications. One hundred (100) mg of the indigested chitin samples were weighed in 50 mL centrifuge tubes. For the negative control there was no indigestible chitin weighed in the 50 mL centrifuge tubes. Ten (10) mL of 10% lactose medium and 7µL of 10% starter culture namely ABY 10 (*Bifidobacterium*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* sbsp *bulgaricus* and *Streptococcus thermophiles*) and ABT 5 (*Bifidobacterium*, *Lactobacillus acidophilus* and *Streptococcus thermophiles*) solution were added into the centrifuge tubes. The centrifuge tubes were then sealed with rubber stoppers and placed in a water bath with constant stirring at 37 °C for 24 and 48 hours. To stop fermentation 2.5 mL of 1 M NaOH was added and latter samples were centrifuged at 2500× g for 10 min. The supernatant was then freeze-dried and the samples were stored in zip-lock bags at room temperature.

Each tube's precipitate from the fermentation kinetics experiment was homogenized for three minutes in 50 milliliters of 0.9% NaCl. Dacron filters (pore size 50 µm) were used to filter all precipitates; prior to use, they were dried to a consistent weight. The unfiltered residues were first cleaned twice with 50 mL of 0.9% NaCl, and then rinsed with 5 mL of acetone to remove any remaining lipids and other

hydrophobic compounds as well as mineral and inorganic residues. After drying at 60 °C, the filter papers were weighed using a gravimetric scale.

$$Unfermented\ indigestibe\ chitin\ (\%) = \left(\frac{\text{Weight (filter + washed residue)} - \text{initial filter weight}}{\text{Weight of fermented sample}}\right) * 100 \quad (8)$$

The percentage of fermented indigestible chitin was then determined as follows:

$$Fermented\ indigestible\ chitin\ (\%) = (100 - unfermented\ chitin) \quad (9)$$

4.2.4 Antioxidant Activity

Using the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical test (Sigma-Aldrich, USA), the antioxidant activity (free radical scavenging ability) of derivatives of in-vitro digestion and fermentation of chitin was assessed as described by Molyneux, (2004). A stable free radical that gives solutions a rich purple hue is called DPPH. When DPPH takes up an electron from an antioxidant, it either turns light yellow or colorless. The variations in absorbance at 517 nm provide a spectrophotometric means of quantifying this neutralizing process. The following methanol (analytical grade) concentrations of derivatives of in-vitro digestion and fermentation of chitin were prepared: 0.3 mg/mL, 1 mg/mL, and 5 mg/mL. Similar to the derivatives, in the same amounts, vitamin C was utilized as an antioxidant standard. In a test tube, 1 mL of the derivatives was added to 3 mL of methanol, followed by 0.5 mL of 1 mM DPPH in methanol. A control sample was made with the same amount of methanol and DPPH. Methanol was used to zero the spectrophotometer and the absorbance was read at 517 nm after 5 min in UV-Vis spectrophotometer (Shimadzu model UV-1601 PC, Kyoto, Japan). The radical scavenging activity was calculated as follows:

$$\%\ \text{inhibition of DPPH} = \left(\frac{AB - AA}{AB}\right) * 100 \quad (10)$$

Where AB is the absorbance of the control sample and AA is the absorbance of the derivatives of in-vitro digestion and fermentation of chitin. The results were expressed as percentage inhibition of DPPH.

4.2.5 Antimicrobial Activity

4.2.5.1 Test Microorganism for Antimicrobial Assay

The antimicrobial activity of derivatives of *invitro* digestion and fermentation of indigestible chitin was tested against Gram-negative; *Escherichia coli*, *Vibrio cholerae, Bacteroides fragilis, Enterobacter agglomerans, Shigella dysenteriae* and Gram-positive; *Staphylococcus aureus* and *Bacillus cereus*. The test microorganisms were obtained from the Kenya Medical Research Institute (KEMRI) in Nairobi, Kenya.

4.2.5.2 Inoculum and Sample Preparation

Inoculum and sample preparation was done according to a method by Martín-Carrón & Goñi, (1998). Each bacterial strain was sub-cultured overnight at 37 °C in nutrient broth (Sigma Aldrich, St. Louis, MI, USA). Bacterial colonies were thereafter suspended in 9 mL of sterile saline solution, and the suspension was adjusted to achieve turbidity equivalent to the 0.5 McFarland standard, which is equivalent to 1 × 108 colony-forming units (CFUs)/mL. Mueller–Hinton agar (Sigma Aldrich, St. Louis, MI, USA) was then prepared, and 12 mL of the medium was dispensed to the Petri dishes and allowed to solidify.

4.2.5.3 Antimicrobial Susceptibility Assay

The Kirby Bauer disc diffusion method was employed in this study for antimicrobial assay (Biemer, 1973). Typically, 20 µL of freshly prepared McFarland bacterial cultures of *Escherichia coli, Vibrio cholerae, Bacteroides fragilis, Enterobacter agglomerans* and *Shigella dysenteriae, Staphylococcus aureus* and *Bacillus cereus* were then inoculated and spread uniformly onto Mueller Hinton agar plates. Discs were prepared by impregnating 50 µL (250 µg/disc) of derivatives of in vitro digestion and fermentation of chitin at a concentration of 2 mg/mL, 5 mg/mL and 10

mg/mL on sterile filter paper discs (6 mm), followed by air-drying. The discs were then placed on the top of the agar plates followed by incubation of the agar plates at 37 °C for 24 hour using an IS62 incubator (Genlab, Tokyo, Japan). The presence of inhibition zones was measured around each disc in millimetres (mm) and was considered as evidence of antimicrobial activity. The experiments for each test organism were carried out in triplicate.

4.2.6 Data Analysis

Data was reported in means and standard deviations. To determine the effect of the chitin sample, fermentation time and sample concentration on antioxidant and antimicrobial activity data was subjected to a three-way analysis of variance (ANOVA) followed by mean separation by Bonferroni's method at $P \leq 0.05$. Data was analyzed using Stata version 17 and presented using graphs and tables.

4.3 Results and Discussion

4.3.1 Fermented Indigestible Chitin

The type of starter culture used and the source of chitin had a significant influence on the amount of indigestible chitin that was fermented ($p<0.05$) as shown in Figure 4.1. The highest values of fermented chitin were observed in *Hermetia illucens* chitin fermented using ABY 10 while the lowest values were observed in *Acheta domesticus* chitin fermented using ABT 5. The observed differences in the fermentability of chitin samples could be due to purity levels that are characterized by the presence of protein residues in the extract.

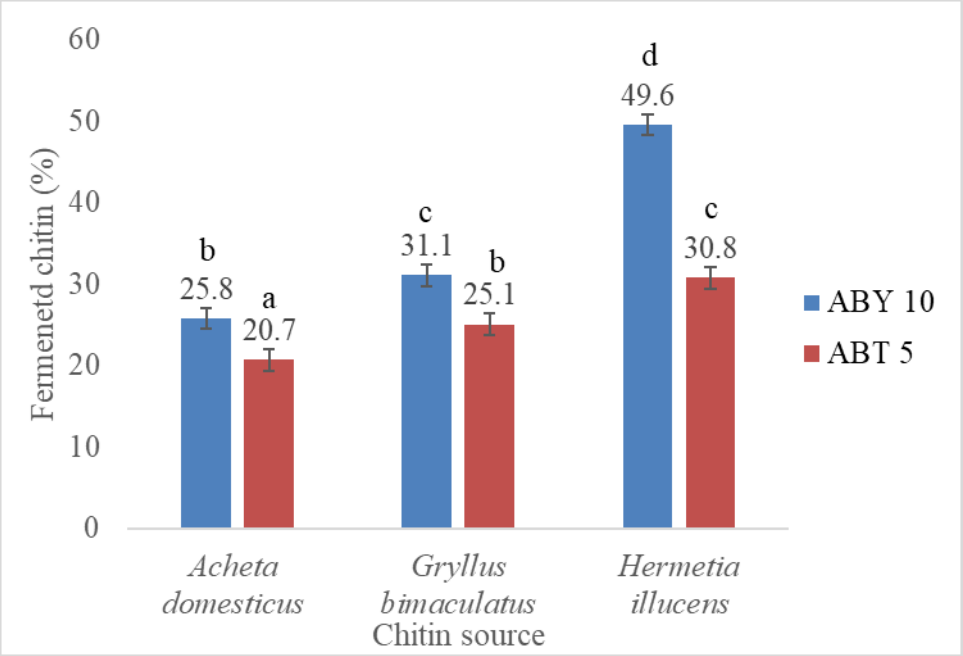

Figure 4.1: Indigestible Chitin (%) Fermented by ABY 10 and ABT 5 Starter Cultures

Evidently, ABY 10 starter culture was able to ferment more chitin as compared to ABT 5 starter culture, potentially because ABY 10 is composed of four bacteria strains i.e. *Bifidobacterium*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckiii subsp. Bulgaricus* and *Streptococcus thermophilus* while ABT 5 has three strains i.e. Bifidobacterium, *Lactobacillus acidophilus* and *Streptococcus thermophilus*. A study by Fu et al., (2022) reported that the diversity and concentration of gut microflora are key factors that enhance the breakdown of fiber in the gut. Probiotics have been shown to have the potential to break down chitin as an energy source while at the same time degrading chitin to metabolites. For instance, a study investigating the prebiotic potential of chitin reported that chitin enhanced the growth of *Lactobacillus fermentum* and *Lactobacillus acidophilus* (Kipkoech et al., 2021). *Hermetia illucens* chitin was readily fermented by both starter cultures, followed by *Gryllus bimaculatus, whereas Acheta domesticus* chitin was not readily fermented. The values of fermented chitin obtained in the present study were slightly lower than the values (65.5%) of fermented Aloe Vera polysaccharides (Tornero-Martínez et al., 2019). Similarly, polysaccharides obtained from straw mushrooms were found to have a fecal fermentability efficiency of 69.15% after 48 hours of fermentation (Hu

et al., 2023). The observed differences could be explained by variations in the samples and inoculum used. Soluble fibres are fermented to a greater extent by colonic bacteria as compared to insoluble fibre (Elleuch et al., 2011). The unfermented fibre normally becomes part of the fecal mass in the colon and is then excreted (Mudgil & Barak, 2013).

