THE PREBIOTIC EFFECTS OF AMADUMBE (*Colocasia Esculenta*) AND OKRA (*Abelmoschus esculentus*) MUCILAGE

Submitted in complete fulfilment for the Degree of Master of Applied Sciences in Food Science and Technology at the Department of Biotechnology and Food Science, Faculty of Applied Sciences,

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Declaration

The work presented in this dissertation is original work conducted by the author, confirming that it has not been previously submitted for a degree at any other Higher Education Learning Institution. Where others' work was used, it was properly acknowledged within text. The research presented in this dissertation was carried out at the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of Prof. Eric Oscar Amonsou, and Dr. Nokuthula Peace Mchunu.

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Dedication

This dissertation is dedicated to my late grandmother Mrs Dasodia Belochun, who passed on during my Masters studies. It was through her unconditional love, support and encouragement that I have managed to attain much of my success.

"Matha Pitha Guru Deivam"

The completion of this dissertation is due to my late parents Ramesh and Serena Gajadhar whom I had promised to make proud by the achievement of this monumental academic goal. I hope that I have fulfilled that promise.

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Preface

The dissertation is arranged into five chapters. Chapter one covers introduction (defines problem statement, aims and contribution to knowledge pertaining to the research) while Chapter two presents literature review (reviews previous related work and highlights potential knowledge gaps, research aims and objectives). Chapter three (Research objective 1) highlighted the chemical composition and functional properties of amadumbe and okra mucilage. This chapter investigates the chemical composition of amadumbe and okra mucilage. Using the knowledge of the composition it further investigates the functional properties of amadumbe and okra mucilage to better understand its fermentation and possible applications. Chapter four (Research objective 2) enumerated the prebiotic effect of amadumbe and okra mucilage. This chapter investigates the prebiotic potential of amadumbe and okra mucilage in stimulating gut microbial composition. Chapter five contains conclusion (general discussion of key research findings, limitations, recommendations and future work).

Abstract

Prebiotics have been shown to aid in the improvement and maintenance of human health through positive manipulation of gut microbiota. Diet-induced changes in gut microbial diversity has been recognized as a factor which contributes to the rising epidemics of chronic illnesses in both developed and developing countries.

Traditional crops, amadumbe (*Colocasia esculenta*) and okra (*Abelmoschus esculentus* (L.) Moench) offer nutritional security to many communities in South Africa. These crops are rich in mucilage and are presumed prebiotics. Structural composition and functional properties of polysaccharides like mucilage are suggested to influence their fermentability by gut microbiota and potential health effects. The purpose of this study was to investigate the prebiotic effects of amadumbe and okra mucilages for potential application as dietary supplements.

Mucilage was extracted from amadumbe and okra by cold water extraction. Purified mucilage was obtained by Sevag method, lipid removal and thereafter dialyzed. The composition and structure of crude and purified mucilage were analyzed using Fourier transform infrared spectroscopy (FT-IR), size exclusion chromatography (SEC) and high pressure liquid chromatography (HPLC). Functional properties including water and oil holding capacity, swelling and solubility were determined. The prebiotic potential of amadumbe and okra mucilage was carried out by *in- vitro* fermentation using human faecal sample.

Glucose was the common monosaccharide present in both amadumbe and okra mucilage. Monosaccharides present in amadumbe mucilage were arabinose, mannose and xylose, while galactose, ribose and rhamnose were the main monosaccharides present in okra mucilage. The presence of β-glucan was found to be higher 0.20 g/100 g in amadumbe mucilage than in okra mucilage 0.07 g/100 g. The resistant starch content in amadumbe mucilage was higher 4 g/100 g than in okra mucilage 0.7 g/100 g. Asparagine, proline, glutamine, and threonine were the most common amino acids found in both amadumbe and okra mucilage samples. Purified amadumbe and okra mucilage displayed the same characteristic peaks as crude amadumbe and okra mucilage in the FT-IR spectrum but at a lower intensity suggesting that purification contributed to a more stable and uniform structure. The FT-IR spectrum indicated the presence of uronic acid and hydroxyl groups which confirm the existence of carbohydrate in both amadumbe and okra mucilage.

The molecular weight of crude amadumbe and okra mucilages ranged between 219 and 224 kDa while molecular weight of purified amadumbe and okra mucilage ranged between 220 and

244 kDa. The purification process was seen to improve functional properties such as the water holding capacity, swelling and solubility of mucilages. In comparison to okra mucilage, crude and purified amadumbe mucilage showed low water holding capacity 5 and 9 g/100 g and high percentage solubility 61 and 73%. Amadumbe mucilage had a slightly higher oil holding capacity 11 g/100 g in comparison to okra mucilage 10 g/100 g.

During *in-vitro* fermentation, inulin (positive control) rapidly decreased the pH of the fermentation medium from 7.0 to 6.5, in comparison to amadumbe (7.0 to 6.7) and okra (7.0 to 6.8) mucilage. At the end of fermentation inulin had maximum gas production of 233.19 mL, followed by amadumbe mucilage 158.98 mL and okra mucilage 113.98 mL. These results suggest inulin is more easily fermented by microbes compared to amadumbe and okra mucilage. Gut microbiota analysis at phylum level showed that amadumbe mucilage stimulated the proliferation of Actinobacteria and reduced the presence of Firmicutes in comparison to okra mucilage. At species level, okra mucilage promoted the growth of *Bacteroidaceae bacteroidetes*, *Bacteroides ovatus* and *Bacteroides uniformis*. These species are known to assist in protection of the gut and are capable of providing nutrients to other microbial species. This suggest that amadumbe and okra mucilages are fermented differently by gut microbiota possibly due to differences in their structure and composition.

This study concluded that amadumbe and okra mucilages has potential to be utilized as an emerging prebiotic in food applications or as supplements.

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List of Abbreviations

GOS - Galacto-oligosaccharides

FOS - Fruto- oligosaccharides

GI - Gastro-intestinal

SCFAs - Short chain fatty acids

NCDs - Non-communicable diseases

WHO - World Health Organization

SSA - sub-Saharan Africa

IBS - Irritable bowel syndrome

IBD - Inflammatory bowel disease

NGS - Next generation sequencing

WHC - Water holding capacity

OHC - Oil holding capacity

SI - Solubility index

SP - Swelling power

FISH - Fluorescent *In Situ* Hybridisation

qPCR - Quantitative Polymerase chain reaction

TGGE - Temperature gradient gel electrophoresis

DGGE - Denaturing gradient gel electrophoresis

HRB - Human residential Bifidobacteria

SFB - Segmented filamentous bacteria

AMPs - Antimicrobial peptides

ACTH - Adrenocorticotropic hormone

RSs - Resistant starches

NF - Nuclear factor

HDI - Human development index

AD - Alzheimer's Disease

IBS - Irritable bowel syndrome

CRC - Colorectal cancer

AICR - American Institute for Cancer Research

MAC - Microbiota accessible carbohydrates

DP - Degree of polymerization

ITFs - Inulin-type fructans

NDP - Non-digestible polysacharies

CBPs - Carbohydrate-based compounds

PLs - Polysaccharide lyases

GH - Glycoside hydrolases

TSLP - Thymic stromal lymphopoietin

PCOA - Principal co-ordinate analysis

OTU - Operational taxonomic unit

CHAPTER ONE

1. Introduction

Consumers have become increasingly conscious of their health and the foods they consume. This consciousness has generated increased demand and potential for functional foods and ingredients such as prebiotics (Quigley 2019). Prebiotics are non-digestible carbohydrates that serve as nourishment for beneficial bacteria located in the human gut by promoting their growth, thus contributing to human health (Davani-Davari *et al*. 2019). In addition to their health benefits, prebiotics are suggested to contribute towards improving sensory and textural properties in food and beverage products (Sebastián, Ariel and Eduardo 2019). The prebiotic market has been projected to reach $9.4 billion by 2026, increasing by 8.7% between 2021 and 2026 (Region 2018). The global growth of the prebiotic market is highly driven by its health benefits an industrial application in food and beverages as well as pharmaceuticals and cosmetics (Amiri *et al*. 2021).

Prebiotics are naturally derived from fruits, roots, seed and some microorganisms (Wang *et al.* 2019a). They include various oligosaccharides (galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS)), inulin and structurally different polysaccharides such as mucilages, β-glucans, arabinoxylan, galactomannan, celluloses and pectins (Cruz-Rubio *et al*. 2018). They differ in structure, composition and functionality based on their plant origin, cultivar, anomeric configuration, chain lengths, linkage patterns, sugar composition and composition of branch chains (Maji 2019). A group of polysaccharides suggested to have the most promising potential as prebiotics are mucilages. These are high molecular weight polysaccharides, structurally composed of various monosaccharide units, galacturonic or uronic acids with varying branched structures. Mucilage may be associated with other compounds such as glycoprotein, lipids, tannins and steroids (Monrroy *et al*. 2017). Due to their bioavailability, non-toxicity, cost efficiency and vast application potential, there has been increased research interest in mucilage from traditional and novel plant sources (Amiri *et al*. 2021).

Taro (*Colocasia esculenta*), is a rooted vegetable crop native to South-East Asia, however, its cultivation has spread across most tropical and sub-tropical regions (Rashmi *et al*. 2018). In South Africa, Taro commonly known as amadumbe is considered a traditional crop widely grown in the province of Kwa-Zulu Natal (Shange 2004). The corms are consumed boiled, as porridge, in stews, fried as chips or processed into flour (Plucknett, 2019). Amadumbe is

known to be rich in mucilage (Andrade, Nunes and Pereira 2015). Mucilage derived from the amadumbe corn has previously been reported to be composed of mainly carbohydrates, proteins, a glycoprotein and accompanying starch which is present as an impurity (Njintang *et al*. 2014). Due to its suggested structure and composition, amadumbe mucilage have been applied as emulsifiers in dough formulation and bread making (Bisulca, Odegaard and Zimmt 2016), fortifying agent and hydrocolloid in pudding-like products (Hendek Ertop, Atasoy and Akın 2019) and as biopolymer for the encapsulation of pharmaceutical ingredients (Singh and Kumar 2016).

Okra (*Abelmoschus esculentus* (L.) Moench), is a flowering vegetable crop belonging to the Malvaceae family cultivated primarily for its edible pods (Badrie 2016). Okra is known to be rich in mucilage, neutral sugars, minerals and protein (Gemede *et al*. 2018). Okra mucilage has been reported to be made up of D-galactose, L-rhamnose and L-galacturonic acid with some proportions of glucose, mannose, arabinose and xylose (Gbenga and Zulikha 2013). It has been reported to have various functional properties such as foaming and emulsifying capabilities, water binding and gelling (Jideani and Bello 2009). Many studies have reported on the application of okra mucilage in food and pharmaceutical products (Etaware and Etaware 2019; Nampuak and Tongkhao 2020; Olawuyi *et al*. 2020).

Amadumbe and okra mucilages have been found to differ in structure and composition (Gbenga and Zulikha 2013; Andrade, Nunes and Pereira 2015), which are suggested to strongly influence their functional properties (Kontogiorgos *et al*. 2012). Pham *et al*. (2017) reported that the presence of other compounds in mucilage can influence their functional properties. For example, the presence of protein and lipids in okra mucilage were observed to increase its ability to absorb oil, by the hydrophobic fractions that attach to the hydrocarbon molecules of oil (Thanatcha and Pranee 2011).

In addition to its application in food and non-food products, polysaccharides such as mucilage have also been found to positively influence human intestinal health (Tang *et al*. 2019). Inclusion of prebiotic polysaccharides in diets have been suggested to have positive impact on gut microbiota species diversity and composition (Jefferson and Adolphus 2019). Structure, composition and functional properties of polysaccharides have been reported to influence their ability to be fermented by gut microbiota. These factors also influence the diversity and composition of gut microbiota, the production of short chain fatty acids and their conferred health benefits (Shi *et al*. 2020). Pectin derived from okra was observed to increase the viability

of probiotic strains *Bifidobacterium longum* and *Lactobacillus rhamnosus* and encouraged the production of short chain fatty acids (Yeung *et al*. 2021).

Amadumbe and okra mucilages vary in structure and composition, and can be hypothesized that these substrates will vary in their functionality and will stimulate gut microbial growth differently. Just like most traditional foods, there is limited evidence supporting the use of mucilages from Southern African amadumbe and okra varieties as prebiotics. Although amadumbe and okra mucilages are presumed prebiotic, there are limited studies addressing their prebiotic potential which is essential in directing their use as targeted supplements or in the developmentof dietary prebiotic supplements. Therefore, there is need to investigate the prebiotic effects of amadumbe and okra mucilages on gut microbiota species diversity and composition.

CHAPTER TWO

2. Literature Review

2.1 The human gastrointestinal microbiome

There has been greater understanding of microbes that inhibit humans, particularly those located in the gastro-intestines. A variety of micro-organisms have been found to inhabit the human body. These microorganisms dwell both within and outside of the human body. The gastrointestinal system effectively houses what is referred to as a complex community of microorganisms (Shang *et al*. 2018). Gut microbiota is known to be composed of diverse community of archaea, bacteria and eukarya that exist within the human gastrointestinal tract (mainly the colon) and interact with the host in a mutually beneficial manner (Thursby and Juge 2017). This community is expected to have 10^{10} to 10^{11} bacterial cells per gram of faecal material and the microbiome (genes from microbiota) having approximately 9.9 million genes - ~100 times more than the human genome (Bussolo de Souza 2019).

Previously, the majority of knowledge existing on human gut microbiota was derived from time-consuming culture-based methods (Mizrahi-Man, Davenport and Gilad 2013). However, the ability to screen gut microbiota has greatly increased with the development of culture-independent methods, such as high-throughput and affordable sequencing techniques (Poretsky *et al*. 2014). Most developed technologies are based on the principle of 16s ribosomal RNA gene sequencing. There are various culture independent molecular techniques that are utilized in the evaluation of gut microbiota such as Quantitative Polymerase Chain Reaction (qPCR); Temperature / Denaturing Gradient Gel Electrophoresis (TGGE / DGGE); Terminal - Restriction Fragment Length Polymorphism (T-RFLP) and Fluorescent *In Situ* Hybridisation (FISH) (Gerritsen *et al*. 2011).

Aside from just obtaining the classification of gut microbiota, it has also become possible to evaluate their complete genetic make- up, with the aid of metagenomics (Almeida *et al*. 2019). Initiatives like the Human Microbiome Project and Meta-Hit data have combined the use of various technologies to produce the most comprehensive depiction of the human-associated microbial repertoire (Li *et al*. 2014). Data from these investigations were compiled identifying 2172 species isolated from humans, divided into 12 different phyla, 93.5% of which belonged to Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria. Only one

species isolated from humans were found in three of the 12 recognized phyla, including gastrointestinal species, *Akkermansia muciniphila*, the only reported representative of the Verrucomicrobia phylum. Of the reported species in humans, 386 are strictly anaerobic and will thus be found in mucosal regions such as the mouth cavity and the gastro-intestinal (GI) tract (Hugon *et al*. 2015).