4.3.2 Antioxidant Activity

The antioxidant activity of the fermentate of in vitro digestion and fermentation of the indigestible chitin was significantly affected by fermentation time and sample concentration ($p<0.05$) as shown in table 4.1. It is worth noting that vitamin C (positive control) had significantly higher antioxidant activity as compared to the fermentate of in vitro digestion and fermentation of indigestible chitin. Among the indigestible chitin samples fermented using ABY10, *Hermetia illucens* samples fermented for 48 hrs and at a concentration of 5 mg/mL had the highest antioxidant activity while the least activity was reported in the negative control. *Gryllus bimaculatus*, *Acheta domesticus* and *Hermetia illucens* indigestible chitin samples fermented using ABY 10 had significantly high antioxidant activity as compared to the negative control ($p<0.05$). This implies that in vitro digestion and fermentation of the chitin samples yielded compounds with antioxidant activity. In samples fermented using ABT5, *Acheta domesticus* samples fermented for 48 hours and at a concentration of 5 mg/mL had the highest antioxidant activity, while the negative control had the least activity. It is worth noting that antioxidant activity increased with an increase in concentration and fermentation time, whereby the highest antioxidant activity was observed in samples fermented for 48 hours and at a concentration of 5 mg/mL.

Table 4.1: Antioxidant Activity of Fermentate of Chitin In Vitro Digestion and Fermentation

Chitin Vitamin C (+ control)	Fermentation time (hrs)	Derivatives concentration (mg/mL)	Antioxidant activity (%) ABT 5	Antioxidant activity (%) ABY 10
			100 µg/ml	**47.38±3.89^{j}**	**47.38±3.89^{g}**
Control (-)	24	0.3	0.02 ± 0.00^{a}	0.06 ± 0.00^{a}
		1	0.02 ± 0.01^{a}	0.10 ± 0.00^{b}
		5	0.03 ± 0.00^{a}	0.20 ± 0.01^{b}
	48	0.3	0.03 ± 0.01^{a}	0.04 ± 0.00^{a}
		1	0.04 ± 0.01^{a}	0.05 ± 0.00^{a}
		5	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}
Gryllus bimaculatus	24	0.3	17.35 ± 0.36^{bc}	22.76 ± 0.86^{c}
		1	39.52 ± 0.41^{e}	41.54 ± 1.20^{e}
		5	52.68 ± 1.86^{g}	57.95 ± 0.69^{h}
	48	0.3	16.57 ± 0.76^{b}	25.27 ± 0.06^{d}
		1	39.76 ± 1.75^{e}	44.57 ± 3.52^{f}
		5	57.82 ± 3.43^{h}	61.11 ± 2.95^{i}
Acheta domesticus	24	0.3	16.15 ± 0.50^{b}	23.59 ± 0.54^{c}
		1	39.48 ± 4.07^{e}	43.67 ± 2.27^{ef}
		5	59.29 ± 1.35^{h}	61.79 ± 3.13^{i}
	48	0.3	19.20 ± 1.70^{bc}	25.20 ± 1.47^{d}
		1	44.59 ± 1.88^{f}	47.27 ± 2.69^{g}
		5	61.57 ± 1.72^{i}	63.88 ± 4.73^{i}
Hermetia illucens	24	0.3	15.07 ± 0.80^{b}	23.26 ± 0.29^{c}
		1	36.54 ± 0.78^{d}	40.58 ± 1.53^{e}
		5	54.94 ± 3.00^{g}	56.85 ± 5.20^{h}
	48	0.3	21.16 ± 0.007^{c}	25.75 ± 0.63^{d}
		1	41.42 ± 2.10^{ef}	45.19 ± 7.58^{fg}
		5	63.37 ± 2.51^{i}	61.63 ± 2.00^{i}

Values are means ± SD. Means with different superscript letters along the columns are significantly different at $p<0.05$.

According to Mendoza-Salazar et al., (2021) the antioxidant activity of chemically extracted chitin, chitosan and derivatives obtained after fermentation of edible insect flours has been investigated. It is however, worth noting that this study is among the first studies to report on the antioxidant and antibacterial properties of a fermentate obtained after in vitro digestion and fermentation of *Acheta domesticus*, *Gryllus bimaculatus* and *Hermetia illucens* chitin. Evidence shows that chitin is degraded enzymatically to produce derivatives such as chitosan and further enzyme activity produces chitooligomers (Ngo & Kim, 2014). In the present study, chitin was subjected to in vitro digestion and fermentation using probiotic starter cultures, which indicates that chitin was potentially enzymatically degraded.

Chitin, chitosan and their derivatives such as COS exhibit antioxidant activity and hence their utilization in medical and pharmaceutical industries (Ying, Xiong, Wang, Sun, & Liu, 2011). The antioxidant activity of derivatives of in vitro digestion and fermentation of the three edible insect chitin at a concentration of 1 mg/mL and 5 mg/mL was comparable to the activity of grasshopper chitosan at a concentration of 10 mg/mL (28.4%- 52.3%) (Kaya, Baran, et al., 2015). The antioxidant activity of chitin oligomers has been associated with the degree of acetylation and molecular weight of the oligomers (Ngo & Kim, 2014). In vivo studies have shown that COS have inhibitory effects on myeloperoxidase activity in human myeloid cells as well as inhibiting protein and DNA oxidation (Ngo et al., 2009). The evidence provided by the present study suggests that consuming chitin-based products may have a positive impact on human health. For instance, the antioxidant properties observed in chitin fermentation derivatives provide a mechanistic basis for these positive effects, as oxidative stress is a key factor in the pathogenesis of numerous chronic diseases (Ngo & Kim, 2014). Additionally, the study documents the potential of application of *Acheta domesticus, Gryllus bimaculatus* and *Hermetia illucens* chitin and its derivative products in the functional food sector.

4.3.3 Antimicrobial Activity of Fermentate Obtained after In Vitro Digestion and Fermentation Using ABT 5 Starter Culture

The antimicrobial activity of derivatives of in vitro digestion and fermentation of *Gryllus bimaculatus*, *Acheta domesticus* and *Hermetia illucens* chitin using ABT 5 probiotic starter culture is shown in Table 4.2. The fermentate exhibited significantly different antibacterial activity against the tested pathogenic microorganisms ($p<0.05$). It is however worth noting that the activity of the fermentate was considerably lower than that of the positive control (Oxacillin 100 µg). The antimicrobial activity of the fermentate increased with an increase in its concentration. Similarly, the inhibitory effect of chemically and biologically extracted *Hermetia illucens* chitosan was reported to increase with an increase in sample concentration (Lagat et al., 2021).

Table 4.2: Inhibition Zone (mm) of Selected Pathogenic Bacteria by Fermentate Obtained from In Vitro Digestion and Fermentation Using ABT 5

Chitin	**Fermentation time (hrs)**	**Concentration (mg/mL)**	***Escherichia coli***	***Staphylococcus aureus***	***Vibrio cholera***	***Bacillus cereus***	***Bacteroides fragilis***	***Enterobacter agglomerans***	***Shigella dysenteriae***
Oxacillin (+Control)		**100 µg**	**14.78±1.23^{f}**	**10.60±2.56^{f}**	**33.25±0.99^{h}**	**ni**	**15.61±2.74^{e}**	**9.96±1.03^{g}**	**21.17±2.83^{c}**
Control (-)	24	2	3.00±0.24^{a}	0.90±0.001^{a}	0.40±0.05^{a}	ni	0.50± 0.01^{a}	0.50±0.02^{a}	ni
		5	3.50±0.19^{ab}	1.80±0.58^{b}	0.20±0.02^{a}	0.20±0.01^{a}	1.08±0.21^{b}	0.80±0.05^{a}	ni
		10	3.32±0.42^{a}	2.13±0.64^{b}	0.40±0.02^{a}	0.30±0.02^{a}	1.36±0.45^{a}	1.52±0.16^{b}	0.10±0.05^{a}
	48	2	2.60±0.71^{a}	0.80±0.28^{a}	1.02±0.01^{b}	ni	ni	0.70±0.01^{a}	0.20±0.01^{a}
		5	3.01±0.31^{a}	1.27±0.87^{ab}	1.24±0.05^{b}	0.20±0.01^{a}	ni	1.87±0.47^{b}	0.50±0.01^{a}
		10	3.94±0.82^{b}	1.38±0.48^{ab}	2.00±0.52^{b}	0.40±0.05^{a}	0.20±0.05^{a}	1.75±0.51^{b}	1.51±0.18^{b}
Gryllus bimaculatus	24	2	20.18±0.10^{c}	22.98±0.16^{c}	19.07±0.89c	20.71±0.52^{b}	23.49±0.41^{b}	22.06±1.35^{d}	21.91±0.15^{c}
		5	21.20±0.59^{c}	25.22±1.14^{d}	20.06±0.93^{c}	21.75±0.77^{bc}	24.82±0.23^{b}	23.37±1.31^{d}	23.66±0.55^{d}
		10	29.96±1.96^{e}	24.72±1.56^{cd}	23.86±0.67^{d}	23.89±0.65^{cd}	26.59±0.58^{c}	24.69±1.18^{de}	24.92±0.55^{d}
	48	2	25.54±0.48^{d}	22.51±1.33^{c}	26.79±0.783^{f}	20.21±0.91^{b}	25.47±0.69^{bc}	26.54±0.50^{e}	27.26±0.22^{f}
		5	26.78±0.47^{d}	24.30±1.05^{cd}	27.17±1.19^{f}	22.64±0.57^{c}	27.38±1.20^{cd}	27.96±0.62^{ef}	28.73±0.93^{f}
		10	29.96±1.96^{e}	27.10±0.14^{e}	29.59±1.19^{g}	23.98±0.79^{cd}	29.52±1.38^{d}	29.16±0.27^{f}	31.34±0.32^{g}
Acheta domesticus	24	2	20.16±0.82^{c}	22.94±0.60^{c}	19.87±0.55^{c}	20.57±0.38^{b}	24.60±0.90^{b}	23.67±0.40^{d}	20.79±0.65^{c}
		5	22.20±1.40^{c}	24.70±0.54^{cd}	21.75±0.67^{cd}	21.84±0.42^{bc}	25.90±0.32^{bc}	25.74±0.65^{de}	22.85±0.39^{cd}
		10	23.55±1.30^{cd}	26.44±0.45d^{e}	23.59±0.60^{d}	24.32±0.66^{d}	28.01±0.42^{d}	28.34±0.95^{f}	23.78±1.05^{d}
	48	2	23.17±0.10^{cd}	25.58±0.41^{d}	25.50±0.51^{e}	21.94±0.33^{bc}	25.05±0.28^{bc}	21.25±0.26^{cd}	21.93±0.38^{c}
		5	24.65±0.09^{d}	27.05±0.34^{e}	26.92±0.38^{ef}	23.27±0.92^{cd}	27.06±0.09^{cd}	22.48±0.48^{d}	23.44±1.15^{d}
		10	23.55±1.30^{cd}	27.26±1.66^{e}	28.77±0.30^{g}	24.79±1.14^{d}	28.46±0.49^{d}	23.66±0.09^{d}	25.06±0.81^{e}
Hermetia illucens	24	2	24.30±0.21^{d}	23.17±0.40^{c}	25.37±0.72^{e}	20.81±0.62^{b}	25.58±0.92^{bc}	19.15±0.50^{c}	23.74±0.78^{d}
		5	25.72±0.90^{d}	24.17±0.69^{cd}	26.54±1.15^{ef}	22.32±0.29^{c}	27.02±0.85^{cd}	20.27±0.34^{c}	25.61±0.57
		10	20.57±12.67^{c}	24.23±1.04^{cd}	28.97±2.1^{g}	24.21±0.22^{d}	29.08±0.50^{d}	22.97±0.62^{d}	27.76±0.50^{f}
	48	2	24.86±0.19^{cd}	23.31±0.22^{c}	23.75±0.30^{d}	19.83±0.73^{b}	25.58±0.54^{bc}	24.81±0.30^{de}	28.14±0.17^{f}
		5	26.66±0.63^{d}	25.87±0.52^{d}	25.41±0.60^{e}	21.58±0.32^{bc}	26.85±0.43^{c}	26.69±0.57^{e}	30.00±0.62^{g}
		10	28.72±0.58^{e}	27.86±0.41^{e}	28.11±0.20^{g}	23.66±0.57^{cd}	28.59±0.83^{d}	28.55±1.08^{f}	31.30±0.34^{g}