The human gut microbiota is composed of a relative proportion of major bacterial phyla with diverse species composition. Gut microbiota share a symbiotic relationship with humans and are responsible for various metabolic and immune functions (Thursby and Juge 2017). Gut microbiota play a crucial role in the digestion and absorption of various nutrients, as well as the synthesis of metabolites such as short chain fatty acids, bile acids and lipids (Rowland *et al*. 2018). They have a significant role in human health as well as disease condition. Various studies have observed that imbalance in gut microbiota composition and diversity is also responsible for the development or cause of various non-communicable diseases (Noce *et al*. 2019). These include gastrointestinal diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and colon cancers (Marchesi *et al*. 2016; Pittayanon *et al*. 2019). Disruptions in the symbiotic host-gut microbiota connection results in immune system dysfunction and diseases. To ensure optimal immunological and metabolic function, as well as disease prevention, the composition of human gut microbiota must be balanced and remain as such (Rinninella *et al*. 2019).

2.2 Gut microbiota development

Development of microbiota is widely accepted to begin at birth with the manner of delivery being a crucial factor in influencing microbiota composition. Studies depict that microbiota of vaginally delivered newborns are composed of significant abundance of *Lactobacilli* during the first few days (Avershina *et al*. 2014), whilst the microbiota of newborns born via cesarean delivery is deficient and delayed in the colonization of the *Bacteroides* genus, however is populated by facultative anaerobes such as *Clostridium* species (Jakobsson *et al*. 2014). The microbiota is often limited in diversity during the initial phases of development and is characterized by two major phyla, Proteobacteria and Actinobacteria (Bäckhed 2011). As development occurs, the microbial diversity rises and the microbiota composition converges into a distinct adult-like microbial profile with time-based patterns specific to each newborn (De Muinck and Trosvik 2018). Thereafter, the infant's microbiota diversity, composition and

functional capabilities begin to take shape resembling those of adult microbiota as depicted in Figure 2.1. The GI tract is suggested to swiftly colonize with life events such as dietary changes, illness and antibiotic treatment resulting in alterations of microbiota (Koenig *et al*. 2011).

The diversity and composition of gut microbiota present in adults varies greatly from individual to individual. This includes variations in genera and species as well as the relative ratios of major phyla found in an individual (Rinninella *et al*. 2019). According to multiple research conducted primarily in developed countries, gut microbiota of a healthy adult is composed of a two-phylum combination, consisting of Firmicutes and Bacteroidetes (Claesson *et al*. 2011). The next most prevalent phylum is Actinobacteria, which consists primarily of *Bifidobacterium*. Even within predominant phyla exists diverse species and strains. At this precise scale, in terms of strain identity and relative abundance, an individual's microbiota is as customized as a fingerprint (Dethlefsen *et al*. 2006).

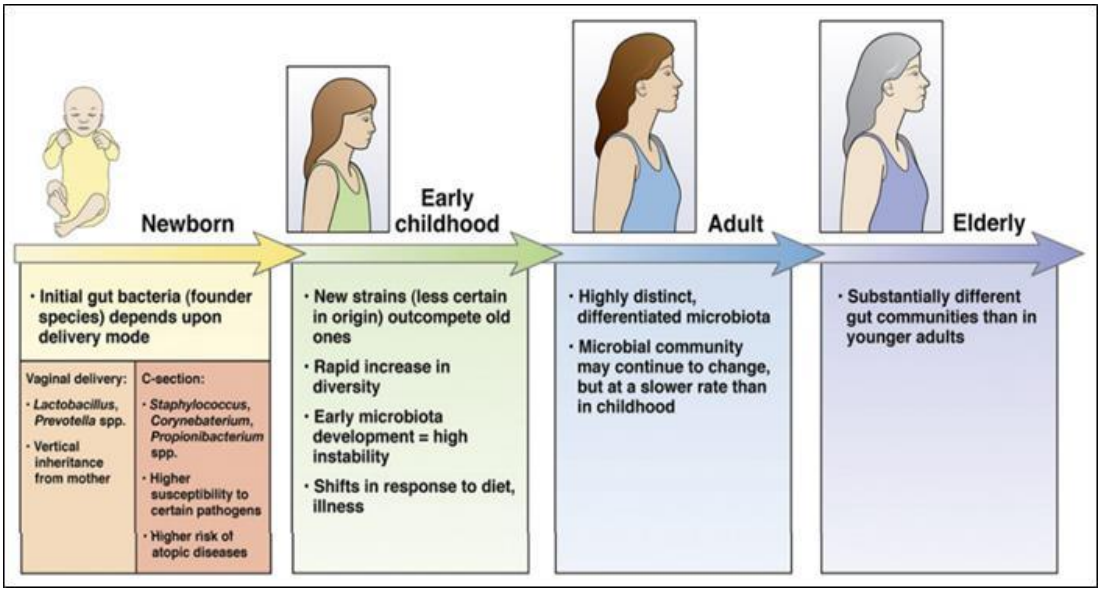

Figure 2. 1 Depicts the evolution of the microbiota from the initial inoculum as an infant to the present day, as influenced by nutrition, genetics and the environment (Dominguez-Bello *et al*. 2011).

2.3 Gut microbiota composition and diversity

The human GI tract is known to house diverse and complex microbial species. These microbial species are of anaerobic or facultative anaerobic nature. The gut microbiota is made up of many species of microorganisms such as yeast, bacteria and viruses. Bacteria are categorized taxonomically into phylum, classes, orders, families, genera and species (Laterza *et al*. 2016).

Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia are the predominant gut microbial phyla as shown in Figure 2.2. Firmicutes and Bacteroidetes are the two phyla that have been suggested to make up 90% of gut microbiota composition (Arumugam *et al*. 2011). Bacteroidetes include major genera, *Bacteroides* and *Prevotella*. The Actinobacteria phylum is less numerous and is mostly characterized by *Bifidobacterium* genus (Rinninella *et al*. 2019). More than 200 different genera make up the Firmicutes phylum, including *Lactobacillus*, *Closridium*, *Bacillus, Enterococcus* and *Ruminicoccus*, with the *Clostridium* genera accounting for 95% of the Firmicutes phyla (Arumugam *et al*. 2011).

2.3.1 Bacteroidetes

Bacteroides species are among the dominant beneficial bacteria in the gut. These Gram-negative, rod shaped, obligate anaerobes play a variety of roles in the human gut microbiome (Flint *et al*. 2012). They are capable of fermenting oligosaccharides and polysaccharides obtained from diet, providing nutrients and vitamins to the host as well as other gut microbial occupants (Zafar and Saier Jr 2021). Preliminary studies demonstrated that *Bacteroides* species are capable of degrading a wide range of plant polysaccharides, such as galactomannan, pectin, alginate, arabinogalactan, xylans and laminarin (McCarthy, Kotarski and Salyers 1985). However, findings have expanded it to include rhamnogalacturonans I and II, β-glucans, xyloglucan and glucomannan (Martens *et al*. 2011). The ability to break down such wide range of polysaccharides and amino acids further explains their predominance within the gut. Fermentation of plant polysaccharides by this genus result in the production of metabolites such as succinic acid, acetic acid and valeric acid (Wexler 2007).

Prevotella is described as one of the more important groups of bacteria in the human gut and has been described as having a high genetic diversity (Tett *et al*. 2021). *Prevotella* bacteria, which are prevalent colonizers of agrarian communities, aid in polysaccharide breakdown in the gut (Precup and Vodnar 2019). Many studies addressing *Prevotella* have made observations with regards to the connections between diversity of species, ecosystems, genomes, dietary habits, health and disease (Mancabelli *et al*. 2017). The use of next-generation sequencing techniques has further assisted in revealing *Prevotella* species predominance in healthy humans within the gut ecosystems (Mancabelli *et al*. 2017). Various reports suggest that some *Prevotella* strains have positive effects in the gut, such as reducing the risk of cardiovascular disease and improving metabolism of glucose (Wang *et al*. 2016), *Prevotella* also have pathobiontic capabilities that promote illnesses including

obesity, inflammatory diseases and inflammatory bowel disease (Larsen 2017). Based on cultivated isolates of *Prevotella*, has been subdivided into four distinct species: *P. bryantii*, *P. brevis*, *P. albensis* and *P. ruminicola* being the most dominant species. Most cultivated isolates can metabolize starch, xylan, pectins, as well as other compounds generated by degradation of polysaccharides in the gut (Flint 2014).

2.3.2 Firmicutes

Firmicutes is a bacterial phylum that includes three classes *Clostridia*, *Bacilli* and *Mollicutes*, which comprises of a total of 235 genera and all lactic acid bacteria species. Species of this phylum differ in morphology, physiology and Gram-staining properties (Vos *et al*. 2011). According to 16S rRNA studies, two Firmicutes families, Ruminococcaceae and Lachnospiraceae are particularly prevalent in the human large intestine, accounting for 50–70% of bacteria found in fecal samples from healthy human (Rey *et al*. 2010). These families include species that are strictly anaerobic and are responsible for critical metabolic interactions within the gut microbiome (Flint *et al*. 2007). They are identified as the primary butyrate-producing species (Louis *et al*. 2010), capable of converting lactate to butyrate or propionate (Duncan, Louis and Flint 2004). Species in these families can also undergo reductive acetogenesis (Rey *et al*. 2010).

Within the taxonomic class *Bacilli*, exists two orders namely *Bacillales* and *Lactobacillales* (Ludwig, Schleifer and Whitman 2015). *Bacilli* are facultative aerobes (Wolf *et al*. 2004), reported to make up a lesser fraction of gut microbiota (Rajilić-Stojanović and De Vos 2014). *Bacilli* have been observed to actively influence the gut microbial community including other species due to their abilities of secreting a wide range of metabolites (Siraj *et al*. 2015). As part of gut microbiota, *Bacilli* help in the metabolism of dietary components, xenobiotics and medications, thereby aiding in the maintenance of intestinal homeostasis and maintenance of human health (Rowland *et al*. 2018). *Lactobacilli* have long been regarded as being an essential component of the human gut microbiota. Emerging evidence indicates that few *Lactobacillus* species are real occupants of the human gastrointestinal tract and that the majority of *Lactobacilli* present are derived from food (Walter *et al*. 2001). Attempts to categorize *Lactobacilli* as indigenous or transitory pose as a challenge since diet has a substantial impact on their composition in the colon. *Lactobacilli* are present in the duodenum and jejunum (Kerou and Schleper 2015), while their composition increases from the duodenum to the colon (Derrien and van Hylckama Vlieg 2015). *Lactobacillus* and *Bacillus* species have

previously been identified in various traditional fermented food products, conferring beneficial properties for the gut (Sornplang and Piyadeatsoontorn 2016) and are now widely marketed as commercial probiotics (Marco *et al*. 2017).

Bacilli are recognized as having a wide range of complex enzymes such as cellulases, lipases, amylases and proteases which they release aiding in the digestion of dietary components in the gut (Keller *et al*. 2017). Fermentation of dietary components, particularly carbohydrates, by *Bacilli* genera produces short chain fatty acids (SCFAs). This genus is known to favor the synthesis of lactate and acetic acid, but it is also capable of producing propionic and butyric acid (Nyangale *et al*. 2015). Clostridia is a taxonomic class that includes *Clostridium* and other genera. Species belonging to the class Clostridia are known to be anaerobic and *Clostridia* species are frequently but not always Gram-positive and can generate spores (Baron 1996). The genera *Clostridium* is composed of two main groups, namely the *C. leptum* group, which has four members and the *Clostridium coccoides* group, which is composed of 21 different species (Gomes, Hoffmann and Mota 2018).

Clostridium species are situated in the large intestine, particularly in the mucosal folds of the ascending colon, coexisting with *Enterococcaceae*, *Bacteroidaceae* and *Lactobacillaceae*. They derive their energy from the fermentation of dietary carbohydrates, producing substantial quantities of short chain fatty acids (SCFAs), which plays an important role in gut homeostasis (Nagano, Itoh and Honda 2012). Not all species within this class are regarded as pathogenic and disease causing. *In-vitro* and *in-vivo* studies have observed *F. prausnitzii* to be effective in protecting against inflammation by inhibiting NF-B activation and IL8 production (Sokol *et al*. 2008). Zhou *et al*. (2021) observed *F. prausnitzii* produces metabolites able to protect mice from colitis and improve gut dysbiosis by increasing bacterial diversity. However, *Clostridium* species such as *C. perfringens*, *C. botulinum* and *C. difficile* have been reported as the main cause of anaerobic intoxications. For instance, *C. perfringens* is known to produce necrotic enteritis B- like toxin and enterotoxin which can result in gas gangrene, necrotizing enteritis and other complications as well as mortality (Li *et al*. 2013). Necrotizing enterocolitis in premature infants were found to been linked to species *C. paraputrificum, C. tertium* and *C. butyricum (*Kiu *et al*. 2017).

2.3.3 Actinobacteria

The Actinobacteria phylum are gram-positive bacteria that have a filamentous appearance, with

high cytosine and guanine concentrations in their DNA (Puttaswamygowda *et al*. 2019). Actinobacteria is reported to be made up of 219 genera, 48 families and 5 orders. The bulk of them reside in the soil but a few groups can be found in healthy individuals. *Propionibacterium, Corynebacterium, Actinomyces, Rothia* and *Bifidobacterium* are the most common found in this genus (Cho and Blaser 2012). The earliest micro-organisms reported to colonize the human gut were members of *Bifidobacterium*. Emerging evidence indicates that certain species are natural inhabitants of the human gut and have been identified as Human- Residential *Bifidobacteria* (HRB) (Wong, Odamaki and Xiao 2020). *Bifidobacteria* are classified as anaerobic, Gram-positive, non-spore forming polymorphic rods of the family Bifidobacteriaceae. *Bifidobacteria* have been observed to have a variety of cell morphologies, including short, curved and bifurcated Y shapes (Milani *et al*. 2014; Ventura *et al*. 2014).

The genus *Bifidobacterium* has roughly 80 species, which include species *Bifidobacterium longum, Bifidobacterium animalis, Bifidobacterium thermacidophilum* and *Bifidobacterium pseudolongum* that are further subdivided into subspecies (Parte 2018; Sakanaka *et al.* 2020). Their presence in the human gut coveys some health advantages such as production of metabolites like short chain fatty acids and vitamins, immune system development and the prevention of gastrointestinal disorders (O'callaghan and Van Sinderen 2016). Even though *Bifidobacteria* are not predominant in the gut microbiota of adults, their functional biological involvement in the metabolism of host derived dietary glycans is well recognized (Milani *et al*.2014). *Bifidobacteria* exhibit saccharolytic behavior and their potential to colonize and thrive in the gastrointestinal tract is strongly reliant on their ability to ferment and utilize dietary carbohydrates (Milani *et al*. 2016). They have gained increased popularity due to their probiotic benefits and species within this phylum have been utilized in the food industry extensively in beverages and foods. The by-products of fermentation by *Bifidobacteria* result in the production of lactate as well as acetate, however, it has been found that *Bifidobacteria* are incapable of producing butyrate or propionate (Fukuda *et al*. 2011).