Values are means ± SD. Means with different superscript letters along the columns are significantly different at $p<0.05$. [ni] no inhibition.

The highest activity against *Escherichia coli* was observed in a fermentate of in vitro digestion and fermentation of *Gryllus bimaculatus* for 24 and 48 hours and *Hermetia illucens* chitin fermented for 24 hours at a concentration of 10 mg/mL. *Staphylococcus aureus* was more sensitive to a fermentate of in-vitro digestion and fermentation of *Gryllus bimaculatus* and *Hermetia illucens* chitin for 48 hours at a concentration of 10 mg/mL. Additionally, *Staphylococcus aureus* was more sensitive to 5 mg/mL and 10 mg/mL of a fermentate of in vitro digestion and fermentation of *Acheta domesticus* chitin for 48 hours. Similarly, chitosan synthesized from *Hermetia illucens* exuviae at a concentration of 5 mg/ml exhibited the highest inhibitory activity (23mm) against *S. aureus* (Peng et al., 2022).

Vibrio cholera was more sensitive to a fermentate at a concentration of 10 mg/mL obtained after in-vitro digestion and fermentation of *Gryllus bimaculatus* and *Acheta domesticus* chitin for 48 hours and fermentation of *Hermetia illucens* chitin for 24 and 48 hours. *Bacillus cereus* was more sensitive to a fermentate of in-vitro digestion and fermentation of *Acheta domesticus* chitin for 24 and 48 hours at a concentration of 10 mg/mL and a fermentate of *Hermetia illucens* indigestible chitin fermented 24 hours at a similar concentration. A fermentate at a concentration of 10 mg/mL obtained after in-vitro digestion and fermentation of *Gryllus bimaculatus* chitin for 48 hours and fermentation of *Acheta domesticus* and *Hermetia illucens* chitin for 24 and 48 hours exhibited the highest activity against *Bacteroides fragilis. Enterobacter agglomerans* was more sensitive to a fermentate at a concentration of 10 mg/ml obtained after in vitro digestion and fermentation of *Gryllus bimaculatus* and *Hermetia illucens* chitin for 48 hours, and *Acheta domesticus* chitin fermented for 24 hours. A fermentate at a concentration of 10 mg/mL obtained after in vitro digestion and fermentation of *Gryllus bimaculatus* chitin for 48 hours and a fermentate at a concentration of 5 mg/mL and 10 mg/mL obtained after in vitro digestion and fermentation of *Hermetia illucens* chitin for 48 hours had the highest activity against *Shigella dysenteriae*. Evidently, increasing fermentation time and fermentate concentration seemed to enhance the antimicrobial activity of the samples. Potentially an increase in fermentation time enhanced more

degradation of chitin by the probiotic microorganisms and thus generation of more derivatives. Similarly, the extent of fibre fermentation is highly dependent on intestinal transit time (Timm et al., 2010).

4.3.4 Antimicrobial Activity of Derivatives Obtained after in Vitro Digestion and Fermentation Using ABY 10 Starter Culture

The antimicrobial activity of the Fermentate of in Vitro digestion and fermentation of *Gryllus bimaculatus, Acheta domesticus* and *Hermetia illucens* using ABY 10 probiotic starter culture is shown in table 4.3. The fermentate obtained after in vitro digestion and fermentation of the different chitin samples exhibited significantly different antimicrobial activity against the tested pathogenic microorganisms ($p<0.05$). A fermentate of in vitro digestion and fermentation of *Hermetia illucens* chitin for 48 hours at a concentration of 5 mg/mL and 10 mg/mL exhibited the highest activity against *Escherichia coli*. *Staphylococcus aureus* was more sensitive to a fermentate from in vitro digestion and fermentation of *Gryllus bimaculatus* chitin for 48 hours at a concentration of 10 mg/mL (34.03 mm and 33.87 mm).

Table 4.3: Inhibition Zone (mm) of Selected Pathogenic Bacteria by a Fermentate Obtained from In Vitro Digestion and Fermentation of Chitin by ABY 10

Chitin samples Oxacillin (+Control)	**Fermentation time (hrs)**	**Concentration (mg/mL) 100 µg**	***Escherichia coli*** **14.78±1.23^{e}**	***Staphylococcus aureus*** **10.60±2.56^{f}**	***Vibrio cholera*** **33.25±0.99^{ef}**	***Bacillus cereus*** **ni**	***Bacteroides fragilis*** **15.61±2.74^{g}**	***Enterobacter agglomerans*** **9.96±1.03^{f}**	***Shigella dysenteriae*** **21.17±2.83^{g}**
Control (-)	24	2	3.04±0.07^{a}	1.56±0.16^{ab}	0.50±0.03^{a}	ni	0.20±0.01^{a}	0.30±0.05^{a}	ni
		5	3.30±0.12^{a}	1.68±0.24^{ab}	0.80±0.04^{a}	0.30±0.08^{a}	0.4±0.02^{a}	0.50±0.01^{a}	0.20±0.01^{a}
		10	4.09±0.12^{a}	2.09±0.38^{b}	1.03±0.03^{b}	0.70±0.04^{a}	0.8±0.36^{a}	0.80±0.01^{a}	0.50±0.02^{a}
	48	2	2.76±0.53^{a}	1.09±0.61^{a}	1.29±0.02^{b}	0.70±0.05^{a}	0.49±0.15^{a}	1.26±0.45^{b}	2.38±0.02^{b}
		5	3.07±0.41^{a}	2.34±0.72^{ab}	2.38±0.25^{b}	0.60±0.05^{a}	0.42±0.01^{a}	1.58±0.29^{b}	2.68±0.01^{b}
		10	3.54±0.006^{a}	2.05±0.15^{ab}	2.97±0.12^{b}	0.30±0.01^{a}	0.5±0.03^{a}	1.97±0.01^{b}	2.21±0.01^{b}
Gryllus bimaculatus	24	2	27.98±0.37^{b}	30.76±0.58^{cd}	26.78±0.64^{c}	26.77±0.48^{b}	29.74±0.43^{cd}	28.32±0.85^{b}	28.37±0.26^{c}
		5	29.45±0.17^{bc}	32.06±0.02^{d}	28.14±0.76^{d}	29.07±0.61^{c}	31.36±0.16^{de}	31.07±1.64^{cd}	30.77±0.31^{d}
		10	31.51±0.24^{c}	33.53±0.30^{de}	28.91±0.36^{d}	30.38±0.70^{cd}	32.70±0.16^{e}	32.05±1.55^{d}	32.56±0.36^{e}
	48	2	29.92±0.67^{bc}	29.47±0.59^{c}	30.78±0.30^{de}	30.41±1.29^{cd}	27.91±0.71^{b}	30.82±0.19^{c}	28.60±0.60^{c}
		5	30.96±0.54^{c}	32.04±0.03^{d}	32.55±0.13^{e}	32.00±1.16^{de}	30.55±0.33^{d}	33.07±0.10^{d}	30.73±0.41^{d}
		10	32.88±0.82^{cd}	34.03±0.42^{e}	34.61±0.87^{f}	33.76±1.76^{e}	32.31±1.24^{e}	34.66±0.64^{e}	32.80±0.65^{e}
Acheta domesticus	24	2	27.78±0.25^{b}	28.87±0.56^{c}	26.26±0.25^{c}	29.06±0.36^{c}	30.64±0.50^{d}	28.07±0.53^{b}	30.37±0.46^{d}
		5	29.20±0.42^{bc}	30.98±0.52^{cd}	27.95±0.81^{c}	30.78±0.44^{cd}	32.68±1.04^{e}	29.62±0.18^{bc}	31.71±0.70^{de}
		10	31.64±1.04^{c}	32.49±0.20^{d}	28.79±0.80^{d}	32.53±1.23^{de}	33.07±1.40^{ef}	31.44±0.46c^{d}	32.80±0.27^{e}
	48	2	29.98±0.24^{bc}	28.41±0.29^{c}	30.77±0.31^{de}	30.62±0.37^{cd}	28.64±0.34^{c}	28.64±0.74^{b}	29.67±0.44^{cd}
		5	32.46±0.38^{cd}	29.72±0.85^{c}	32.12±1.69^{e}	31.78±0.79^{d}	31.14±0.57^{de}	31.11±0.73c^{d}	32.91±1.60^{e}
		10	33.21±0.24^{cd}	31.50±0.40^{cd}	33.43±1.18^{ef}	33.49±0.57^{e}	34.06±0.46^{f}	32.80±0.84^{d}	33.88±1.83^{ef}
Hermetia illucens	24	2	27.49±0.37^{b}	29.90±0.73^{c}	27.85±0.44^{cd}	31.13±1.00^{d}	26.74±0.74^{b}	28.56±0.41^{b}	28.57±0.50^{c}
		5	29.03±0.53^{bc}	31.16±0.71^{cd}	29.92±0.82^{d}	32.41±0.87^{de}	28.38±0.59^{c}	30.22±0.09^{c}	30.55±0.46^{d}
		10	30.61±0.37^{c}	32.83±0.61^{d}	31.60±0.33^{e}	32.41±1.01^{de}	29.87±0.91^{cd}	31.17±0.68^{cd}	32.60±0.70^{e}
	48	2	32.63±0.50^{cd}	29.15±0.06^{c}	32.91±0.33^{e}	29.79±0.43^{c}	28.02±1.19^{c}	31.01±0.59^{cd}	30.27±0.78^{d}
		5	34.61±0.59^{d}	31.63±0.37^{cd}	34.37±0.72^{f}	30.65±1.08^{cd}	30.57±1.05^{d}	31.74±0.32^{cd}	31.96±0.74^{de}
		10	34.43±1.52^{d}	33.00±0.46^{de}	36.22±0.22^{g}	33.89±0.28^{e}	32.86±0.75^{e}	34.29±0.79^{e}	33.14±0.15^{ef}