2.3.4 Proteobacteria

Proteobacteria are Gram-negative bacteria and facultative anaerobes. They include several pathogenic genera with *Salmonella, Escherichia, Vibrio, Yersinia, Helicobacter* and *Legionellales* as some of the pathogenic genera belonging to this phylum (Bennett, Dolin and Blaser 2014). Various studies have observed that gut microbiota imbalance is due to the presence of Proteobacteria (Shin, Whon and Bae 2015). A study of children's gut microbiota

revealed higher presence of Proteobacteria in European children whose diets were calorie-dense, consisting of low-fiber and high fat in comparison to children from Burkina-Faso who consumed a low-fat, high-fiber diet (De Filippo *et al*. 2010).

In-vivo studies have shown Proteobacteria have obesogenic potential, lending credence to the link between Proteobacterial proliferation and metabolic disease (Jeong, Jang and Kim 2019). It has further been observed that Proteobacteria derive their energy from simple monosaccharides and high sugar diets have been found to induce higher abundance of Proteobacteria (Do *et al*. 2018). Within the phylum, class gamma-Proteobacteria and the family Enterobacteriaceae are known to have lipopolysaccharide (LPS, endotoxin) molecules which are key regulators of inflammatory responses (Mukhopadhya *et al*. 2012).

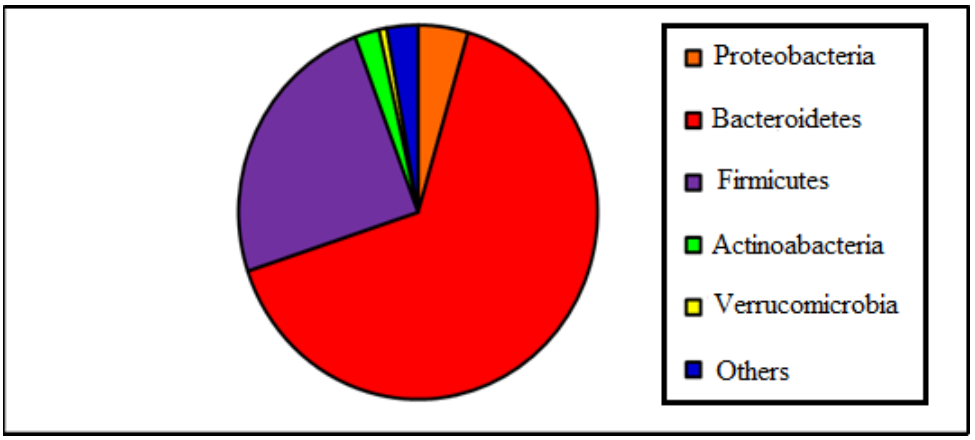

Figure 2. 2 Relative abundance (%) of major phyla gut microbiota in healthy individual (Shin, Whon and Bae 2015).

2.4 Function of gut microbiota in relation to human health

Various animal and human studies have provided evidence to support the importance of gut microbiota in human health and wellbeing (Beaumont *et al*. 2016; Falony *et al*. 2016; Danneskiold-Samsøe *et al*. 2019). Gut microbiota and the host interact in a robust system that controls and maintains their symbiotic relationship. These interactions include metabolic, immunological and neuroendocrine interplay (Kho and Lal 2018), which are reviewed subsequently in detail.

2.4.1 Metabolism

In terms of human metabolism, the main function of gut microbiota is the fermentation of

dietary carbohydrates (Kumar, Rani and Datt 2020). This process enhances the composition and diversity of gut microbiota while generating essential metabolites such as SCFAs (Gentile and Weir 2018). Numerous studies have revealed that particular gut microbiota are capable of fermenting specific types of dietary carbohydrates and their by-products serve as sources of energy for other bacteria in the gut microbiome (Cockburn and Koropatkin 2016). Alteration in gut microbiota composition and metabolite abundance caused by dietary carbohydrate consumption may prevent and reduce disease outcome. Roytio *et al*. (2018) for instance, discovered a strong correlation between consumption of dietary carbohydrates and the diversity and richness in microbial species within the gut. The mechanism of fermentation of dietary carbohydrates by gut microbiota resulted in increased composition of *Bifidobacterium* and *Lactobacillus* species within the gut. Relative abundance of these species has been linked to improve digestion, alleviated constipation, resistance infections, eliminated traveler's diarrhea and relieved inflammatory bowel disease (Gibson *et al*. 2010; Makizaki *et al*. 2018; Chen, Chao and Pan 2020).

Azcarate-Peril *et al*. (2017) investigated the effect of highly pure galacto-oligosaccharides (GOS) on gut microbiota in lactose intolerant humans. The study found a rise in the relative abundance of *Faecalibacterium*, *Bifidobacterium* and *Lactobacillus* in response to GOS. Proceeding this intervention, dairy products were introduced which enhanced the abundance of *Roseburia* species. The study concluded with findings that lactose fermenting bacteria collaborate with GOS to promote dairy product tolerance in humans. SCFAs produced as by-products of microbes contribute 70% of ATP generation in the colon, with butyrate being the preferred fuel for colonocytes (Louis and Flint 2017). These metabolites are thought to provide a source of energy for the host and contribute to other metabolic activities within the host (LeBlanc *et al*. 2017). Butyrate enables epithelial cells to consume substantial amounts of oxygen via β-oxidation, resulting in hypoxia which sustains oxygen balance in the gut and prevents gut dysbiosis (Byndloss *et al*. 2017).

According to controlled experiments, increased SCFA production correlates with decreased diet-induced obesity (Lin *et al*. 2012) and lower insulin resistance (Zhao *et al*. 2018). Butyrate and propionate have been shown to influence gut hormones, lower hunger and food intake in mice (Fluitman *et al*. 2018), with further findings that gut microbial enzymes assist in bile acid metabolism by producing unconjugated and secondary bile acids, which serve as signaling molecules for other active pathways within humans (Long, Gahan and Joyce 2017).

2.4.2 Immune response

The human immune system is critical in safeguarding the body from potentially hazardous substances, pathogens and cell alterations (Zhou *et al*. 2020). The primary functions of the immune system in the body is to combat disease-causing pathogens such as viruses, parasites, bacteria, or fungi and eliminate them from the body, identify and neutralize hazardous substances in the environment and to counteract disease-causing alterations in the body such as cancer cells (Medina 2016). Gut microbiota provides critical health advantages to its host by controlling immunological homeostasis. Furthermore, it has been demonstrated that changes in gut microbial composition and species diversity might promote immunological dysregulation, leading to autoimmune diseases (Wu and Wu 2012). In establishing the significance of gut microbiota in shaping both native and adaptive immunity, the use of germ- free models in which animals are raised in a sterile environment with no exposure to microorganisms, has been an effective tool in demonstrating that signals derived from gut microbiota are vital for immune system development and function (Smith, McCoy and Macpherson 2007).

To prevent infection, pathogens must be neutralized and eradicated as soon as possible. When the barrier is breached, the innate immune system acts as a first line of defense against pathogens (Huitema *et al*. 2021). Research has also shed light on the significance of gut microbiota in enhancing innate immune response against infections (Gallo and Nakatsuji 2011). For instance, *Bacteroides fragilis* and symbiotic *Clostridium clusters IV* and *XIVa* have been shown in rodent studies to promote scurfin (Foxp3) + Regulatory T cells (Treg) and contribute in the development of immunological tolerance (Atarashi *et al*. 2011). Antimicrobial peptides (AMPs) serve an important role in innate immunity by responding under homeostatic conditions and killing pathogenic microorganisms via a variety of ways such as inducing cell death via interaction with DNAs and RNAs (Zhang *et al.* 2021). AMPs have been found to be secreted by gut microbiota. *S. epidermidis* has been observed to secrete phenol-soluble modulin γ and δ, both of which show antibiotic properties against *S. aureus* (Cogen *et al*. 2010). Atarashi *et al*. (2015) observed segmented filamentous bacteria (SFB) in combination with twenty bacterial isolates from ulcerative colitis patients, including *Clostridium* and *Bifidobacterium* species and promotes osteoclastogenesis (Th17 activity).

2.4.3 Pathogen colonization

The potential of gut bacteria to suppress pathogen colonization is regulated by a variety of

methods, namely direct killing, competition for limited resources and immune response improvement (Pickard *et al*. 2017). According to Hammami *et al*. (2013), growth inhibition or killing may play a dominant role in pathogen colonization resistance. Bactericidal compounds such as Bacteriocins are produced by gut microbiota. *Lactobacillus* strains were observed to protect mice against infection by *Listeria monocytogenes*, which was dependent on *Lactobacillus* bacteriocin (Corr *et al*. 2007). Various studies have acknowledged the role of gut microbial metabolic by-products being effective in inhibiting and hindering the growth of pathogenic organisms (Schulthess *et al*. 2019). SCFAs were early reported as a critical factor in inhibiting *Salmonella typhimurium* growth in mice (Lawhon *et al*. 2002), are also efficient against pathogenic *Escherichia coli* (Tobe, Nakanishi and Sugimoto 2011) and *Clostridium difficile* (Seekatz *et al*. 2018).

Bile acids are amphipathic, cholesterol derived compounds that are released into the small intestine (Pickard *et al*. 2017). Gut bacteria such as *Lactobacillus*, *Bifidobacterium* and *Bacteroides* have been reported to possess bile salt hydrolases encoding genes (Molinero *et al*. 2019). Theriot *et al*. (2014) established a link between secondary bile acid deoxycholic acid and *C. difficile* resistance. Remarkably, the major bile acid chenodeoxycholic acid was also able to provide indirect protection by triggering innate defenses in the small intestine via its receptor, FXR (Inagaki *et al*. 2006).

2.4.4 Neuroendocrine system

The neuroendocrine system is traditionally defined as a structured group of cells with neural determination that create hormones or neuropeptides (Farzi, Fröhlich and Holzer 2018). According to recent studies, the neuroendocrine system and gut microbiota communicate bi-directionally (Martin and Mayer 2017). The early development of gut microbiota has been shown to influence various aspects of brain function and behavior, including neuroendocrine responses to stress (Berens, Jensen and Nelson 2017). *Lactobacilli* have been shown to be effective against a variety of stress regimens including neurochemical alterations. A two-week intervention of female mice with *Lactobacillus farciminis* followed by partial restraint stress can inhibit stress-induced elevations in adrenocorticotropic hormone (ACTH) and in regulating corticosterone (Ait-Belgnaoui *et al*. 2014). It has been proposed that gut microbiota composition varies in individuals with anxiety or depression than that in healthy individuals (Jiang *et al*. 2018).

The direct effect of gut microbiota on emotional behaviors has been demonstrated in studies which found that anxiety-like behaviors varied between germ-free mice, mice raised in a microbiota-free environment and animals with administered normal gut microbiota (Nishino *et al*. 2013). It was observed that colonization of mice with normal gut microbiota had improved behavioral differences (Clarke *et al*. 2013). Gut microbiota composition is a crucial factor that contributes towards better immune function, serving as protection against pathogens and supports brain sanity (Pickard *et al*. 2017). Evidence presented shows that alterations in gut microbial composition and diversity leads to imbalance and have been linked to contracting of autoimmune, infectious and inflammatory disease (Toor *et al*. 2019).

2.5 Influence of prebiotics on secondary metabolite production

Nutrient intake and energy regulation in humans are influenced by various factors including environmental and lifestyle factors with diet being the most important factor. Dietary intake is suggested to have profound effects on gut microbiota composition and diversity (Kolodziejczyk, Zheng and Elinav 2019). Gut microbiota promotes the biosynthesis of a wide range of chemical substances due to their ability to ferment complex dietary carbohydrates such as pectin, cellulose, lignin and other polysaccharides (Baj *et al*. 2015). The fermentation of these substrates results in the formation of metabolites known as SCFAs. SCFAs are a subset of fatty acids, volatile in nature, composed of 1 to 6 carbon molecules existing in straight or branched conformation (Koh *et al*. 2016). The primary metabolites include propionate, butyrate and acetate (Pascale *et al.* 2018).

Fermentation is a cytoplasmic anaerobic redox process in which organic substances act as both electron donors and acceptors (Liu, Qin and Lin 2017). In the gut, the fermentation of dietary carbohydrates and complex polysaccharides involves various species present within the microbiome. The fermentation products of certain species often become substrates for fermentation or integrated as intermediate metabolites into the metabolic pathways of other species. This results in substrates being sequentially fermented. Ethanol, lactate and pyruvate levels are reduced following bacterial consumption and SCFA synthesis (Markowiak-Kopeć and Śliżewska 2020). The primary end-products of sugar catabolism are SCFAs which account for 85–95% of total SCFAs in the colon (Perez Chaia and Oliver 2003). SCFAs have substantial impact on human energy metabolism. The existence of these metabolites specifically butyric, acetic and propionic acids, in adequate amounts in the human body is crucial for the host's

health and well-being. SCFAs play a vital role in the maintenance of immunological and intestinal homeostasis (LeBlanc *et al*. 2017).

2.5.1 Butyrate

Butyrate, a four-carbon short-chain fatty acid is generated in the lower digestive tract via microbial fermentation of dietary fiber (Liu *et al*. 2018a). It is the least abundant SCFA, accounting for 15% of total short chain fatty acid production (Conlon and Bird 2015). *Faecalibacterium prausnitzii* in *Clostridial cluster IV* and *Roseburia* spp. in the *Clostridial cluster XIVa* are two of the most important butyrate producing groupings (Mokhtari, Gibson and Hekmatdoost 2017). Butyrate has been reported to be vital in sustaining health through regulating the immune system (Furusawa *et al*. 2013), maintaining the epithelial barrier and promoting satiety after meals (Mikkelsen *et al*. 2015). It may be beneficial in the prevention of illnesses including colon cancer, inflammatory bowel disease, graft-versus-host disease, diabetes and obesity (Mathewson *et al*. 2016).

Dietary carbohydrates that enter the intestines are degraded into smaller soluble particles, which are then fermented producing SCFA and gases by secondary degraders such as butyrate-producing bacteria in the gut (Baxter *et al*. 2019). Many studies have demonstrated the fermentation of various dietary carbohydrates and its ability to produce butyrate. Venkataraman *et al*. (2016) demonstrated that diet supplemented with unmodified potato starch and un-gelatinized starch showed higher relative abundance of resistant starch (RS) - degrading gut microorganisms as well as in butyrate production. As Brouns, Kettlitz and Arrigoni (2002) observed that fermentation of RSs typically results in a substantial production of butyrate ranging between the molar quantity of 20 and 28%. Acidic polysaccharides are a type of polyanionic molecules with uronic acid or sulfate groups and fermentation of these polysaccharides have also been reported to increase gut microbial diversity and produce SCFAs (Fu *et al*. 2019). The fermentation of pectins by gut microbiota have been shown to be associated with anti- inflammatory properties (Onumpai *et al*. 2011). Pectin have been observed to promote the growth of *Clostridium, Lachnospira* and *Dorea* species belong to *Clostridium cluster XIV*. These species are known as butyrate producing species capable of increasing butyrate levels by fermentation of pectin (Bang *et al*. 2018). Butyrate is a biological mediator that regulates various functions of gut cells and beyond, including gene expression, gut tissue development, immunological modulation, cell differentiation and diarrhea management (Bedford and Gong 2018). Butyrate has been established in numerous trials to be an anti-

inflammatory agent (Mowat and Agace 2014). Numerous investigations have demonstrated that butyrate inhibits Nuclear Factor NF- κB signalling pathways via restoring the redox machinery and regulating reactive oxygen species, which promote NF- κB activation (Russo *et al*. 2012).