Values are means ± SD. Means with different superscript letters along the columns are significantly different at $p<0.05$. [ni] no inhibition

Fermentate at a concentration of 10 mg/mL obtained after in vitro digestion and fermentation of *Hermetia illucens* chitin for 48 hours had the highest inhibition zones (36.22 mm) against *Vibrio cholera. Bacillus cereus* was more sensitive to a fermentate at a concentration of 10 mg/mL obtained after in vitro digestion and fermentation of *Gryllus bimaculatus*, *Acheta domesticus* and *Hermetia illucens* chitin for 48 hours. The highest inhibition zone (34.06 mm) against *Bacteroides fragilis* was observed in a fermentate at a concentration of 10 mg/mL obtained after in-vitro digestion and fermentation of *Acheta domesticus* for 48 hours. A fermentate at a concentration of 10 mg/mL obtained after in vitro digestion and fermentation of *Gryllus bimaculatus* and *Hermetia illucens* chitin for 48 hours exhibited the highest activity against *Enterobacter agglomerans*. Similarly, a fermentate at concentrations of 10 mg/mL obtained after in vitro digestion and fermentation of *Acheta domesticus* and *Hermetia illucens* chitin for 48 hours showed the highest antimicrobial activity against *Shigella dysenteriae*. Evidently, the antibacterial activity of the derivatives increased significantly with an increase in concentration and fermentation time.

The antimicrobial activity of samples obtained after in vitro digestion and fermentation of the chitin samples could be attributed to the metabolites of chitin breakdown. For instance, chitosan a metabolite of chitin deacetylation has been documented to have antimicrobial properties against *Staphylococcus aureus* and *Candida albicans* (Higazy et al., 2010). Chito-oligosaccharides (COS) another derivative of chitin degradation has been utilized as an antibacterial agent against *Escherichia coli, Vibrio cholera*, *Shigella dysenteriae* and *Salmonella typhimurium* (Benhabiles et al., 2012b). The concentration of metabolites of chitin degradation has a positive correlation with antimicrobial activity. For instance, chitosan at a concentration of 100 ppm exhibited significantly higher antimicrobial activity against *Escherichia coli* as compared to chitosan at a concentration of 20 ppm (Liu et al., 2006).

The antimicrobial activity of derivatives of chitin breakdown has been associated with their degree of deacetylation, polymerization and molecular weight (Costa et al., 2012). The mechanisms of antimicrobial activity of chitosan may include bacterial cell

permeabilization which results in cell death. Permeabilization results from the interaction between the positively charged amino group in the chitosan with the negatively charged molecules on the bacterial cell surface as demonstrated in organisms such as *Escherichia coli* and *Pseudomonas aeruginosa* (Costa et al., 2012; Li et al., 2016). Another mechanism suggests that chitosan inhibits the expression of DNA by binding to specific nucleic acids (Galván Márquez et al., 2013). It is further suggested that chitosan and derivatives of its breakdown inhibit bacteria growth through chelating essential metals and nutrients, including *Escherichia coli***,** *Staphylococcus aureus*, and *Bacillus subtilis* (Mohandas et al., 2018).

Given the study findings, the fermentate of chitin in vitro digestion and fermentation may have beneficial effects on the gut such as altering the intestinal flora composition and conferring gut health. Chitin and its derivatives are the functional dietary fibre that can reduce LDL-cholesterol levels in the blood, promote the growth of beneficial gut microbiota, and enhance immune system function (Caparros Megido et al., 2014; Choi et al., 2012).

4.4 Conclusion

The results indicate that the ABY 10 starter culture was more efficient in fermenting the chitin samples as compared to ABT 5. Furthermore, *Hermetia illucens* chitin was easily fermented by the starter culture, followed by *Gryllus bimaculatus* chitin while *Acheta domesticus* chitin was less easily fermented. It is clear that chitin in vitro digestion and fermentation yielded derivatives with significant antioxidant and antimicrobial properties. The derivatives of in-vitro digestion and fermentation of the chitin samples showed significant inhibition of the growth of gram-negative and gram-positive pathogenic bacteria. Evidently, fermentation time and sample concentration significantly influenced the antioxidant and fermentation capacity of chitin-derived products during in vitro digestion and fermentation. Based on this evidence, we conclude that chitin could have beneficial health effects in the gut. Therefore, chitin inclusion in food products as an ingredient or consumption of whole insects would potentially promote gut health.

CHAPTER FIVE

IDENTIFICATION AND QUANTIFICATION OF DERIVATIVES OF IN VITRO DIGESTION AND FERMENTATION OF EDIBLE INSECT'S CHITIN

5.1 Introduction

Globally, the prevalence of diet related chronic diseases has been on the increase, thus consumers have become more nutrition savvy and there is a consistent demand for nutritious and healthy diets (Lamas et al., 2016; Miranda et al., 2015). Further, there has been an increase in the consumption of foods containing bioactive compounds for purposes of enhancing good health (Roca-saavedra et al., 2017). However, the consumption of foods with high dietary fiber (DF) is considerably low despite DF having a broad spectrum of health benefits which include preventing obesity, diabetes type 2 and carcinogenesis among other health benefits (Hijová et al., 2019). The major sources of DF for many consumers are cereals, nuts, beans, fruits and vegetables (Cai et al., 2020). It is worth noting that there are some non-vegetable sources of DF such as tunicates and edible insects which contain chitin (Lopez-Santamarina et al., 2020). Chitin content in edible insects' ranges from 5 to 15% with the variance being dependent on insect species as well as stage of development (Spranghers et al., 2017). Chitin has been found to be nontoxic, biodegradable and film-forming (Ali et al., 2024). Further studies indicate that chitin and its derivatives, such as chitosan have antimicrobial and antioxidant capabilities.

An in-depth understanding of the derivatives of chitin modification and their structural and functional properties is critical for their application for the welfare of human health (Ishraque et al., 2020). Studies have documented enhanced growth of beneficial bacteria in the gut after consumption of cricket powder as well as synthesis of SCFA and branched-chain fatty acids after the consumption of *Tenebrio molitor* insect flour (DeCarvalho et al., 2019; Stull et al., 2018). Acetate, propionate and butyrate which are the primary SCFAs produced by the fermentation process, serve as key signaling

molecules that modulate host metabolism, immune function and gut barrier integrity. Few studies employing in vitro colonic fermentation models have shown that insect chitin selectively enhances the growth of *Ruminococcaceae*, *Lachnospiraceae* and *Faecalibacterium* genera (Kipkoech et al., 2021). The selective microbial modulation occurs without significant reduction in total SCFA thus suggesting that chitin may exert prebiotic effects primarily through biodiversity enhancement rather than quantitative increases in fermentation end products (Reichardt et al., 2017). Other than SCFA, the enzymatic degradation of chitin produces chitooligosaccharides (COS) of varying degrees of polymerization and acetylation patterns which show different bioactivities such as antimicrobial and antioxidant properties (Refael et al., 2022). The production of these derivatives is influenced by the crystallinity, degree of acetylation and particle size of chitin which affect enzyme accessibility and fermentation kinetics (Tao et al., 2026). Evidence further shows that COS metabolism involves cross-feeding interactions among bacterial species where primary degraders release oligosaccharide intermediates that are subsequently utilized by secondary fermenters, hence shaping bacteria community composition and metabolic output (Mohan, Ganesan, Ezhilarasi, Kondama, et al., 2022).

Evidently, from a health and nutrition perspective, very few studies have documented the colonic fermentability of edible insects and chitin which is a major fiber component (Refael, Riess, et al., 2022). Further studies suggest that for the purposes of considering chitin as a nutraceutical that enhances gut health, it would be prudent to understand its functional impacts which requires analysis of bioactive compounds of chitin metabolism by gut microbiota (Zhang et al., 2018). Therefore, this study aimed to investigate the derivatives of in vitro digestion and fermentation of chitin extracted from crickets and BSF cocoons.

5.2 Methods

5.2.1 Chitin Extraction, in Vitro Digestion and Fermentation

Chitin extraction, in vitro digestion and fermentation was done as described in section 3.21, 4.2.2 and 4.2.3 respectively.

5.2.2 Fatty Acid Composition

Gas chromatography-mass spectrometry (GC-MS) was used to analyze the fatty acid composition with methyl esterification being done by a method described earlier by Cheseto and others (Cheseto et al., 2020). A portion of the freeze dried supernatant obtained after fermentation of the indigestible chitin (0.1 grams) was mixed with sodium methoxide in methanol (0.5 mL) at a concentration of 15 mg/mL. The mixture was vortexed and then sonicated for 10 sec and 5 min, respectively. The reaction mixture was placed in an incubator maintained at 60°C for 1 hour before the addition of deionized water (100 µL) and vortexing for 1 min. A GC-grade hexane (1 mL) was used to extract the resultant methyl esters before a 5 min centrifugation at 14,000 rpm was effected. Anhydrous sodium sulphate was then used to dry the supernatant before its transfer to vials. An auto sampler 7683 (Agilent Technologies, Inc., Beijing, China) was used to inject methyl esters (1 µL), in the splitless mode, into a GC-MS (7890A gas chromatograph; Agilent Technologies, Inc., Santa Clara, CA, USA) fitted with a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC's inlet temperature was maintained at 270°C, while that of the transfer line was set at 280°C. The column oven's temperature was preset to rise from 35°C to 285°C, with the former temperature being held for 5 min followed by a 10°C increase for every minute until the temperature reached 280°C, where it was maintained for 20.4 min. A low bleed capillary column (HP5) of miniature 30 m × 0.25 mm i.d., 0.25 µm (J&W, Folson, CA, USA) was used together with helium as a carrier gas, flowing at the rate of 1.25 mL/min. The mass selective detector was maintained at quadrupole (180°C) and ion source (230°C) temperatures. Electron impact (EI) mass spectra was obtained at the

acceleration energy of 70 eV with fragment ions being analyzed over 40–550 m/z mass range in the full scan mode, having set a 3.3-min delay time for the filament. Fatty acids were identified via their methyl esters using comparisons of their fragmentation patterns and retention times to those of known fatty acid methyl ester standards where available and tentatively from their reference spectra published by library–MS databases: National Institute of Standards and Technology (NIST) 05, 08, and 11. Standard methyl octadecenoate (≥ 95 % purity, Sigma-Aldrich, St. Louis, MO)of known concentrations (0.2, 5, 25, 50, 75, 100 and 125 ng/µL) was subsequently analyzed under the same GC-MS conditions to obtain a linear calibration curve (y = 7E + 06x – 4E + 07(R2 = 0.9757) that was used for external quantification of the fatty acid methyl esters. The quantities were presented in percentage of total fatty acids.