Butyrate, through its function as an HDI (human development index) can perform various cellular homeostasis-related actions (Jahns *et al*. 2015). Dysbiosis is the most common cause of diarrhea caused by an antibiotic disruption of gut microbiota, which reduces fermentation (Whelan and Schneider 2011). Butyrate absorption has been demonstrated to improve potassium, salt and water absorption, thereby contributing to its antidiarrheal qualities (O'Keefe *et al*. 2011). Paparo *et al*. (2017) also described butyrate to play a critical role in protecting the heart against ischemia and pathologic hypertrophy.

2.5.2 Acetate

Acetate is suggested to account for a majority of SCFAs produced by gut microbiota (Ríos-Covián *et al*. 2016). Considering that therefore, its production is critical to the overall health and well-being. There are two major biological processes for acetate generation by gut microbiota which have been identified. Firstly, is that most acetate production are as result of dietary carbohydrate fermentation by most gut microbiota. Secondly, approximately one-third of colonic acetate comes from acetogenic bacteria, which can synthesis it from hydrogen, carbon dioxide and formic acid (Louis, Hold and Flint 2014). Nordgaard *et al*. (1996) showed increase acetate synthesis in previous colonic cancer patients that were administered with fiber derived from *Plantago ovuta* seeds. *Bifidobacteria* and *Lactobacilli* are the main producers of acetate however, it can be produced by *Akkermansia muciniphila* (Fukuda *et al*. 2012; Daisley *et al*. 2020). Factors shown to influence SCFAs production includes gut microbiota composition and species diversity in addition to the type of dietary fibers provided to the microbes through diet.

2.5.3 Propionate

In the human gut, propionate has been suggested as a significant metabolite produced as a result of microbial fermentation, that may have positive impact on health beyond the gut epithelium (El Hage *et al*. 2019). Propionate has been shown to slow down lipogenesis, other tissue carcinogenesis and serum cholesterol levels (Hosseini *et al*. 2011). Many studies have shown that specific prebiotic foods are responsible for increased production of propionate. For instance, Wu *et al*. (2019) found that grape seed proanthocyanidins, which are natural

flavonoids increased the synthesis of propionate and altered microbial composition. Monsma *et al*. (2000) observed a substantially higher fraction of propionate production during oat bran fermentation in comparison to wheat bran. In addition, when compared to wheat bran, oat bran was shown to increase the bacterial mass, further maintaining it for a longer duration.

2.6 Functions of short chain fatty acids in human health

SCFAs play a pivotal role in pH regulation, enhancing calcium, iron and magnesium absorption, promoting glucose and protein metabolism in the liver (Kuczyńska *et al*. 2011). Furthermore, these acids have an effect in maintaining the overall structure, integrity and function of the gastrointestinal tract. They are the principle source of energy for colonocytes (Koh *et al*. 2016). Furthermore, by encouraging the growth of saprophytic microflora, SCFAs restrict the development of pathogenic microbes like *Campylobacter*, Salmonella and *E. coli*, that compete for colonization sites (Pickard *et al*. 2017). Butyric acid has been found in studies to boost the expression of the Mucin 2 (MUC2) gene in cell lines as well as synthesize mucin. The sticky film it generates protects the intestinal epithelium (Hamer *et al*. 2010).

SCFAs have also been found to play significant roles in prevention and treatment of metabolic disorders such as obesity (Lu *et al*. 2016; Li *et al*. 2018). According to some studies, SCFAs has potential in the prevention and treatment of obesity-related insulin resistance (Zhang *et al*. 2021). It has been shown in rodent studies that administered prebiotics induce a shift in gut microbiome toward enhanced butyrate production. This was found to have positive effects related with higher levels of glucagon-like peptide-1 (GLP-1) (Barrea *et al*. 2019), as well as hypothalamic expression of pro-opiomelanocortin, which influence the hunger-satiety cycle (Ahmadi *et al*. 2019). The effect of SCFA in blood pressure regulation has been studied primarily in animal models. It has been observed that acetate and propionate appear to regulate blood pressure through a complex interaction involving renin activation via olfactory receptor 78 (Olfr78) and counter-regulation via free fatty acid receptor 3 (FFAR3) (Natarajan *et al*. 2016).

According to growing evidence, SCFAs may also alter crucial neuropathological processes underlying Alzheimer's disease (AD) (Walsh *et al*. 2015). AD is the most common type of dementia characterized by increasing cognitive impairment (Prince *et al*. 2016). SCFAs have been found to interfere with protein complexes between amyloid-β peptides (Aβ), further altering their conformation into neurotoxic oligomers (Ho *et al*. 2018), which are the primary

toxins responsible for synaptic disruption and cognitive deficits in AD (Ferreira *et al*. 2015). Depression is one of the most typical mood disorders, having a negative effect on patients' quality of life and ranking among the primary causes of social disability. Untreated depression increases the risk of illness and mortality, such as suicide. Clinical evidence supports these findings, demonstrating that fecal SCFA concentrations are lower in those with depression than in non-depressed counterparts (Skonieczna-Żydecka *et al*. 2018). Furthermore, current research indicates that butyrate has antidepressant-like effect capable of reversing behavioral changes such as low energy (Wei *et al*. 2015), anhedonia and cognitive (Sun *et al*. 2016) and social abnormalities as observed in rodent studies (Deng *et al*. 2019).

2.7 Effects of prebiotics on gas production

Although prebiotics are considered advantageous in stimulating gut–friendly microbiota and in the production of SCFAs, one of their limitations is the excessive production of gas which is rarely taken into account in the development and design of prebiotic supplements (Leis *et al*. 2020). Although a majority of intestinal gases are absorbed into the blood and expelled via the lungs, it can nevertheless have physiological consequences on a person's body (Kalantar-Zadeh *et al.* 2019). The amount of gas produced due to consumption of dietary prebiotic fiber may have an impact on how quickly it moves through the colon by constricting the colonic wall (Azpiroz 2005). Chronic constipation and irritable bowel syndrome (IBS) may be as result of methane production, which has been shown to lower serotonin levels and decrease intestinal transit in the digestive system (Sahakian, Jee and Pimentel 2010). Considering gas production while choosing prebiotics may be crucial, mainly due to bloating which is a common sign of various gastrointestinal diseases including IBS (Seo, Kim and Oh 2013).

Various studies have suggested that the chemical composition of the prebiotic and variations in microbiome diversity may directly affect the formation of various gases by gut bacteria during prebiotic fermentation (Yu *et al*. 2020). Smiricky-Tjardes *et al*. (2003) using swine faecal microbiota via *in-vitro* fermentation experiment, found that α-gluco-oligosaccharides produce less gas than galacto and fructo-oligosaccharides. Sarbini *et al*. (2014) found that dextrans of low molecular weight ferment more rapidly, taking less time to reach maximum gas production. This observation was attributed to the simple structure that make low molecular weight prebiotics easily fermentable to gut microbiota. In addition to the structure and composition of prebiotics, gut microbial composition can also be a factor which influences gas generation.

Sánchez-Moya *et al*. (2017) observed whey protein that is composed of α-lactalbumin, serum albumin, β- lactoglobulin and immunoglobulins to cause increased gas production in normal weight individuals in comparison to obese individuals. This was attributed to the composition of gut microbiota present in the respective groups.

2.8 Alterations in gut microbial species diversity and composition

There are many definitions that exist which define dysbiosis of the gut, however the most accepted and well understood definition is that it is any alteration in the composition and or diversity of resident commensal communities, relative to that found in healthy persons (Petersen and Round 2014). Dysbiosis is caused by a combination of gut pathogen colonization and host inflammatory responses. Inflammation is a mechanism utilized by pathogens to breach the gut barrier leading to the contracting of various diseases (Yoo *et al*. 2020). Many factors such as antibiotics and geographical location can lead to gut dysbiosis (Strzępa *et al*. 2017), however, changes in diet is considered a major factor. Various studies to date, have compared the effects of diet and dietary changes on gut microbiota composition and diversity. A study undertaken by De Filippo *et al*. (2017) compared the gut microbiota of European children with that of rural African children. It was observed that children in industrialized Europe had larger abundance of Bacteroides and a lower abundance of Prevotella in comparison to rural African children who ate an agrarian diet, with lower Bacteroides and greater Prevotella proportions.

Moreover, studies have shown that dietary alterations can promote a rapid but transient change in gut microbial composition and diversity within 24 h (David *et al*. 2014). Ruiz-Ojeda *et al*. (2019) observed that human participants who were subjected to diets rich in polyols such as maltitol, lactitol and isomalt had an abundance of *Bifidobacteria* but lower *Bacteroides*. In mice, a combination of a high-fat and high-sugar diet was observed to cause dysbiosis, with increased abundance of *Bacteroides* spp. and *Ruminococcus* (Agus *et al*. 2016). Consumption of "Western-style" diets, which are often low in fiber and high in fat and digestible sugars, negatively shift the composition of the gut microbiota, leading to various non-communicable diseases (NCDs). Taken together, these findings imply that dietary changes can influence and shift human gut microbiota in either a positive or negative way and is a “factor” which can be manipulated to prevent and reduce disease and illness.

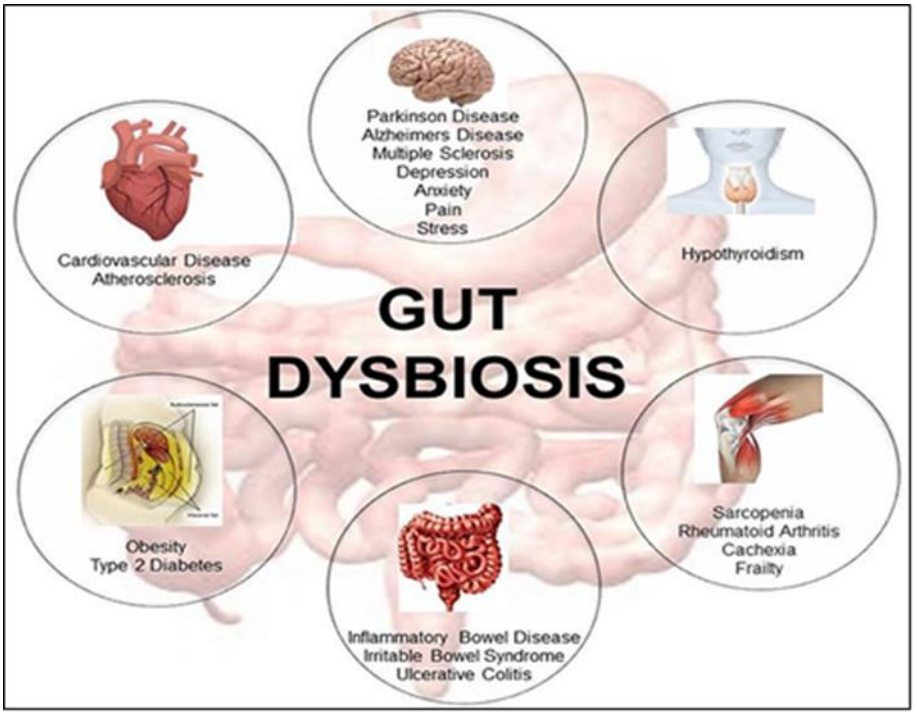

Figure 2. 3 Represents metabolic diseases, neuromusculoskeletal conditions, endocrine pathologies, cardiovascular diseases and gastrointestinal disorders, as result of gut dysbiosis (Buford 2017).

2.9 Diet

2.9.1 The influence of gut microbiota in non-communicable diseases (NCDs)

Over the years, the predominant cause of deaths is as a result of non-communicative diseases (NCDs) and obesity. By definition NCD is chronic illness or disorder that is non- infectious and is not transmittable, linked to nutrition and lifestyle habits (Adjaye-Gbewonyo and Vaughan 2019). NCDs are considered to be progressive disorders with long durations or with gradual development (WHO 2014a). Cardiovascular disease, diabetes, cancer, chronic respiratory disease and obesity are the most common NCDs. At least two-third of global deaths reported is due to chronic cardiovascular disease, diabetes mellitus, stroke, obesity and various cancers (Beaglehole, Bonita and Alleyne 2011). According to the World Health Organization (WHO), the trend of NCDs is estimated to account for 60% of disease burden and 73% of deaths globally by 2020 (Spires *et al.* 2016). Global estimates suggest that the greatest rise in deaths caused by NDC, occur in middle and low-income countries, with South Africa being no exception (World Health Organization (WHO) 2011). The rate of mortalities as a result of NCD

have been projected to increase to 52 million by 2030, of which 80% are predicted to occur in developing countries (Day *et al*. 2014; Nojilana *et al*. 2016b). It has been projected that NCDs are likely to surpass communicable, maternal, neonatal and nutrition diseases as the leading cause of death in sub-Saharan Africa by 2030 (WHO 2014b).

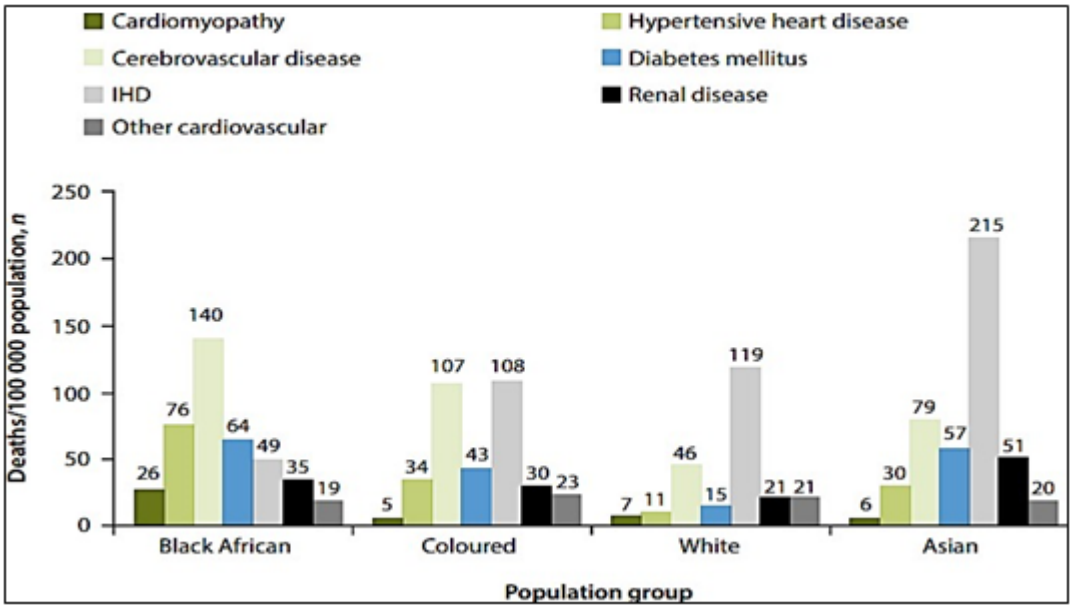

Figure 2. 4 ASDRs for coronary disease, diabetes mellitus and kidney disease by demographic group, SA 2010 (Nojilana *et al*. 2016a).

In South Africa, NCDs pose as a rising burden on the health sector as well as the economy. Over recent years, the rate of obesity and overweight have significantly increased and is classified as the second leading metabolic risk factor in NCD mortalities as depicted in Figure 2.4 (Armstrong, Lambert and Lambert 2011; Kruger *et al*. 2012). The shift from infectious diseases to NCDs in developing countries is strongly influenced and driven by economic development, a change from organic and traditional diets to highly processed and convenient food products (Hancock, Kingo and Raynaud 2011). It is currently estimate that more than 45% of men and women over 35 years of age are classified as either being overweight or obese (Bradshaw *et al*. 2011). According to Sanpath (2016), research conducted at the Wits University School of Public Health, revealed that 13.1% of deaths are now caused by cardiovascular diseases and diabetes, in addition to the observed increase in relatively young individuals suffering from high blood pressure, cardiovascular disease and various types of cancers. Although NCDs have traditionally impacted on western population, these diseases are also affecting other demographic groups (Puoane *et al*. 2012).

Diet is regarded as the leading risk factor for NCDs. Poor diet is reported to be the current cause of more diseases than alcohol, smoking and physical inactivity. This is based on a shift in dietary pattern by the population (Malhotra, Noakes and Phinney 2015). The "nutrition transition" is marked as a change from conventional traditional diets focusing primarily on staples such as vegetables, fruits, starchy roots, grains and legumes to high calorie foods consisting of excessive sugar, high animal protein, fat and extensively processed foods (Popkin 1994; Kennedy, Nantel and Shetty 2004). This modern diet generally referred to as the "Western diet" is composed mainly of inexpensive, heavily promoted high calorie, easily digestible and nutrient-poor foods (Swinburn *et al*. 2011). As mention by da Costa Louzada *et al*. (2015), vast populations derive their energy from ultra-processed foods which are convenient, can be eaten anywhere and without moderation. These foods are highly marketed and sold in the form of ready-to-eat dishes, snacks and drinks making them more advantageous over home-prepared meals (da Costa Louzada *et al*. 2015).

This leads to "mindless consumption" and is likely to harm mechanisms that regulate satiety and appetite (Ogden *et al*. 2013). Ultra-processed foods are known to be of high glycaemic value when solid and are described as being energy dense due to insufficient amounts of carbohydrates and water (Monteiro *et al*. 2010). Foods of high glycaemic value may induce an intensified insulin reaction, which may facilitate weight gain by diverting nutrients away from muscle oxidation and into fat storage (Brand-Miller *et al*. 2009). As South Africa is increasingly growing, matching up with developed countries, the "Western lifestyle" is becoming the norm among all sectors of the society (Spires *et al*. 2016). Contributing to this lifestyle shift and the growing problem of obesity and NCDs, is "Big Foods" as they increase the supply, affordability and acceptability of products. Foods such as cakes, cookies, carbonated drinks, desserts and fast foods are found to be consumed on average four days a week by the youths (Reddy *et al*. 2010).

Among urban South African youths (12-24 years of age), carbonated beverages were ranked third most consumed products (Theron *et al*. 2007). It was found that 11.3% of the population rely on street foods sold by vendors whilst 68% consume fast foods on a regular basis (Steyn and Labadarios 2011). Another reason for this trend is due to supermarkets having increasingly market regular and processed foods as they are more affordable (Weatherspoon and Reardon 2003) and healthier product and food choices being more expensive (Temple *et al*. 2011). Lifestyle change, dietary patterns and food conditions are key factors required to successfully reduce and prevent NCDs. Pathway and cause of the mentioned diseases are suggested to be

multifactorial, however, it has been increasingly recognized that leading cause is due to alter and decrease in beneficial gut microbial species diversity and colonization. This has been linked to physiological, immunological and metabolic dysregulation seen in many NCDs (West *et al*. 2015). There is a distinct relationship that exists between host metabolism, gut microbiome and diet. Disruptions in these interactions upset natural homeostasis. Alterations in gut microbiota composition creates an imbalance referred to as dysbiosis which is an evolving characteristic in NCDs as shown in Figure 2.5 (West *et al*. 2015). Various studies have established the significance of diet on health comparing the effects of Western diet to Mediterranean diet (Sofi *et al*. 2013).

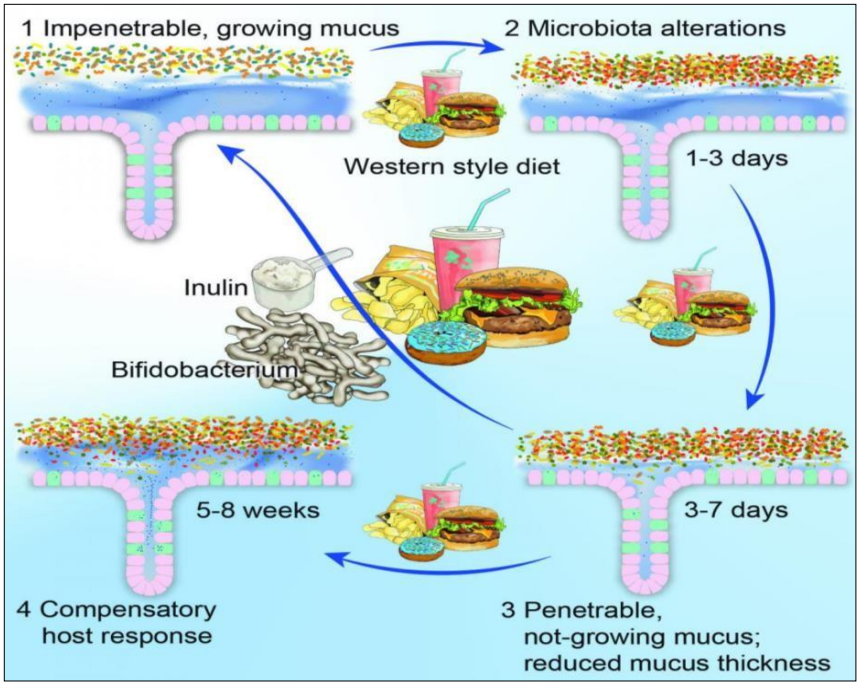

Figure 2. 5 Representation of the colon and gut bacterial changes after consumption of a low- fibre, western -type diet and thereafter proceeding with consumption of subsequent diet supplemented with fibre (Schroeder *et al*. 2018).

2.9.2 The effect of diet on human gut microbiota and health

The western diet, more commonly recognized as the "American Standard Pattern Diet," is one that includes the consumption of food and food products that are excessively high in sugar, sodium, animal protein and saturated fats (Appanna 2018). Worldwide, western diet consists mainly of ultra-processed food and take away foods. These foods are suggested to account for average of 25-60% of a person's daily energy intake (Monteiro *et al*. 2019). Ultra-processed foods also called "cosmetic foods", contain excessive ingredients such as salt, sugar, trans fats,

artificial colours and flavours. They are created with the purpose of convenience and are either ready to heat and eat or to drink. Ultra-processed foods include ready-to-eat and frozen meals, reconstituted meats, sodas and energy drinks, instant soups, candy, chips, cookies and packaged breads (Monteiro *et al*. 2013). Increased consumption of ultra-processed foods are associated with gut microbiota which produce high levels of endotoxins resulting in metabolic endotoxemia (Pendyala, Walker and Holt 2012; Ghosh *et al*. 2014) as shown in Figure 2.6. Various studies have investigated the consumption of ultra-processed foods and the changes that occur in gut microbiota composition, with high intakes of ultra-process foods having shown to increase number of *Firmicutes* species thus reducing the genus *Bacteroidetes* (Sonnenburg *et al*. 2010). This causes excessive storage of lipids in liver and adipose tissues as well as hyperinsulinemia (Lee 2013). Other studies show that western diets high in fat content induces metabolic endotoxemia (Martinez, Leone and Chang 2017).

According to Ott and Schreiber (2006), there is growing occurrence of inflammatory bowel disease (IBD) in western countries where diet consists mainly of processed foods, high animal protein and excessive sugar. Process foods have been found to decrease gut microbiota diversity, in particular bacteria that are responsible for butyrate production which as a result leads to increases in IBD. Studies conducted at the institute of Biomedical Sciences at New York Institute of Technology College of Osteopathic Medicine (NYITCOM), exposed rodents to a typical western diet, proceeding this treatment and it was observed that the blood vessels of these rodents showed disruption and elevated blood pressure, typical characteristics in diabetics (Technology 2017). Colorectal cancer (CRC) is classified as the third most globally spread cancer with IBD being a contributing factor (Byndloss *et al*. 2017). The rise in CRC incidences are associated with reduced consumption of dietary fiber (Aune *et al*. 2016). O'Keefe *et al*. (2015b) observed that by increasing fiber consumption among African American population, improved gut microbiome, increased butyrogenesis thus decreasing the threat of cancer biomarkers. Various studies have shown that dietary changes resulting in the increased intake of saturated fats, simple carbohydrates and animal protein not only disrupt the composition and diversity of gut microbiota but also lead to the development of neurodegenerative disease (Aguayo-Patrón and Calderón de la Barca 2017).

Recognizing diet as a crucial factor capable of changing gut microbiota composition and diversity was De Filippo *et al*. (2010). The intestinal microbiota of children from Europe and Burkina-Faso were studied and compared. The findings of multiple DNA sequencing and

biochemical tests revealed significant differences in gut microbiota composition and diversity between the two groups. Burkina-Faso kids showed enrichment in *Bacteroidetes,* a depletion in *Firmicutes*, and had increases in *Preotella* and *Xylanibacter* bacteria. These microbes are known to include bacterial genes responsible for cellulose and xylan hydrolysis, which children in Europe lacked. The study also showed that the diet of children from Burkina-Faso were low in fat and in animal protein but were high in fiber and plant polysaccharides. Burkina-Faso children consumed meals primarily made of millet grains, lentils and vegetables. High fiber diets have been frequently report to improve gut microbial diversity which is associated with lower weight gain (Menni *et al*. 2017). Such results were confirmed by Schnorr *et al*. (2014) who demonstrated that richness in microbial diversity was higher in Hadza hunters in comparison to urban Italian controls. Gut diversity of the Hadza hunter consisted of increased species of *Bacteriodetes,* decrease in *Firmicute* species with the absence of Actinobacteria phylum (Schnorr *et al*. 2014). Further clinical intervention study have demonstrated how increased intake of soluble fermentable fibres are capable of reducing obesity by administrating oligofructose enriched inulin in overweight and obese kids for a duration of 16 weeks to reduce their fat mass (Nicolucci *et al*. 2017). It is known that obesity is linked to the development of diabetes- type 2. However unlike obesity, diabetes is related to a decrease in fibre degrading bacteria (Qin *et al*. 2012; Karlsson *et al*. 2013).

It has also been shown how consumption of bread made from barley kernels, high in β-glucans, improves the metabolism of glucose in healthy humans (Nilsson, Johansson-Boll and Björck 2015). In addition to skin cancer, breast cancer has been reported to be the most prevalent cancer among women living in the USA. An additional 250,000 case are diagnosed each year (Park *et al.* 2021). In 2014, 10 guidelines for cancer prevention were proposed by the American Institute for Cancer Research (AICR). The concluding remark of the study was that dietary consumption of predominantly whole plant-based foods decreases the likelihood of cancer (Shams-White *et al.* 2019). Diet is also known to be crucial in infant development with feeding strategies suggested to influence the composition of gut microbiota diversity. The proliferation of *Bifidobacterium* species was found to be higher in breast-fed infants (Yu, Chen and Kling 2012), in comparison with infants fed infant-formula (Bezirtzoglou, Tsiotsias and Welling 2011).

In rural Africa, the diet of infants is characterized by intakes of fibre, starch and polysaccharides derived from plants. These infants reported to harbour microbiota that is rich in species such as

Actinobacteria and *Bacteroidets* (De Filippo *et al*. 2010). As consequent this led to African infants having maximized energy intake and being less susceptible to noninfectious colonic diseases and inflammations (De Filippo *et al*. 2010). Intake of plant proteins such as pea and whey protein have been reported to increase beneficial gut microbiota in particular *Lactobacillus* and *Bifdobacterium* species. In addition, plant proteins have been shown to decrease pathogenic species such as *Clostridium perfringens* and *Bacteroides fragilis* (Dominika *et al*. 2011). Kim, Park and Kim (2014) further demonstrated that pea protein was observed to increase SCFAs production, known to be essential for mucosal maintenance. As suggested by Sonnenburg and Sonnenburg (2014), a diet with reduced quantities and diversity of Microbiota-Accessible Carbohydrates (MACs) is the leading reported cause of depletion of beneficial gut microbiota in industrialized and urban populations.

It can be concluded from the cited studies that NCDs are as a result of poor diets leading to imbalances in gut microbiota composition and diversity. Robust evidence shows that plant-based diets bare strong relation in shaping gut microbiota positively further improving human health. Therefore, there is increased motivation for dietary interventions such as functional foods and food ingredients, capable of reducing and preventing NCDs. Inclusion of prebiotics are an efficient way to improve human health, thereby reducing the likelihood of NCDs.