5.2.3 Determination of Chitosan

Chitosan was determined using FMOC-Cl derivation and HPLC determination as described by (B. Li et al., 2013; Zhou et al., 2005; X. Zhu et al., 2005b).

5.2.3.1 Hydrolysis

Hydrolysis was done according to a method described by. The freeze dried supernatant (10 Milligrams) was accurately weighed and transferred to a hydrolysis tube. Five milliliters of water and 5 mL of mixed acid solution of HCl–H3PO4 (prepared with 75 mL HCl and 25 mL 12 M H3PO4) were added to the tube, such that the final concentrations of HCl and H3PO4 were 4.5 and 1.5 M, respectively. The tube was vacuum-treated, flushed with N2 and then fuse-sealed to avoid the influence of oxygen. Acid hydrolysis was performed at 110 °C for 24 h to generate GlcN. The tube was then cooled and kept at 4 °C till derivation.

5.2.3.2 Derivation

For chitosan samples, the hydrolysate (2.0 mL) was transferred to a volumetric flask and mixed with 1.0 mL internal standard (IS, 0.8 mg/mL a-aminobutyric acid solution). The

mixture was neutralized with 2 M NaOH in an ice bath, mixed with 25 mL acetonitrile, and then diluted to 50 mL with water. The solution was filtered with 0.45 lm membrane. Then 200 µl of the filtrate was mixed with 200 µl 0.25 M borate buffer (pH 8.0) to adjust pH of the solution. Glucosamine and IS was then derived with the addition of 100 ll FMOC-Cl solution (20 mM, in acetonitrile). The mixture was allowed to react at room temperature for 5 min. After the derivation was complete, the reaction was stopped with the addition of 200 µl acetonitrile-acetate acid solution (8:2, v/v) to remove excess FMOC-Cl. For GlcN reference, 2.0 mL GlcN solution (1 mg/mL) was mixed with 1.0 mL IS solution and 25 mL acetonitrile. The solution was then diluted to 50 mL with water and treated in the same manner as hydrolyzed chitosan solutions.

5.2.3.3 LCMS Determination

Separation and detection of the FMOC-Cl derivatives were performed on a Waters liquid chromatography system consisting of a 1525 binary pump, a 717 plus autosampler, and a 2487 dual absorbance detector. Data were collected and processed using the Waters Breeze software. Reversed-phase separation was carried out on a Symmetry C18 column (4.6 × 150 mm, 5 lm, Waters Co.). A solution of NH4Ac buffer (10 mM, pH 5.0) and acetonitrile (80:20) was used as mobile phase A and acetonitrile was used as mobile phase B, with gradient program shown in Table 4. The temperature of the column was maintained at 35 °C and detection was performed at 262 nm. The IS was eluted at 9.4 min, and GlcN showed two peaks at 5.5 and 6.1 min after derivation, presumably representing the anomers of FMOC-GlcN.37 GlcN concentration was quantified with the area ratio of GlcN at 6.1 min to IS. Chitosan content was calculated using the following equation.

$$Chitosan\ content = \frac{Gsam \times F \times 0.899}{w \times (1 - Cwater} \qquad (11)$$

Whereby *Gsam* is the amount of GlcN in hydrolysate, w is the amount of sample weighed, *Cwater* is the water content of the chitosan and *F* is the factor of GlcNAc.

5.2.4 Determination of Chito-Oligosaccharides (COS)

Chito oligosaccharide content was determined using a method described by Chang et al., (2000). The freeze-dried samples were hydrolyzed as described in section 5.2.2.1. The identification and amount of COS was determined using HPLC analysis with a Waters liquid chromatograph (Millipore Corp., Milford, MA, USA). The HPLC system was fitted with two Waters Model 510 HPLC pumps and a Waters 490E programmable multi-wavelength detector. A reversed phase LiChrospher 100NH2 (5 µm, 4 × 250 mm) column from E. Merck (Darmstadt, Germany) was used. Eluents containing different ratios of acetonitrile to water were used to determine the optimal mobile phase. The flow rate was 1 mL/min; the injection volume was 20 µL. The UV absorbance at 205 nm was monitored. For the quantification of hydrolyzed samples, standard solutions of COS (containing 2000 ppm of each COS) were used for calibrations. The peak areas of COS of the standard solution and hydrolyzates were integrated by Chromatography Data Station software (Scientific Information Service Co., Taipei, Taiwan). The ratios between the peak areas of samples and standards were used to calculate the weight of COS in the original hydrolysate solution.

5.2.5 Determination of Antimicrobial Peptides (AMPs)

The sample were analyzed by Agilent Liquid Chromatography Mass Spectrometry Mass Spectrometry (LCMSMS) as described by Alkotaini et al., 2014 and Chen et al., (2019). Amphipathic AMPs were extracted with 66.7% ethanol. Liquid chromatography was accomplished on a Poroshell 120 C18 5µm, 5.0mm×250mm with the mobile phase being 0.1% formic acid in water for Solvent A and 0.1% formic acid in acetonitrile for Solvent B. Elution was done at a gradient of 1–30% of solvent B in solvent A for 15min at a flow rate of 1ml/min, followed by a 5-min rinse with 70% of solvent B in solvent A. The eluted sample was directly exposed to positive electrospray ionization performed using capillary and cone voltages at 3kV and 20V, while the source temperature was maintained at 80°C. Collision energies were set at 4V and 15–40V for both MS and MS/MS. Peptide sequencing was performed by data directed analysis (DDA), where the

only input required was the intensity threshold to trigger MS/MS acquisition and the MS/MS acquisition time. During the course of MS/MS acquisition, the collision energy was ramped up with a pre-defined collision energy profile, maximizing data quality and information while minimizing user input and the time required. The standard used was HPLC peptide standard mixture, purchased from sigma Aldrich Germany.

5.2.6 Determination of Vitamin A

Determination of vitamin A was done according to the methods described by Cheseto et al., 2020 and Jermacz et al 2008. About 300 Milligrams of freeze dried supernatant obtained after fermentation of the indigestible chitin was transferred into a 10 mL glass vial containing a mixture of hexane, methanol and distilled deionized water (2:1:2, 5 mL), vortexed for 30 s, sonicated for 30 min and centrifuged at 14,000 rpm for 5 min. The supernatant was dried over anhydrous Na2SO4, evaporated to dryness under a gentle stream of N2 (g) before derivatizing any residual fatty acids to fatty acid methyl esters to limit the matrix interference. About 1.0 µL of the sample was analyzed using GC-MS on a 7890. A gas chromatograph linked to a 5,975 C mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA). The GC was fitted with a (5%-phenyl) - methylpolysiloxane (HP5 MS) low bleed capillary column (30 m × 0.25 mm i.d., 0.25µm; J&W, Folsom, CA, USA). Helium at a flow rate of 1.25 mL min−1 served as the carrier gas. The oven temperature was programmed from 35°C to 285°C, with the initial temperature maintained for 5 min, with a rise at 10°C min−1 to 280°C, and then held at this temperature for 20.4 min. The mass selective detector was maintained at ion source temperature of 230°C and a quadrupole temperature of 180°C. Electron impact (EI) mass spectra was obtained at the acceleration energy of 70 eV. Fragment ions was analyzed over 40–550 m/z mass range in the full scan mode. The filament delay time was set at 3.3 min. Serial dilutions of the authentic standard retinol (≥ 95.5% purity) (0.1–100 ng/µL, Sigma- Aldrich, St. Louis, MO) was analyzed by GC-MS in full scan mode to generate a linear calibration curves (peak area vs. concentration) which gave a coefficient of determinations R2 = 0.9999. The regression equation was used for the external quantification of vitamin A. These compounds were identified by comparison

of mass spectral data and retention times with those of authentic standards and reference spectra published by library–MS databases: National Institute of Standards and Technology (NIST) 05, 08, and 11. The samples were analyzed in triplicate, with each replicate collected from a different batch of respective samples.

5.2.7 Determination of B Vitamins (B2 and B12)

The methods used by Chase, Landen, and Soliman, (1993); Ekinci and Kadakal, (2005); Kammman, Labuza, and Warthesen, (1980) was modified and used to determine concentration of riboflavin and cyanocobalamin. These concentrations were determined in triplicates. A 20 mL of deionized water was added to a 50 ml volumetric flask containing 5g of freeze dried supernatant. The resulting solution in each case was evenly mixed using a homogenizer (PCU11/98000-18, Kinematica AG Littau, Switzerland) at medium speed for 1 min. The mixture was centrifuged for 15 min at 1500 rpm and water soluble vitamins extracted using Sep-Pak C18 (500 mg) cartridges method as described by Cho, Ko, and Cheong, (2000). Vitamin extracts were passed through FP 30/45 CA-S filters (Schleicher and Schuell, Darmstadt, Germany) with 0.45 μm micropore membrane. A syringe was used to inject 0.45 μm of the filtrate into HPLC column (20A Series, Shimadzu Co-operation, Kyoto, Japan) with a set flow rate of 0.1 mol L-1. The column measurements for this chromatograph was C18 150 mm x 4.6 mm. The mobile phase comprised of KH2PO4 at PH 7.0 and methanol in a ratio of 90:10 and was set in isocratic mode at 0.7 mL min-1. The vitamins were identified by comparing their retention times and UV-visible spectra with those of standards stored in a data bank at 266 nm for riboflavin and 360 nm for cyanocobalamin.

5.2.8 Determination of Total Polyphenols

Total polyphenols were analyzed using a method described earlier by Waterman and Mole (Waterman & Mole, 1994) with gallic acid as a standard. A 10 grams fermentate sample was mixed with50%aqueousmethanol (20mL) at 80°Cfor 1h. The solution was then filtered, and volume made to 50mL. A portion of the solution (1 mL) was

transferred to a volumetric flask (50mL) before adding 20 mL of water, Folin Denisreagent (2.5mL) and17% sodium carbonate (10mL). The mixture was then homogenized, before being topped up to 50 mL using distilled water after which it was left undisturbed for 20 min. Its absorbance was then measured in a 1-cm glass cell at 765 nm on a Uv-visible spectrophotometer (SP65, Gallenkamp, UK). Total polyphenols were calculated as gallic acid equivalents (GE) from a linear calibration curve obtained from gallic acid absorbance readings.

5.2.9 Data Analysis

Data was reported in means and standard deviations. To determine the effect of the chitin sample, fermentation time and sample concentration on fatty acid content, chitosan, chito-oligosaccharides, AMPS and vitamins data were subjected to a three-way analysis of variance (ANOVA) followed by mean separation by Bonferroni's method at $P \leq 0.05$. Data was analyzed using Stata version 17 and presented using graphs and tables.

5.3 Results and Discussion

5.3.1 Derivatives of in Vitro Digestion and Fermentation of Chitin

5.3.1.1 Fatty Acids

Short Chain Fatty Acids

Close to three quarters of the total fatty acids in the fermentate obtained after in vitro digestion and fermentation of chitin samples were SCFA followed by Lauric acid and then trace amounts of other saturated and unsaturated fatty acids. In the control sample the predominant fatty acid was propionic acid. The predominant SCFA in all the samples was acetic acid, followed by propionic acid then butyric acid as well as trace levels of 4 methyl valeric acid, iso- valeric acid and valeric acid as shown in table 5.1. Similarly, in a study where indigestible cricket chitin was subjected to faecal

fermentation acetate was the major SCFA followed by propionic and butyrate acid with valeric acid detected in minimal levels (Refael, Riess, et al., 2022). Evidence further suggests that acetate accounts for about 60% of total SCFA followed by propionate and butyrate acid while lactate isomers and other branched SCFA such as isobutyric and iso valeric are generated in minimal levels in our gut metabolome (Topping & Clifton, 2001). The physiological benefits of these metabolites are highly concentration-dependent; in a healthy gut environment SCFAs typically represent approximately 75% to 90% of the total fatty acid profile. This relative dominance ensures that absolute SCFA concentrations remain within the range of 50 to 150 mmol/L in the proximal colon, where they serve as critical signaling molecules for immune regulation and intestinal barrier integrity (Ríos-covián et al., 2016). At these levels, butyrate specifically acts as the primary energy source for colonocytes while the collective pool of SCFAs helps maintain a slightly acidic luminal pH, which suppresses the overgrowth of pH-sensitive pathogenic bacteria.

Table 5.1: Short Chain Fatty Acids (% of Total Fatty Acids) Obtained after In Vitro Digestion and Fermentation of Chitin

			Short chain fatty acids						Other fatty acids					
Chitin samples	**Starter culture**	**Fermentation time (hrs)**	**Acetic acid**	**Propionic acid**	**Butyric acid**	**4 methyl valeric acid**	**Iso-valeric acid**	**Valeric acid**	**Lauric acid**	**Stearic acid**	**Palmitic acid**	**Palmitoleic acid**	**Oleic acid**	**Linoleic acid**
Control	ABY 10	24	3.26±0.32^{a}	95.24±0.3^{f}	0.06±0.03^{a}	0.01±0.01^{a}	nd	nd	Nd	nd	nd	nd	nd	nd
		48	6.00±0.15^{b}	90.65±2.5^{e}	0.71±0.20^{b}	0.27±0.06^{a}	nd	nd	Nd	nd	nd	nd	nd	nd
	ABT 5	24	0.85±0.26^{a}	96.57±2.14^{f}	0.03±0.01^{a}	0.02±0.01^{a}	nd	0.01±0.01^{a}	Nd	nd	nd	nd	nd	nd
		48	2.91±0.45^{b}	92.42±2.25^{e}	0.61±0.34^{b}	0.09±0.02^{a}	nd	0.01±0.01^{a}	Nd	nd	nd	nd	nd	nd
Gryllus bimaculatus	ABY 10	24	60.28±1.15^{g}	9.58±0.89^{a}	10.37±0.98^{d}	2.69±1.17^{cd}	nd	0.41±0.49^{b}	10.95±0.91^{d}	3.76±0.76^{b}	1.95±0.10^{a}	1.41±0.22^{a}	1.96±0.05^{a}	0.43±0.88^{a}
		48	59.40±1.25^{g}	13.89±1.44^{b}	15.33±1.63^{e}	2.62±0.69^{cd}	nd	1.27±0.75^{c}	6.15±1.22^{b}	0.64±0.56^{a}	0.66±0.56^{a}	0.33±0.27^{a}	0.67±0.58^{a}	0.87±0.72^{a}
	ABT 5	24	48.05±4.02^{d}	15.93±3.49^{c}	7.51±1.32^{c}	2.71±0.49^{cd}	nd	1.42±0.34^{c}	10.95±1.35^{d}	1.84±0.41^{a}	1.06±0.42^{a}	2.01±0.01^{ab}	2.60±0.53^{ab}	0.20±0.24^{a}
		48	54.16±1.00^{f}	17.34±1.05^{d}	8.45±1.26^{c}	1.01±0.15^{b}	nd	1.45±0.95^{c}	11.15±0.89^{d}	1.78±0.29^{a}	1.04±0.07^{a}	1.61±0.46^{a}	1.68±0.60^{a}	1.99±0.66^{a}

			Short chain fatty acids					Other fatty acids						
Chitin samples	**Starter culture**	**Fermentation time (hrs)**	**Acetic acid**	**Propionic acid**	**Butyric acid**	**4 methyl valeric acid**	**Iso-valeric acid**	**Valeric acid**	**Lauric acid**	**Stearic acid**	**Palmitic acid**	**Palmitoleic acid**	**Oleic acid**	**Linoleic acid**
Acheta domesticus	ABY 10	24	52.90±1.69^{ef}	13.75±3.89^{b}	9.24±1.06^{cd}	2.07±0.68^{c}	nd	0.69±0.44^{b}	10.37±1.14^{d}	1.20±0.10^{a}	2.36±1.35^{a}	3.57±0.52^{b}	2.16±0.38^{ab}	1.17±1.87^{a}
		48	55.78±0.58^{f}	15.24±1.73^{c}	10.88±1.26^{d}	2.41±0.18^{c}	0.76±0.17^{b}	1.03±0.05^{c}	12.21±2.89^{d}	1.10±0.21^{a}	0.59±0.40^{a}	1.66±0.51^{a}	0.84±0.32^{a}	0.25±2.61^{a}
	ABT 5	24	40.86±1.57^{c}	13.15±0.06^{b}	8.66±0.94^{c}	2.33±1.11^{c}	nd	0.52±0.50^{b}	12.17±1.25^{d}	5.11±0.24^{c}	1.34±0.59^{a}	6.92±0.19^{c}	5.07±0.91^{c}	3.28±1.91^{b}
		48	43.50±1.66^{c}	14.41±1.16^{bc}	8.03±1.63^{c}	2.25±0.34^{c}	0.55±0.40^{b}	nd	11.45±1.06^{d}	6.72±1.25^{c}	1.04±0.66^{a}	7.99±1.02^{c}	3.09±0.28^{b}	0.94±0.17^{a}
Hermetia illucens	ABY 10	24	63.94±2.10^{h}	8.76±1.60^{a}	8.91±1.71^{c}	1.87±0.80^{b}	0.03±0.01^{a}	0.78±0.21^{b}	8.13±2.13^{c}	0.93±0.08^{a}	1.74±0.86^{a}	2.89±0.25^{ab}	2.35±1.50^{ab}	1.37±2.63^{a}
		48	64.75±0.31^{h}	12.15±0.98^{bc}	11.86±1.63^{d}	2.59±0.45^{d}	0.53±0.19^{b}	0.59±0.45^{b}	4.18±0.79^{a}	0.90±0.1^{a}	0.47±0.89^{a}	0.97±0.05^{a}	0.11±0.16^{a}	0.98±0.96^{a}
	ABT 5	24	50.15±1.02^{e}	9.97±1.68^{a}	10.40±0.54^{d}	3.88±1.41^{d}	nd	nd	11.47±1.01^{d}	3.96±0.09^{b}	1.58±0.51^{a}	2.83±0.29^{ab}	3.96±0.98^{b}	2.76±0.46^{ab}
		48	52.47±0.59^{e}	18.06±1.39^{d}	9.34±1.13^{cd}	2.00±0.06^{c}	nd	0.66±0.18^{b}	11.32±1.32^{d}	1.71±0.61^{a}	0.72±0.54^{a}	0.98±0.09^{a}	1.35±0.26^{a}	1.11±0.18^{a}

Values are means ± SD. Means with different superscript letters along the columns are significantly different at $p<0.05$. [nd] not detected. [Control] (ABY 10 and ABT 5 cultured using lactose alone)

The SCFA content was significantly influenced by the type of indigestible chitin, starter culture used and fermentation time ($p<0.05$). The highest levels of acetic acid were reported in *Hermetia illucens* indigestible chitin fermented using ABY 10 starter culture for 24 and 48 hours while the least levels were reported in the control. The control yielded the highest levels of propionic while *Gryllus bimaculatus* indigestible chitin fermented using ABY 10 for 24 hours yielded the least levels. The control yielded higher values for propionic acid, potentially because of the substrate lactose which was converted to lactic acid and then potentially to propionic acid. *Gryllus bimaculatus* indigestible chitin fermented using ABY 10 for 48 hours yielded the highest levels of butyric acid followed by *Hermetia illucens* indigestible chitin fermented using ABY 10 for 48 hours while the control had the least yield.

Evidently, fermentation of indigestible chitin samples for 48 hours using ABY 10 starter culture yielded significantly high levels of the SCFA. This observation could be explained by the composition of the ABY 10 starter culture which had an additional prebiotic strain. This therefore demonstrates that diversity of probiotics colonizing the gut may potentially influence the utilization of prebiotics in the gut such as indigestible chitin and thus consequently influence the levels of metabolites generated from the degradation process. The increase in yield of SCFA with an increase in fermentation time to 48 hours indicates that the more the time the probiotics interact with the indigestible chitin, the more the metabolism. Chitin in the gut is degraded to chitosan and further to chito-oligosaccahrides. Chito-oligosaccharides are considered as potential prebiotics and are further degraded by gut microflora to produce SCFA and other bioactive compounds (Selenius et al., 2018). For instance, faecal fermentation of COS using mice fecal matter yielded SCFA with acetate being the most predominant SCFA (Zhang et al., 2018). Studies suggest that the fermentation of indigestible fiber mostly generates SCFA which are normally biomarkers of gut health (Filippis et al., 2015; Singh et al., 2017). A majority of the SCFA are normally absorbed by the colonic epithelial cells and are further metabolized by the colon cells and only 5-10% of the SCFA are excreted in the faecal matter (Wang et al., 2019). The SCFA in the gut

normally confers health benefits, for instance butyrate is highly metabolized by colon cells and prevents colon carcinogenesis (Wu et al., 2018). Furthermore, the SCFA causes acidification of the large intestines, which further enhances uptake of ammonia by the bacterial mass, which consequently promotes the growth of beneficial bacteria and inhibits the growth of pathogens (Topping & Clifton, 2001). Further generation of SCFA in the GIT has been associated with reduced gut inflammation, improved mucosal gut barrier and reduction in risk of metabolic conditions such as obesity (Wolter et al., 2021; Zmora et al., 2019). Additionally, SCFAs have been reported to regulate the immune system and inflammation by reducing the recruitment and migration of immune cells and the differentiation of B cells and T cells (Yao et al., 2022).