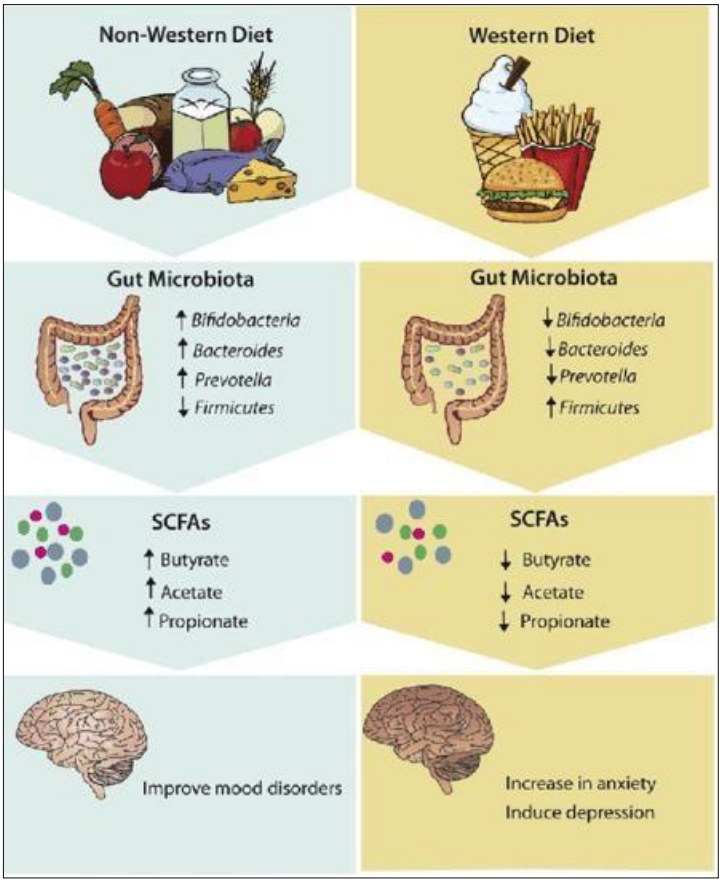

Figure 2. 6 Effects of plant-based diet vs western diet on gut microbiota composition, SCFAs production and brain sanity (Sandhu *et al*. 2016).

2.10 Prebiotics

As the rise in NCDs increase, consumers are becoming more conscious regarding their food choices. The demand for healthier, nutritious foods and food products has encouraged growth in the market of dietary supplements. Gastrointestinal microbiota has profound impact on human health, hence, there is growing interest in using dietary methods to induce beneficial gut species and metabolic activity that contribute to improved health and disease prevention (Holscher 2017). As consumers are becoming increasingly aware of gut health, there is greater demand for prebiotics.

2.10.1 Defining prebiotics

There are many definitions that describe what prebiotics are. The initial proposed definition was by Gibson (1998). However, at this time studies were limited to culture dependent methods and specific bacteria. Progression through technological advancements have assisted in better

understanding of gut microbiome and the effect of diet on microbiota (Holscher 2017). A more acceptable definition is one that describes prebiotics as a substrate resistant to digestion by host enzyme, fermented by gut microbiota, capable of altering the composition and function of gut microbiota thus contributing to human health (Bindels *et al*. 2015b; Thomas, Suzuki and Zhao 2015). The new definition incorporates other types of potential prebiotic compounds and includes those that may function outside the human digestive system (Gibson *et al*. 2017a). Prebiotics as the term "pre" depicts before or prior acts as a fertilizer for microbiota present within the gastrointestinal system. Prebiotics are compounds most employed to maintain healthy microbiome or to re-establish its equilibrium when bacterial homeostasis is disturbed (Quigley 2019).

2.10.2 Types of prebiotics and their sources

Most complex carbohydrates are naturally derived from plants and microorganisms (de Paulo Farias *et al*. 2019). These carbohydrates are resistant to hydrolysis by human digestive enzymes and are known to have prebiotic properties, as they are fermented and utilized by gut microbiota. Prebiotics include resistant starch, resistant dextran's, non-starch polysaccharides (pectin, mucilage, arabinogalactans), non-digestible oligosaccharides, fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides (GOS) and sugar alcohols (Mohanty *et al*. 2018; Colantonio, Werner and Brown 2020). Different types of prebiotic substrates vary in health benefits. Vegetables such as asparagus, onion, Jerusalem artichoke, fruits, legumes and lentils are enriched with oligosaccharides, fibers and sugar alcohols which are prebiotic compounds as illustrated in Table 2.1 (Mohanty *et al*. 2018; Wang *et al*. 2019b). Certain microorganisms are also capable of producing polysaccharides with prebiotic properties, including *Aspergillus oryzae*, *Kluyveromyces marxianus*, *Pseudomonas aurantiaca* and *Saccharomyces cerevisiae* (Mano, Paulino and Pastore 2019).

Few mushroom species including *Agaricus bisporus* have been reported to have possible prebiotic advantages (Hess *et al*. 2018). Extracts from the fruiting body of *Pleurotous* species (pleuran) containing b-glucan, have been employed in many food supplements, reported to have an impact on immunosuppression and the proliferation of gut probiotics in human health (Synytsya *et al*. 2009). Grain legumes such as *Cicer arietinum* L (Chickpea), are a source of dietary fiber and contain α-GOSs which are utilized as functional foods and have been reported to stimulate *Bifidobacterium* species, lowering *Clostridial* species further contributing to gut health and preventing colon infections (Fernando *et al*. 2010). Spices are also known to have

various medicinal and therapeutic effects but have also reported prebiotic effects. Romo-Vaquero *et al*. (2014) reported on Rosemary extracts which were found to limit the progression of *Leuconostoc* and *Pedicoccus* species and increase *Bacteriodes* species. Laminarans (laminarins) derived from *Laminaria/Saccharina* are recognized as dietary components with prebiotic potential (Devillé *et al*. 2004). Laminaran which is a β-polymer of glucose has been shown to regulate gut metabolic reactions through interactions with gut goblet cells. It was also reported to decrease intestinal pH and improves the production of SCFAs (Deville *et al*. 2007). It is highly suggested that there are varieties of prebiotics that exist, that can be derived from different sources, varying in structure and functional properties (Patel and Goyal 2012).

Table 2. 1 Types of prebiotics and their sources from which they are derived (Beisel and Afroz 2016).

Type of prebiotic	**Source of prebiotic**
Galacto-oligosaccharides	Beans, Lentils, Chickpeas, Brussels sprouts
β- glucan	Mushrooms, Seaweed, Yeast, Oatmeal
Fructans (Inulin, Fructo-oligosaccharides)	Chicory, Garlic, Leek, Artichokes
Resistant Starch	Beans, Peas, Lentils, Tubers
Pectin, Cellulose, Gums and Mucilages	Fruits, Vegetables, Roots and Seeds

2.10.3 Fermentation of prebiotics

Prebiotics are recognized as having significant impact on the composition and diversity of gut microbiota. Humans lack digestion enzymes that are capable of hydrolyzing prebiotic polymer bonds and linkages. These substrates are therefore capable of by-passing digestion in the small intestines and reaching the larger colon intact (van der Beek *et al*. 2017). It is in the larger colon, that these prebiotic polymers are fermented by bacteria such as *Lactobacilli* and *Bifidobacteria*. These substrates form the source from which gut microbiota obtain energy, enabling them to grow in composition and increase in diversity (Flint *et al*. 2007). In phylogeny, specific gut microbial species have abilities to ingest specific prebiotic substrates on a regular basis (Scott *et al*. 2013). Functional metagenomics approach recently reported on how specific human microbiota genes are identified for the degradation of numerous prebiotics in the colon. Studies have shown how microbiota such as *Bacteroidetes*, *Actinobacteria* and *Firmicutes* are capable

of fermenting wide range of prebiotic substrates such as fructo-oligosaccharides, galacto-oligosaccharides and xylo-oligosaccharides (Belenguer *et al*. 2006). Other studies have shown that specific species such as *Bifidobacterium* spp. can only ferment specific prebiotic substrates such as fructans and starch (Ryan, Fitzgerald and van Sinderen 2006).

Another important factor that determines the extent to which prebiotic substrates are broken down is their structure and composition. The length of the polymer chain is a factor in differentiating gut species capable of fermenting a given prebiotic. For example, inulin derived from chicory has a degree of polymerization (DP) of 60 and can only be fermented by a few species, but fructo-oligosaccharides with DP of 10 can be degraded by various gut microbes (Scott *et al*. 2014). Fermentation of complex prebiotic substrates often produce by-products, which are utilized as substrates by other gut microbial species. This is referred to as cross-feeding (Belenguer *et al*. 2006). *Ruminococcus bromii*, for example, can breakdown resistant starches and several species can use the fermentation products of this reaction (Welters *et al*. 2002).

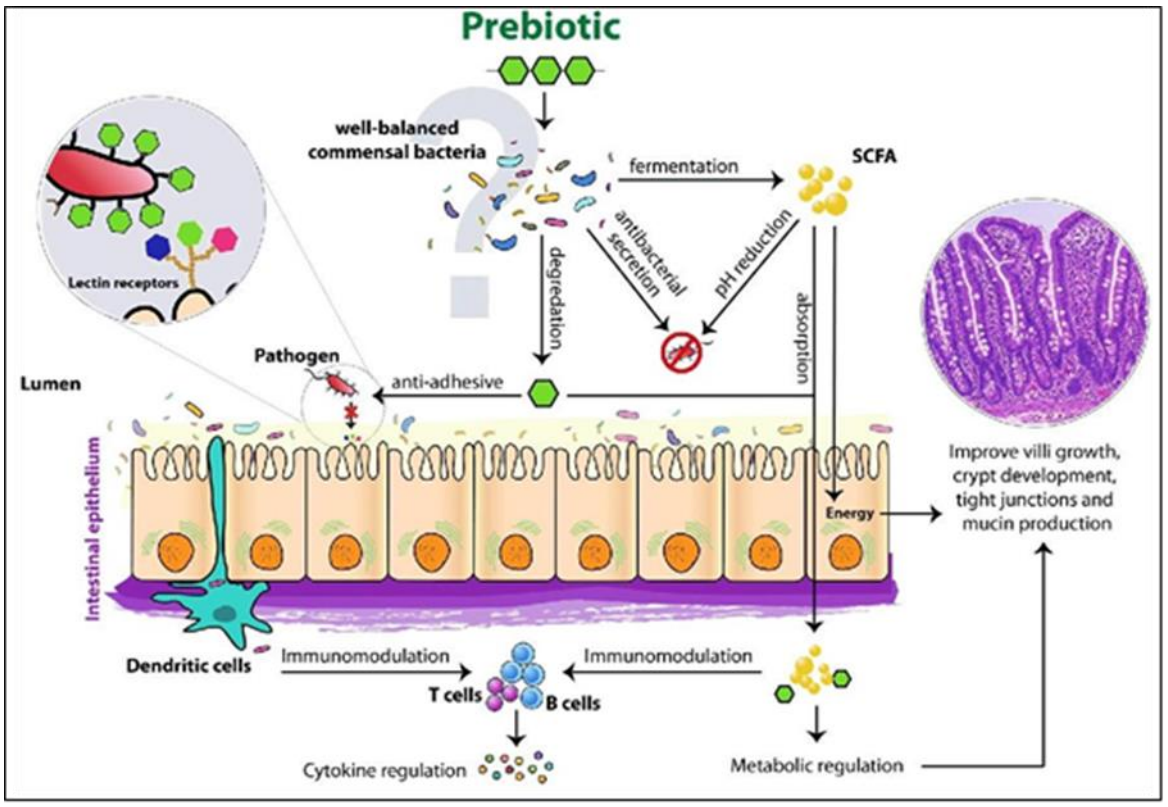

Figure 2. 7 Mode of action of prebiotics. Gut commensal microbiota breaks down prebiotics into secondary metabolites (SCFAs) mainly propionate, acetate and butyrate. This reduces luminal pH, offer energy sources for epithelial cells, and have significant impacts on inflammatory modulators and metabolic regulators. Well- balanced gut community can improve the structure of the gut mucosa. Specific bacterial strains are capable of releasing antimicrobial substances that stimulate the immune system through dendritic cell communication (Pourabedin and Zhao 2015).

2.11 The influence of prebiotic structure and composition on fermentation

Given the numerous potential effects of prebiotics on wellness, selecting appropriate research methodologies is essential to advancing knowledge in this area (Scott *et al*. 2020). Various studies have placed much emphasis on characterizing the gut microbiota (using a variety of microbiome analytical techniques) in order to determine how ingestion of potential prebiotic compounds affects the local microbiota (Donovan 2017; Battson *et al*. 2018). However, compositional analysis of gut microbiota does not provide sufficient evidence to demonstrate that a product or compound is a prebiotic. Moreover, the inclusion of prebiotics into various matrices, like food products, may influence its beneficial function. As a result, structural and functional analysis of potential prebiotic compounds must be performed in addition to microbial analysis (Scott *et al*. 2020). Many studies have also suggested that the physical and functional properties of presumed prebiotic compounds may have an impact on fermentation

and other physiological constraints within the host (Wang *et al*. 2020; Kassem *et al*. 2021). Studies conducted by Tamargo *et al*. (2019) have shown that different plant carbohydrate substrates with different viscosities have different effects on their fermentation and that the viscosity of the substrate may directly correlate with fermentation. The study demonstrated the importance of functional properties of plant carbohydrates in their breakdown and utilization by gut microbiota. Another study examined pectin as a prebiotic substrate with different degrees of methoxylization and viscosities. This study also extensively demonstrated the importance of these two restrictions in addition to the molecular weight of the substrate (Larsen *et al*. 2018). Structure and composition of plant carbohydrates can influence gut microbiota function in many ways. Gut microbiota function and activity tend to correlate with the physicochemical properties of plant carbohydrates. Below are some of the structural and physiochemical properties of plant carbohydrates and their presumed effects (Gill *et al*. 2021):

2.11.1 Branching

Braching is the organization of polymers that contains branch points as opposed to a linear form. This has an impact on both the molecular and macrostructure of the carbohydrate (Payling *et al*. 2020). Depending on the structural properties of the polymer, branching can increase or decrease its fermentability. Centanni *et al*. (2017) found that *Bacteroides cellulosilyticus* degrades xylan from beechwood, which has a low degree of branching, but not xylan from NewZealand flax, which has a high degree of branching.

2.11.2 Molecular weight and degree of polymerization

The number of sugar units in a polymer chain refers to the degree of polymerization (DP). It is understood that compounds with a high DP also have a higher molecular weight (Payling *et al*. 2020). This approach is used in the prebiotic concept to particularly encourage *Bifidobacterium* populations in the gut. Tandon *et al*. (2019) observed that higher DP and molecular weight compounds are fermented more distally in the colon than shorter chain molecules, according to *in silico* models.

2.11.3 Glycosidic linkages

Monosaccharides have multiple hydroxyl groups, hence they can form a variety of glycosidic linkages. The anomeric carbon and oxygen to which the glycosidic linkage is formed determines the linkage structure (Payling *et al*. 2020). These variables influence the specificity of bacterial

enzymes. This has been somewhat confirmed in clinical trials using fructo-oligosaccharide supplementation, where the abundance of faecal *Bifidobacterium* and *Lactobacillus* were observed (Tandon *et al*. 2019).

2.11.4 Monosaccharide composition

Monosaccharide composition refers to the sugar sequencing of the polymer. Homo-polymers are made up of a single monosaccharide, like starch or cellulose, which contain only glucose. Hetero-polymers are composed of a range of monosaccharides (Flint *et al*. 2012). Certain microbiota prefers different sugars that occur in a hierarchical structure. Beisel and Afroz (2016) observed that *Escherichia coli* preferentially uses glucose over lactose, but this preferential hierarchy varies greatly phenotypically. Carbohydrate structure is an important determinant in terms of its functional properties and the metabolism of the gut microbiota.