Other Fatty Acids

Saturated fatty acids detected included considerable levels of Lauric acid and minimal levels of stearic and palmitic acid. Furthermore, two monounsaturated fatty acids detected after fermentation of the indigestible chitin samples were palmitoleic and oleic acid. Additionally, linolenic a polyunsaturated fatty acid was also detected in trace levels. The presence of various fatty acids after fermentation suggests that the process may be breaking down the substrate to yield these fatty acids. Further probiotic microorganisms have been reported to generate fatty acids through pathways such as bifidobacterium pathway (Louis et al., 2007). This could be valuable for potential applications in nutrition or industrial uses. Similarly, in a study assessing bioactive compounds obtained after fermentation of rice bran palmitic, palmitoleic, oleic and linoleic fatty acids were detected (Zhang et al., 2018). These fatty acids contribute to human health by modulating inflammatory responses, supporting cell membrane structure and fluidity, enhancing the absorption of fat-soluble vitamins and improving cardiovascular health through the regulation of lipid profiles (Calder, 2015).

5.3.1.2 Chitosan

The type of chitin source, type of starter culture and fermentation time had a significant effect on the chitosan yields ($p<0.05$) as shown in table 5.2. *Acheta domesticus* indigestible chitin fermented using ABY 10 and ABT 5 for 48 hours and *Hermetia illucens* chitin fermented using ABY 10 for 48 hours yielded the highest chitosan levels. Evidently, *Acheta domesticus* indigestible chitin was easily converted to chitosan by the 2 starter cultures while *Hermetia illucens* indigestible chitin was easily converted to chitosan by ABY 10. Further a fermentation time of 48 hours was optimal for the conversion process. The values obtained in the present study were significantly lower than 13.1 g/100g which was obtained through fermentation of chitin using 10 g/100g *Serratia marcescens* culture for 4 days (Zhang et al., 2021). Similarly, in vitro conversion of chitin using *Alcaligens faecalis,* a chitin deacetylase producing bacteria yielded 19 g/100g chitosan which was considerably higher than the values obtained in the present study (Rakshit et al., 2023). The type of bacteria used, bacterial culture concentration and fermentation time could probably explain the observed difference.

The human gut has some digestive enzymes that are able to break down chitin to some extent (Selenius et al., 2018). Chitosan has been associated with enhanced gut health for instance, chitosan has been documented to have antimicrobial activity against *Escherichia coli*, *Vibrio cholerae*, *Shigella dysentriae* among other pathogenic bacteria (Piccolo et al., 2017). Chitosan has been cited as one of the most significant chitin derivatives due to its prebiotic potential, biocompatibility, low toxicity and mutagenic properties (Zoysa, 2017). Evidence suggests that chitosan promotes the growth of lactic acid and SCFA genera such as Bifidobacterium (Hamed et al., 2016).

Table 5.2: Derivatives Obtained after In Vitro Digestion and Fermentation of Chitin

Chitin	Starter culture	Fermentation time	Chitosan (g/100g)	COS (mg/g)	Defensin like AMP (mg/g)	Alpha helical like AMP (mg/g)	Proline like AMP (mg/g)	Vitamin A (mg/100g)	Vitamin B2 (mg/100g)	Vitamin B12 (mg/100g)	Total polyphenols (mg GAE/g)
Control	ABY 10	24	nd	nd	nd	nd	nd	nd	nd	0.01 ± 0.01^{a}	nd
		48	nd	nd	0.01 ± 0.01^{a}	nd	0.01 ± 0.01^{a}	nd	nd	0.30 ± 0.01^{b}	nd
	ABT 5	24	nd	nd	nd	nd	nd	nd	nd	0.03 ± 0.02^{a}	nd
		48	nd	nd	nd	nd	nd	nd	nd	0.30 ± 0.02^{b}	nd
Gryllus bimaculatus	ABY 10	24	1.86 ± 0.13^{a}	7.94 ± 0.08^{c}	12.11 ± 0.100^{e}	3.34 ± 0.67^{c}	1.93 ± 0.13^{c}	1.13 ± 0.26^{ab}	0.79 ± 0.01^{bc}	1.00 ± 0.01^{c}	1.18 ± 0.16^{a}
		48	2.61 ± 0.12^{b}	9.82 ± 0.01^{e}	19.22 ± 0.22^{g}	4.33 ± 1.43^{d}	1.20 ± 0.20^{bc}	2.25 ± 0.13^{c}	2.17 ± 0.20^{d}	5.74 ± 0.65^{g}	3.89 ± 0.34^{cd}
	ABT 5	24	1.85 ± 0.23^{a}	8.34 ± 0.25^{de}	11.67 ± 0.53^{e}	0.52 ± 0.08^{a}	0.92 ± 0.09^{b}	nd	nd	0.95 ± 0.01^{c}	2.78 ± 0.71^{b}
		48	2.69 ± 0.25^{b}	8.01 ± 0.05^{de}	16.63 ± 5.45^{f}	1.93 ± 0.11^{b}	0.86 ± 0.06^{b}	nd	0.52 ± 0.42^{b}	1.02 ± 0.03^{c}	3.82 ± 0.20^{cd}
Acheta domesticus	ABY 10	24	2.62 ± 1.21^{bc}	8.73 ± 0.12^{de}	13.24 ± 2.91^{ef}	2.01 ± 0.28^{bc}	1.38 ± 0.17^{bc}	1.66 ± 0.27^{b}	1.91 ± 0.37^{cd}	3.28 ± 0.95^{e}	3.22 ± 0.24^{c}
		48	2.91 ± 0.21^{c}	9.10 ± 0.02^{e}	19.40 ± 0.91^{g}	4.30 ± 0.36^{d}	1.20 ± 0.23^{bc}	1.70 ± 0.56^{b}	2.14 ± 0.12^{d}	3.82 ± 0.43^{e}	4.13 ± 0.44^{d}
	ABT 5	24	2.60 ± 0.10^{b}	9.07 ± 0.28^{e}	11.45 ± 1.42^{e}	1.75 ± 0.40^{b}	0.70 ± 0.30^{b}	1.35 ± 0.21^{b}	nd	1.11 ± 0.39^{c}	1.91 ± 0.20^{a}
		48	2.90 ± 0.29^{c}	9.37 ± 0.42^{e}	11.46 ± 1.47^{e}	2.61 ± 0.36^{c}	1.23 ± 0.09^{bc}	1.47 ± 0.21^{b}	0.91 ± 0.15^{bc}	1.94 ± 0.15^{cd}	2.78 ± 0.20^{b}
Hermetia illucens	ABY 10	24	2.22 ± 0.03^{b}	5.98 ± 0.28^{c}	11.85 ± 1.67^{e}	2.03 ± 0.05^{bc}	1.12 ± 0.07^{bc}	1.30 ± 0.09^{ab}	1.13 ± 0.13^{c}	4.05 ± 0.14^{ef}	3.14±0.15bc
		48	2.90 ± 0.10^{c}	8.67 ± 0.16^{de}	18.32 ± 0.91^{g}	2.45 ± 0.42^{c}	2.79 ± 0.42^{d}	2.13 ± 0.23^{c}	1.96 ± 0.11^{cd}	4.79 ± 0.42^{f}	4.01±0.01
	ABT 5	24	1.68 ± 0.31^{a}	7.69 ± 0.51^{d}	6.91 ± 0.86^{d}	1.10 ± 0.26^{b}	1.96 ± 0.95^{c}	0.51 ± 0.12^{a}	0.07 ± 0.05^{a}	1.02 ± 0.09^{c}	2.82±0.38b
		48	2.69 ± 0.43^{b}	8.48 ± 0.31^{de}	13.90 ± 2.19^{ef}	1.94 ± 0.12^{b}	1.73 ± 0.09^{c}	2.22 ± 0.19^{c}	0.32 ± 0.07^{b}	2.24 ± 0.16^{d}	3.04±0.06bc

Values are means ± SD. Means with different superscript letters along the columns are significantly different at $p<0.05$. [nd] not detected

5.3.1.3 Chito- Oligosaccharides

Chito-oligosaccharides (COS) was another derivative of the indigestible chitin fermentation process. *Gryllus bimaculatus* chitin fermented using ABY 10 for 48 hours, *Acheta domesticus* indigestible chitin fermented using ABY 10 for 48 hours and *Acheta domesticus* indigestible chitin fermented using ABT 5 for 24 and 48 hours yielded the highest levels of COS while no levels were detected in the control. In vivo studies indicated that COS enhanced the growth of *Bifidobacterium, Lactobacillus, Akkermansia* and *Bacteroides* which demonstrated the prebiotic potential of COS (Gomaa, 2020; Hamed et al., 2016; Lopez-Santamarina et al., 2020). Further in vitro studies showed that COS inhibited the growth of pathogenic bacteria such as *Escherichia coli* in the colon and caecum (J. Wan et al., 2017; Yan & Kim, 2011). COS have been used as antimicrobial agents against food borne pathogens such as *Salmonella typhimurium, Shigella dysenteriae* and *Vibrio cholerae* (Benhabiles et al., 2012a).Additionally COS have been shown to have antioxidant activity which is influenced by the degree of deacetylation and molecular weight of the COS. Low molecular weight COS have been shown to have considerably higher radical scavenging activity (D. H. Ngo & Kim, 2014).Due to these benefits COS has been utilized in food products development such as yoghurt, tofu and some fruit juices (Patel, 2011).