2.12 Effects of prebiotics on gut microbial diversity and composition and production of secondary metabolites

Prebiotics are often classified based on their origin, structure and composition (chain length, molecular weight and types of linkage), as well as their functional properties (solubility, viscosity and water binding capacity) (McRorie and Fahey 2013). Each of these characteristics are suggested to influence microbial fermentation and consumption. Inulin-type fructans (ITFs) include inulin, fructo-oligosaccharides and oligo-fructose (Liu *et al*. 2017). They are non-digestible soluble fibers found naturally in a variety of plant foods such as onion, asparagus, chicory roots and artichoke (Franco-Robles and López 2015). ITFs are structurally composed of a linear chain of fructose polymer joined by β (2→1) linkages (Mancilla-Margalli and López 2006). They have been shown to specifically increase beneficial gut microbiota (*Bifidobacteria*) and provide a number of health benefits. Structurally, these carbohydrates have linkages that are easily degraded by b-fructosidase and b-galactosidase enzymes, encouraging the growth and activity of *Bifidobacteria*, *Bacteroides* and *Lactobacilli* species (Wilson and Whelan 2017). Also, the consumption of inulin-type fibers, has been shown in various rodent experiments to lower blood cholesterol, body weight and blood glucose concentrations differentially (Rendón-Huerta *et al*. 2012). Non- digestible polysaccharides (NDP), are derived from plant cell walls and are heterogeneous in nature with differing degrees of molecular weight, chain lengths and types of linkages. They are collectively composed of insoluble fractions (lignin, celluloses and

hemicelluloses) and soluble fraction (pectins, gums, β- glucans and mucilages) (Knudsen 1997). Different classifications are utilized to describe them, including origin, chemical composition and physicochemical properties with additional subcategorization based on the degree of polymerization (e.g. chain length) (Holscher 2017). Importantly, each of these properties can also impact on microbial fermentation. With regard to origin, plant-based fibers can be separated into fibers derived from cereal grains, fruits, vegetables, nuts and legumes. However, it is important to note that the fibers present in different types of plants will also have variable chemical compositions, as well as physicochemical properties.

Non-digestible polysaccharides include but are limited to structurally different hetero-polysaccharides from mucilage, β-glucan, galacto-oligosaccharide (GOS), polysaccharides such as gums, pectins, dextrin, inulin and resistant starch (Cruz-Rubio *et al*. 2018; Mano *et al*. 2018; Colantonio, Werner and Brown 2019). These substrates are degraded and fermented in the colon by gut microbiota and form the source from which gut microbiota derive their energy from. As a result, prebiotics promote the activity of gut microbiota, produce energy and SCFAs (Davani-Davari *et al*. 2019). For instance, cereal β-glucan were found to significantly improve human health. Cereal β-glucan, due to its various functional properties such as high viscosity and water solubility, is readily fermented by gut microbiota and assists in providing functions like modulation of gut microbiota, production of SCFAs and improved immune health (Shoukat and Sorrentino 2021).

Pectin isolated from okra was also observed to have prebiotic effects by increasing probiotic bacteria such as *Bifidobacterium longum* and *Lactobacillus rhamnosus*, in addition to promoting production of short chain fatty acids (Yeung *et al*. 2021). Akbari-Alavijeh *et al*. (2018) found that in comparison to inulin, pistachio hull polysaccharides which was composed of xylose, glucose, arabinose and fructose, promoted the proliferation of *L. plantarum PTCC 1896* and *L. rhamnosus GG in-vitro*. It also increased the synthesis of acetate, propionate and butyrate. Prebiotic activity is characterized by a specific substrates potential to stimulate and support the growth and activity of gut microbiota. *In-vitro* and *in-vivo* approaches are utilized to evaluate candidate prebiotics, hence stimulation in gut species diversity and function are classified as determinants for the selection of prebiotics (Seifert and Watzl 2007; Wang 2009).

2.13 Novel polysaccharides as potential prebiotics

Traditionally, the concept of prebiotics centered around selectively fermented carbohydrates

acting in the colon and modulating levels of resident *lactobacilli* and *bifidobacteria* which are known to have beneficial effects on gut microbiota management (Cremon *et al*. 2018). However, technological approaches such as omics have improved methods such as *in-vitro* and *in-vivo* studies in order to assess the entire scope of prebiotic effects. Prebiotics have since then shown larger spectrum of microbial responses in addition to LAB (Gibson *et al*. 2017b). With the use of prebiotics, microbiota can be fostered and programmed accordingly.

There is currently limited selection of prebiotic compounds that have been scientifically demonstrated. Monopolizing the market are currently galactans and fructans such as inulin, however, the need to stimulate larger groups of commensal organisms has created greater demand for generation of novel potential prebiotics (Cunningham *et al*. 2021). Also gaining much attention, is the use of prebiotics to influence other host microbiomes such as the skin, oral cavity and the female urogenital tract (Bustamante *et al*. 2020). As attention shifts to scalability, sustainability and cost, there is growing research interest in extracting prebiotics from more novel and traditional sources (Bhagea *et al*. 2022). Depending on the desired functionality, prebiotic compounds can potentially undergo structural and chemical modification through the use of high pressure, acid, enzyme, sonication and oxidation processes (Lam and Cheung 2019). Additionally, novel combinations of prebiotics in optimized mixtures may enable the creation of new beneficial profiles.

Over the years, alternative definitions of prebiotics with broader scopes have been brought out from various studies in efforts to better incorporate potential and developing microbiome-modulating substances (Bindels *et al*. 2015a; Yang and Xu 2018). These substances are likely to include varieties of non-carbohydrate substances (polyphenolics, micronutrients), yeast-based compounds as well as carbohydrate-based compounds (gums, mucilages and pectins) (Singh *et al*. 2016; Khorasani and Shojaosadati 2017). Large research focus has been placed on carbohydrate-based compounds as prebiotic candidates. The search for novel carbohydrate-based prebiotic compounds is a continual activity and advancements are still ongoing (Kumar *et al*. 2015). Carbohydrate-based compounds can be obtained from a variety of sources including mammalian and non-mammalian origins. They are most widely isolated and can be purified from plants, bacteria, fungus and marine organisms for utilization in research and commercial markets (Lam and Cheung 2019). Currently, the development of carbohydrate-based compounds is divided into three categories: the search for novel CBPs, innovative uses of existing CBPs and the development of large-scale products. In order to assess the prebiotic

effects of carbohydrate-based compounds, it is necessary to establish the structure-function relationship. Structural characteristics such as the type of glycosidic linkages, degree of branching and sugar composition need to be established in order to better understand the fermentation and utilization of these compounds by gut microbiota. Gut microbiota have a variety of enzymes such as polysaccharide lyases (PLs) and glycoside hydrolases (GH) in their biological genomes which determines their abilities to breakdown and utilize specific carbohydrates (Tailford *et al*. 2015). Some gut microbiota are capable of breaking down a variety of polysaccharides whilst others are selective fermenters (Cockburn and Koropatkin 2016).

Potential carbohydrate components of plants that may be prebiotic include polysaccharides in plant cell walls, such as those of pectins, gums, mucilages and xylans. These compounds are considered popular as potential prebiotic candidates due to their indigestibility in the upper gastrointestinal tract and fermentation by gut microbiota (Scott *et al*. 2020). Plants suggested as being good potential prebiotic sources include garlic, mushrooms, okra, barley and dandelion greens. Legumes which are known to be high in fiber are also presumed prebiotics. For instance, chickpea is structurally composed of α-galacto-oligosaccharide and other components of dietary fiber, observed to stimulate the growth and activity of *bifidobacteria* in the gut, contributing to improved gut health (Hussein *et al*. 2020). Most plants with researched prebiotic capabilities are of western origin, however it is essential to investigate other parts of the world such as Africa and Asia as sources for novel prebiotics (Arshad *et al*. 2018). Traditional crops hold much potential, yet are often neglected. With increased stress on the agricultural system there is renewed interest in identifying and improving uses and applications of various traditional crops (Ficiciyan *et al*. 2018).

2.14 Amadumbe and okra as mucilage rich crops

Sub-Saharan Africa (SSA) consists of variety of traditional crops that have health promoting properties, however remain neglected and under-utilized (Paliwal *et al*. 2021). Most African societies have long relied on the use of homegrown plants for applications as traditional remedies and sources of nutrition. Thus, many ethnopharmacological studies are now aimed at reviewing this strong independence on native plants as applications of indigenous remedies and nutritional sources (Olajuyigbe and Afolayan 2012). Most underutilized and neglected crops in contrast to staple crops have higher nutritional profiles, in terms of micronutrients,

protein and essential vitamins. Hence, traditional crops become an asset in achieving nutritional security (Ebert 2014). Neglected and under-utilized crops hold versatile potential aside from being rich in nutrients, such as being resistant to droughts (well adapted to their environment), offers cultural diversity, greater ecosystem stability and they cater for the consumer demand for functional foods (Mustafa, Mayes and Massawe 2019). In conjunction with indigenous knowledge an advance technology, traditional crops can be transformed into nutrient rich products contributing to the health and betterment of individuals (Maliro 2001).

There are various under-utilized crops in sub-Saharan Africa such as *Colocasia esculenta* (taro), *Curcubita* spp (wild melon), *Vigna subterranea* (bambara groundnut) and *Impomoea batata* (sweet potatoes) (Wani 2021). Over generations, these crops have provided native societies with dietary support, but over time their cultivation has becomes less competitive and less appealing due to Green Revolution (Chivenge *et al*. 2015). Throughout the African region, root and tuber crops form the foundation of a staple food source for many rural communities. These native roots and tubers have been found to account for over 50% of SSA's food production (Maliro 2001). The nutritional profile of these roots and tubers are often rich in starch, carbohydrates, proteins, dietary fiber and minerals extending beyond the more staple crops (Lal *et al*. 2021).

Food is known for its ability to be a source of nutrition and there is growing interest in the functionality of food and food ingredients (van der Goot *et al*. 2016). Functional foods, also defined as modified foods or food ingredients, offer health benefits that go beyond basic nutrient intake (Birch and Bonwick 2019). The relationship between food and health is controversial, but there is increasing evidence that dietary ingredients are highly beneficial to human physiology (Gentile and Weir 2018). Functional foods are now forming an important aspect of scientific research in the prevention and treatment of existing and emerging diseases. However, the development of functional foods remains a scientific challenge especially in terms of identification, safety validations and regulations (Birch and Bonwick 2019).

Consumption of crops are known to assist in maintaining good health and well-being. It contains important components that help build and recover the body. Due to growing popularity among consumers with health related nutrition consciousness, crop production has increased significantly globally (Sharma and Singhvi 2018). Crop production is considered easily accessible and affordable means of obtaining the required micronutrients and is therefore evolving into strategies for reducing nutritional deficits in rural communities (Schreinemachers,

Simmons and Wopereis 2018). The most common challenge faced by SSA in terms of enhancing the popularity of traditional crops and plants is the deficiency in research funding, inadequate research dimensions and the lack of partnership between the respective sectors. These factors hinder the progress and development for viable agriculture in the sub- Saharan region (Govender *et al*. 2017).

2.15 Amadumbe (*Colocasia esculenta*)

Many tropical and sub-tropical plants belong to the Araceae family, of which *Colocasia esculenta* forms the most common consumable variety (Saxby 2020). More commonly referred to as Taro, was thought to have originated in the regions of India and Bangladesh, whereby they exist and are available in many variations. However, it can now be found in various tropical and sub-tropical regions, which are inclusive but not limited to the Pacific Islands, North America and Africa (Ahmed *et al*. 2020). Taro has been utilized for many years, suggesting it as being the oldest crop.

South Africa has 9 provinces and Taro has been found to be essentially cultivated along the Kwa-Zulu Natal coast. Taro in Natal is commonly referred to as amadumbe (Zulu name). Amadumbe is also found in the Eastern Cape, parts of the Mpumalanga and the Limpopo province (Mokhele 2018). It is very rarely cultivated in the Midlands and northern parts of the province. The cultivation of amadumbe seems to hold great agricultural potential especially offering employment opportunities for rural communities. The marketing of amadumbe in KwaZulu-Natal has significantly grown over the past few years. This observed growth can be attributed to small scale farmers marketing amadumbe to leading outlets such as Woolworths Foods and Pick ‘n Pay stores (Mapumulo 2022).

The growth of amadumbe plant reaches height of about 1 to 2 meters and is said to be composed of large leaves that protrude above the ground. Corms are located underground with fibrous roots. Corms are usually covered with a thick dark brown to semi-brown outer layer that acts as protection for the soft and succulent inner starchy flesh as illustrated in Figure 2.8 (Ghumman *et al*. 2019). Amadumbe growth is observed through three phases: the anagen phase, the vegetative stage and the initiation phase, also known as the maturity phase (Ebert and Waqainabete 2018). Amadumbe is usually harvested 8 to 10 months after planting when the leaves of the plant are visibly yellow. Both the leaves and the corms contain considerable amounts of nutrients, but more often the corm is consumed. Amadumbe is usually cultivated and grown in humid environments however it does have the ability to withstand drought conditions (Shelembe 2020).

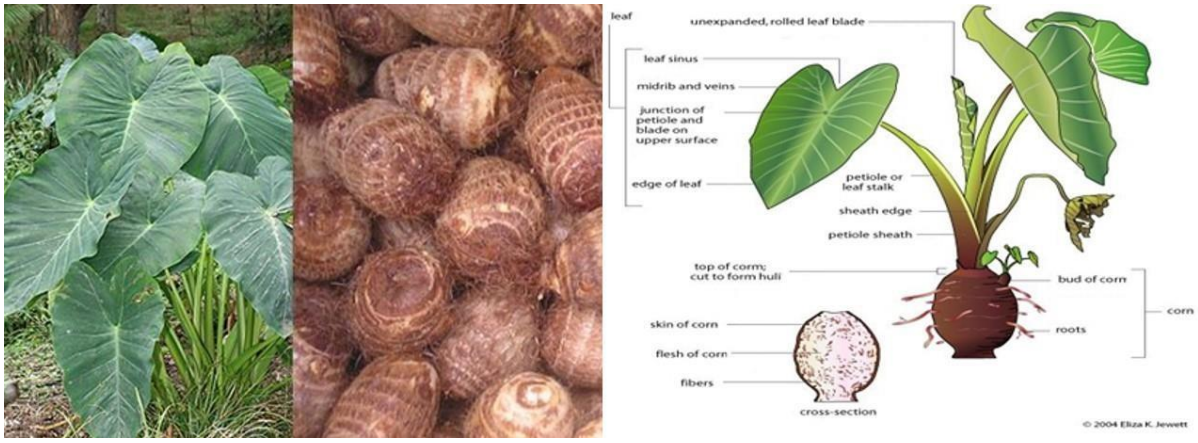

Figure 2. 8 Colocasia esculenta plant and corn (Kahuroa 2008), with labelled structure of taro corn and plant (Jewette 2004).