5.3.1.4 Antimicrobial Peptides

The antimicrobial peptide (AMP) concentration was significantly affected by the type of chitin source, type of starter culture and fermentation time ($p<0.005$). *Gryllus bimaculatus, Acheta domesticus* and *Hermetia illucens* indigestible chitin fermented using ABY10 for 48 hours, yielded the highest defensin like AMP while the control had the least. Furthermore, *Gryllus bimaculatus* and *Acheta domesticus* indigestible chitin fermented using ABY 10 for 48 hours yielded the highest Alpha helical like AMP. Additionally, *Hermetia illucens* indigestible chitin fermented using ABY 10 for 48 hours yielded the highest Proline like AMP. Evidently the ABY 10 starter culture and 48 hours of fermentation time resulted in considerably higher AMP levels. The detection of

distinct AMP types, such as defensin-like, alpha-helical, and proline-like, suggests that the fermentation process can yield a diverse array of antimicrobial compounds. Bioactive AMPs are released after fermentation of fruits and vegetables such as Kimchi, sauerkraut, dairy products and meat and fish products. Studies reporting on the synthesis of AMPs after fermentation of chitin are scarce (Chourasia et al., 2023). The present study is among the first studies to document the production of AMPs after fermentation of indigestible insect chitin with probiotic starter cultures. This observation could be valuable for various applications, such as in the development of natural antimicrobial agents, food preservation, or as alternatives to conventional antibiotics. Similarly, in a metabolomics study of rice bran, peptides were among the secondary metabolites that were identified (Zhang et al., 2018). AMP have broad antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria as well as fungi (Mulder et al., 2012). Further in vivo studies suggest that bioactive peptides exhibit antioxidant, antimicrobial, antidiabetic, antihypertensive, immunomodulatory and anticancer activities upon reaching target cells (Jakubczyk et al., 2017; Kwak & Kang, 2017; Muhialdin et al., 2020; Soleymanzadeh et al., 2019).

5.3.1.5 Vitamins

Chitin source, type of starter culture used and fermentation time had a significant effect on the vitamin A content ($p<0.05$). *Gryllus bimaculatus* and *Hermetia illucens* indigestible chitin fermented using ABY 10 for 48 hours and *Hermetia illucens* indigestible chitin fermented using ABT 5 for 48 hours yielded the highest vitamin A values. Similarly, the highest vitamin B2 content was yielded after fermentation of *Gryllus bimaculatus* and *Acheta domesticus* indigestible chitin fermented using ABY 10 for 48 hours while no vitamin B2 was detected in the control. Equally, Gryllus *bimaculatus* indigestible chitin fermented using ABY 10 for 48 hours yielded the highest vitamin B12 content while the control samples yielded the least content. Evidently, the starter culture ABY 10 seems to be particularly effective in increasing vitamin content especially when used for 48 hours of fermentation. Further fermentation of *Gryllus bimaculatus* indigestible chitin resulted in considerably high values for all three vitamins

as compared to indigestible chitin from the other edible insects. The differences observed between insect species (*Gryllus bimaculatus*, *Hermetia illucens*, *Acheta domesticus*) in terms of vitamin production could be due to variations in their chitin structure.

Evidence indicates that fiber such as chitin facilitates the growth and activity of bacteria which in turn yields vitamins such as vitamin A, K and B vitamins (Contreras et al., 2012; Leblanc et al., 2013; Srinivasan & Buys, 2019). Studies have shown that lactic acid bacteria and Bifidobacterium are highly involved in the production of vitamins as metabolites (Pompei et al., 2007; Stanton et al., 2005). When some of these vitamins are produced in the gut they may directly contribute to human nutrition and health through their absorption (Albert et al., 1980). For instance, the vitamins act as important precursors for the formation of cofactors that are involved in metabolic and regulatory processes in humans as well as in the gut microbiome (Putnam & Goodman, 2020; Z. Wan et al., 2022). Vitamin B2 is critical in the formation of coenzymes used by cellular glutathione reductase which protects cells from ROS (Pham et al., 2021). Studies suggest that high doses of vitamin B2 in the colon enhance the growth of beneficial bacteria such as *Faecalibacterium prausnitzii* and *Roseburia* species while at the same time reducing the population of pathogenic bacteria such as *Escherichia coli* by facilitating the reduction of colonic ROS (Steinert et al., 2016; Zempleni et al., 1996).

5.3.1.6 Total Polyphenols

The total phenolic content was significantly influenced by the chitin source, type of starter culture used and fermentation time ($p<0.05$). *Acheta domesticus* indigestible chitin fermented using ABY 10 starter culture yielded the highest amounts of total polyphenols (4.13±0.44mg/g) while no total polyphenols were detected in the control. Similarly, a study by Mendoza-Salazar et al., (2021) documented that fermentation of Mealworm and Grasshopper flour using *Lactococcus lactis* strains yielded 15.41 mg GAE/ml and 9.69mg GAE/ml total polyphenols which were considerably higher than values obtained in the present study. The variation in total polyphenols observed could

be due to differences in samples used as well as overestimation of total polyphenols due to potential interference of proteins during analysis (Everette et al., 2014). Polyphenols released in the gut may have beneficial effects on humans as well as the gut microflora. The reciprocal interaction between polyphenols and gut microbiota is crucial, as polyphenols can stimulate beneficial bacteria like Bifidobacterium and Lactobacillus while inhibiting pathogenic species, thereby modulating microbial composition and metabolic function (Whitman et al., 2024). Furthermore, the gut microbiota converts dietary polyphenols into bioactive metabolites that exert systemic effects, including enhanced antioxidant capacity and reduced inflammation, which together contribute to the prevention of chronic diseases (Nemzer et al., 2025).

5.3.2 Conclusion

In conclusion the study indicates that the SCFA were the predominant fatty acids obtained after the in vitro digestion and fermentation of chitin with acetic acid being the most predominant, followed by propionic acid and then butyric acid. Further lauric acid was one of the saturated long chain fatty acid detected in considerable amounts. Oleic and linoleic fatty acids were the monounsaturated and polyunsaturated fatty acids identified. *Acheta domesticus* indigestible chitin was easily converted to chitosan and chito oligosaccharides by both ABY 10 and ABT 5. This study demonstrates that insect-derived chitin can be effectively fermented to produce antimicrobial peptides, with specific conditions yielding optimal results. The indigestible chitin fractions from *Gryllus bimaculatus, Acheta domesticus* and *Hermetia illucens,* when fermented using ABY 10 starter culture for 48 hours, consistently produced high levels of AMPs. Evidently the starter culture ABY 10 seems to be particularly effective in increasing vitamin content especially when used for 48 hours of fermentation. Fermentation of *Gryllus bimaculatus* indigestible chitin resulted in considerably higher values for all three vitamins as compared to indigestible chitin from the other edible insects. Additionally, a fermentation time of 48 hours was found to yield considerably high amounts of chitosan, chitooligosaccharides, antimicrobial peptides and vitamins.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study revealed several key findings regarding insect-derived chitin and its properties. The FTIR spectra indicated that the insect chitin samples were similar to commercial chitin. All the chitin samples were more soluble in water than the commercial chitin. The *Gryllus bimaculatus* chitin had significantly higher fat binding capacity, emulsion capacity and emulsion stability as compared to the commercial chitin while the commercial chitin showed superiority in regards to water absorption capacity. Interestingly, all the chitin samples could absorb four times more water and two times more fat than their weight which is indicative of the potential for utilization in baking and the meat industry. The commercial chitin and BSF had the highest DDA while commercial chitin and *Gryllus bimaculatus* had the highest values of purity, followed by BSF. The SEM images showed similar surface morphology in all the chitin samples which were characterized by the presence of microfibers and pores. The similarity between commercial chitin and insect chitin implies that insect chitin is a potential alternative of commercial chitin in the market.

Regarding fermentation ABY 10 starter culture was more efficient in fermenting the chitin samples as compared to ABT 5. Furthermore, *Hermetia illucens* chitin was easily fermented by the starter culture followed by *Gryllus bimaculatus* chitin while *Acheta domesticus* chitin was less easily fermented. It is clear that chitin in vitro digestion and fermentation yielded derivatives with significant antioxidant and antimicrobial properties. The derivatives of in-vitro digestion and fermentation of the chitin samples showed significant inhibition of the growth of gram-negative and gram-positive pathogenic bacteria. Evidently, fermentation time and sample concentration had a significant influence on the antioxidant and fermentation capacity of the derivatives of

chitin in vitro digestion and fermentation. It is with this evidence that we conclude that chitin could have beneficial health effects in the gut.

The fermentation of indigestible chitin with prebiotic starter cultures yielded a number of bioactive compounds. SCFA were the predominant fatty acids obtained after the in vitro digestion and fermentation of chitin with acetic acid being the most predominant, followed by propionic acid and then butyric acid. Further lauric acid was one of the saturated long chain fatty acid detected in considerable amounts. Oleic and linoleic fatty acids were the monounsaturated and polyunsaturated fatty acids identified. *Acheta domesticus* indigestible chitin was easily converted to chitosan and chitooligosaccharides by both ABY 10 and ABT 5. This study demonstrates that insect-derived chitin can be effectively fermented to produce antimicrobial peptides, with specific conditions yielding optimal results. The indigestible chitin fractions from *Gryllus bimaculatus*, *Acheta domesticus* and *Hermetia illucens* when fermented using ABY 10 starter culture for 48 hours, consistently produced high levels of AMPs. Evidently the starter culture ABY 10 seems to be particularly effective in increasing vitamin content especially when used for 48 hours of fermentation. Fermentation of *Gryllus bimaculatus* indigestible chitin resulted in considerably higher values for all three vitamins as compared to indigestible chitin from the other edible insects. A fermentation time of 48 hours was found to yield considerably high amounts of chitosan, chitooligosaccharides, antimicrobial peptides and vitamins.

The study findings collectively suggest that chitin extracted from insects could be a potential alternative to commercial chitin and thus has significant potential for application in food, pharmaceutical, medical, and textile industries. Additionally, the demonstrated beneficial properties indicate that chitin inclusion in food products or whole insect consumption could potentially promote gut health through the production of beneficial compounds, including SCFAs, antimicrobial peptides, and vitamins.

6.2 Recommendations

I recommend:

1. Scaling up of chitin extraction by implementing a pilot-scale processing line capable of handling up to 200 kg of insect meal.
2. Development of standard guidelines for the incorporation of chitin in food products specifying inclusion levels and quality parameters such as sensory and microbial thresholds.
3. Clinical trials to validate the gut health benefits of chitin extracted from insects when consumed as part of a regular diet.

6.3 Areas of Further Research

1. It is necessary to study the bioavailability of chitin-derived compounds in human digestive systems, their absorption rates and actual antimicrobial efficacy within the gut microbiome.
2. Research could examine whether the promising in vitro results translate to measurable health benefits when edible insects are consumed as food.
3. There is a need to investigate how factors such as diet, life stage and processing methods affect chitin composition.

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