2.15.1 Nutritional composition of amadumbe corms

Amadumbes underlying food coherence properties include nutritional and anti-nutrient factors, as well as other phytochemicals with a variety of biological activities (Slavin 2013). Amadumbe is an excellent source of carbohydrates, fiber, sugar starch, ash, vitamin A and provides more energy than sweet potatoes and potatoes. Amadumbe corms contain more magnesium, calcium and potassium than other tubers (Kapoor, Singh and Kumar 2021). On a fresh weight basis, amadumbe corms contains about two-thirds of the water and 13-29% carbohydrate content. Compared to other major roots and tubers such as yam, cassava and sweet potato, amadumbe contains about 7% protein. The typical nutitional composition of amadumbe is shown in Table 2.2. Amadumbe is rich in all other essential amino acids except for low content of lysine,

isoleucine, histidine and methionine. Unlike other roots and tubers, amadumbe is also said to be low in fat (Naidoo, Amonsou and Oyeyinka 2015). The major component of amadumbe is carbohydrates. The presence of water soluble gums, also known as mucilage, is responsible for amadumbe slimy text. Amadumbe also produces a significant amount of mucilage. Amadumbe is reported to contain an average of 3 to 19% mucilage (Njintang *et al*. 2014), however, research on amadumbe mucilage has been limited, despite suggestions that viscous soluble fibers are more beneficial to health (Slavin 2013). Although amadumbe corms are rich in mucilage, they also contain significant levels of starch (Naidoo, Amonsou and Oyeyinka 2015). The levels of starch vary depending on varieties and cultivation environment. The presence of starch is suggested to contribute to its functionality including improved solubility and swelling properties (Falade and Okafor 2013).

Table 2. 2 Proximate composition of amadumbe corm (Kapoor, Singh and Kumar 2021).

Composition	**g/ per 100 g**
Protein	1.5
Ash	1.2
Carbohydrate	26.46
Dietary fiber	4.1
Sugars	0.4
Total lipids	0.2
Moisture	70.64

2.15.2 Utilization of amadumbe

Traditionally, amadumbe corms are consumed boiled, as porridge, in stews, fried as chips or processed into flour (Plucknett 2019) and offer nutritional security to poorer communities in Africa (Mabhaudhi, Modi and Beletse 2014). West African amadumbe are boiled and formed into a paste by pounding. It is thereafter made into balls which are swallowed after being soaked in soups and stews. In Hawaii and Polynesia, taro is processed into a porridge like consistency better known as poi. Poi is commonly consumed by locals but is also fed to tourists. Corms are thinly sliced and fried in hot oil in the United States, giving rise to a product comparable to crisps or potato chips. Taro products according to experimental procedures, can include cereals, flour bread, cake, noodles, infant food and beverage powder, in addition to

canned, flaked or frozen corms (Hrishi and Balagopal 2019; Chukwu 2020; Saxby 2020). Amadumbe consumption currently has many health benefits, including lessening the risk of developing diabetes, lowering the risk of contracting lung or oral cancers, increasing cognitive function, increasing blood circulation and preventing anaemia, excess gas, bloating, constipation and improving overall health and immunity (FAO 2009; Dhaliwal and Sharma 2016; Iwu 2016).

2.16 Okra (*Abelmoschus esculentus* (L.) Moench)

Okra (*Abelmoschus esculentus* (L.) Moench), is a flowering vegetable crop as shown in Figure 2.9, belonging to the Malvaceae family cultivated primarily for its edible pods (Badrie 2016). There is much debate about the exact geographical origin of *Abelmoschus esculentus*, also known as Okra, lady-finger or bhindi. It is thought to have originated from Africa, specifically in the Egyptian region, where it has been cultivated for many years (Dhaliwal and Sharma 2016). Okra is now a widely spread vegetative crop that can be found in most regions, including India, the Philippines, Thailand and Brazil, since it can be grown in most tropical and subtropical regions (Iwu 2016). However, India is thought to be the world's largest producer of okra (Kumar, Kumar and Nadendla 2013). Okra has been reported to be a staple crop in countries such as India and various African regions. Okra production worldwide is estimated to be around 9.96 million tons per year, indicating that it is a valuable crop (Mohammed, Bekele and Kumar 2017).

Despite its diverse economic values, okra is underutilized in SSA. The average pod yield of okra in SSA is 2.5 t/ha, with the crop's potential yield reaching up to 8.8 t/ha (Mohammed, Bekele and Kumar 2017). Okra can be grown as a garden crop or on commercial agricultural farms. In terms of agriculture, okra grows relatively easily and is less susceptible to pests, rodents and diseases than other staple crops (Leach *et al*. 2017). It can withstand unfavorable climate conditions and hence proves to be a sustainable food source. In comparison to other staple crops, okra produces significantly higher yields in addition to having a good nutrient profile (Dhankhar, Deswal and Singh 2012), making it a low cost potential functional food.

Figure 2. 9 Okra plant and pods (Kumar, Kumar and Nadendla 2013).

2.16.1 Nutritional composition of okra

Okra is a nutrient-dense crop that can provide numerous benefits when consumed. The most abundant macronutrients observed are dietary fiber followed by carbohydrates and some levels of proteins as illustrated in Table 2.3 (Romdhane *et al*. 2020). Despite the energy and fat content, okra seeds are suggested to contain unsaturated fatty acids, like linoleic acid, that are essentially considered beneficial in human nutrition (Singh *et al*. 2014). Carbohydrates are present mainly in the form of viscous and slimy mucilage (Dantas, Alonso Buriti and Florentino 2021). Various studies and current bibliographic surveys have been conducted on the bioactive potential of okra mucilage and its rheological properties (Mohan *et al*. 2018; Al-Shawi *et al*. 2021).

Studies have observed and suggested various applications for mucilage derived from okra pods. For instance, mucilage derived from okra head waste was found to have relatively low thermal degradation abilities (Mohan *et al*. 2018). Nie *et al*. (2019) proposed that okra mucilage has vast industrial applications in addition to being a functional ingredient in the food industry.

Table 2. 3 Chemical composition of okra plant and pods (Kumar, Kumar and Nadendla 2013).

Composition	(g/ 100 g)
Carbohydrates	4.86
Dietary fibers	8.16
Protein	3.55
Fat	0.19

2.16.2 Utilization of Okra

Okra is identified as a crop with nutritional and medicinal characteristics. Okra fruit is commonly utilized in culinary dishes such as soups and stews due to its ability to thicken (Nguekouo *et al*. 2018). Okra is also processed into extruded snacks and flours. Seeds of the okra pods are consumed as a non-caffeinated substitute for coffee proceeding roasting and grinding (Gemede *et al*. 2015). Consumption of okra powder was found to control increased blood glucose concentrations (Dubey and Mishra 2017). Some studies have used okra to enrich foods such as cakes and cookies, resulting in innovative and functional products (Brito *et al*. 2017; de Oliveira *et al*. 2020). The partial substitution of wheat flour for okra flour was found to nutritionally enhanced baked products. Brito *et al*. (2017) also produced an okra flour cake and observed good sensory acceptance of the product. Due to the high protein content the majority of okra seeds are processed into high yielding oils (Zhang *et al*. 2019).

2.17 Mucilage as potential prebiotics

Mucilage is a hydrocolloid that is translucent, viscous and sticky in nature. It is commonly found in most plants and produced in varying amounts, however, can also be found in microorganisms and marine organisms (Kassem *et al*. 2021). Mucilage (a polymeric polysaccharide complex) is primarily composed of carbohydrates with highly branched structures composed of sugar units of D-xylose, D-galactose, L-rhamnose and galacturonic acid. They also have glycoproteins and various bioactive components like alkaloids, tannins, and steroids (Fernandes and de las Mercedes Salas-Mellado 2017; Beikzadeh *et al*. 2020). Upon hydrolysis, mucilage generates an indefinite number of sugar units such as xylose, arabinose, galactose, glucose and rhamnose, this however, depending on the type of hydrolysis (Petera *et*

al. 2015). Mucilage has been suggested to have excellent functional properties. Due to hydrogen bonding which occurs between different functional and other polar groups, they also play a significant role in the establishment of emulsions, films, gels and coated metal nanoparticles (Alpizar-Reyes *et al*. 2017).

2.17.1 The influence of extraction on mucilage structure, composition and functionality

Mucilage, can be extracted from any part of the plant, including its roots, leaves or fruit and is considered a valuable source with potential food and pharmaceutical applications (Dybka-Stępień *et al*. 2021). The yield, structural, functional and rheological capacities of mucilage are strongly reliant on the extraction technique and conditions (Andrade *et al*. 2020; Souza *et al*. 2020). In general, all mucilage extraction techniques consist of two sequential procedures which include maceration and precipitation (Andrade *et al*. 2020). The maceration procedure includes soaking the raw material in the preselected solvent at room temperature and agitating it on regular basis. Maceration for mucilage extraction is generally performed with a low solid-liquid ratio and hot water treatment (Rashid *et al*. 2019). To further improve the extraction of mucilage, ammonium oxalate, acid solutions and EDTA are also utilized. The disadvantage of the maceration technique is its lengthy extraction periods, low efficiency (low mucilage yields) and subsequent denaturation (Souza *et al*. 2020). Thus far, there have been several studies reporting on various extraction techniques which include microwave or ultrasound energy, solvent extraction, enzymatic and acid extraction (Adetunji *et al*. 2017; Chemat *et al*. 2017). Extraction procedures are critical as lack of standardized extraction techniques result in variability in chemical structure and composition and affects the purity and performance of the mucilage (Andrade *et al*. 2020).

2.17.2 Structure and composition of mucilage

Mucilage and gum are hydrocolloid subgroups that include simple sugars bond with organic acids and are related due to the hydrophilic and hydrocolloid parts that make a sticky solution or gel in the presence of water (Singh and Barreca 2020). Mucilage from plants are made up of two major polysaccharides, pectin and hemicellulose, both of which contain rhamnogalacturonan and arabinoxylans. Mucilage arabinoxylans are generally comprised of β-1,4-linked xylose backbones that are mostly replaced by 1-3 sugar residues at O-2 or/and O-3 positions. Mucilage rhamnogalacturonan I (RG-I) is a repetitive disaccharide composed of α-

(1,2)-rhamnose and α-d-(1,4)-galacturonic acid (Hesarinejad *et al*., 2018).

Furthermore, there is an indication that rhamnogalacturonan I side chains can be covalently bound to hemicelluloses, resulting in the formation of a super-macromolecular polymeric network (Tosif *et al*. 2021). It has been reported that the neutral sugars present in mucilage are primarily L-arabinose, D-xylose and D-galactose with the ratios and types varying depending on the origin of the mucilage. Mucilage contains both hydroxyl and carboxyl groups as functional groups and as a result of the presence of these functional groups, it acts as a polyelectrolyte (Hesarinejad *et al*. 2018; Tosif *et al*. 2021). Their chemical nature demonstrates that structures are of relatively high molecular weight, consisting of highly branched chains (Ma *et al*. 2020). The structure of mucilage is also dependent on factors such as environment, varieties and method of extraction (Kaewmanee *et al*. 2014; Messina *et al*. 2021).

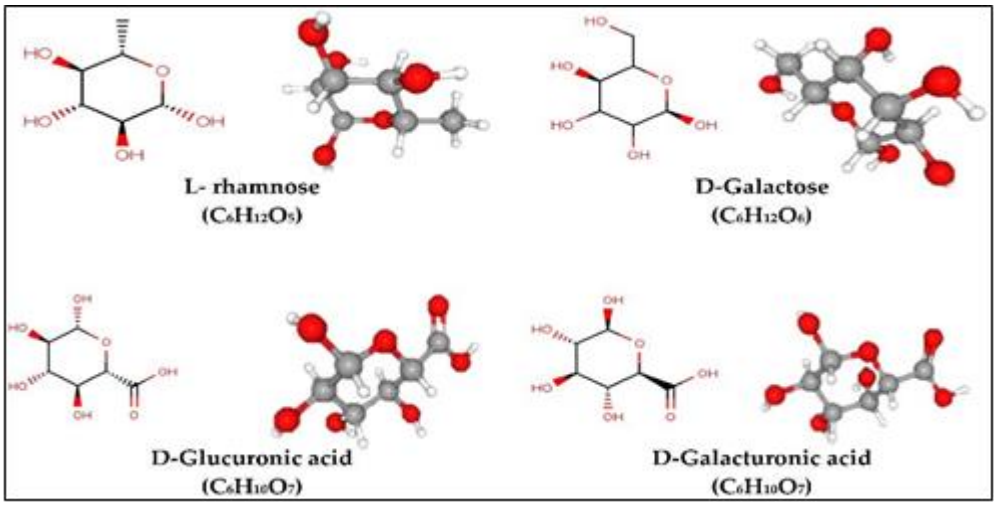

Figure 2. 10 Different mucilage chemical structures (L-rhamnose) ($C_6H_{12}O_5$) methyl-pentose or 6-deoxy-hexose, aldehyde group at C_1 and carboxylic acid group at C_6 make up D-glucuronic acid ($C_6H_{10}O_7$)., D-Galactose ($C_6H_{12}O_6$) Glucose epimer C-4 and D-Glucuronic acid ($C_6H_{10}O_7$) has had its sixth carbon atom oxidized to a carboxylic acid (Ma *et al*. 2020).

2.17.3 Functional properties of mucilage

Hydrocolloids within the food and pharmaceutical industries are typically composed of large molecular biopolymers. Mucilage contains a hydroxyl group, which increases water attraction and causes viscous dispersions (Alpizar-Reyes *et al*. 2017). Due to differences in their structure and composition, their functional properties include water and oil absorption properties, emulsifying, foaming, swelling, solubility and gelling capabilities (Albuquerque *et al*. 2016; Razavi 2019). Therefore, mucilage is commonly utilized as thickeners, stabilizers, as fat

substitutes, whipping agent or as gelling agents (Alpizar-Reyes *et al*. 2017). Furthermore, they have applications in crystallization inhibition, edible films, flavor encapsulation and edible coatings (Ma *et al*. 2020).

The occurrence of hydroxyl groups and protein substituents in mucilage structure is responsible for the high water holding capacity, according to various studies (Alpizar-Reyes *et al*. 2017). Water holding capacity is the ability of a moist polymer or sample to retain water when subjected to an external centrifugal gravity force or compression. It is made up of linked water, physically trapped water and hydrodynamic water, the latter of which correlates more to this capacity (Alpizar-Reyes *et al*. 2017). A low water holding capacity is suggested to be related to mucilage high solubility at high concentrations, which results in the inability of mucilage to form a gel. Various studies have reported on the water holding capabilities of mucilages derived from different plant sources. For instance, it was reported that the water holding capacity of sweet basil seed and *Cordia myxa* mucilage was 2.4 g /g and 2.44 g/g respectively (Keshani-Dokht *et al*. 2018; Avlani *et al*. 2019). Another study reported on the water holding capacity (WHC) of tamarind seed mucilage at varying temperatures. It was observed at 25 °C the WHC was 0.014 g/g, at 45 °C the WHC was 0.167 g/g and at 65 °C 0.025 g/g. This study demonstrated that the water holding capacity of tamarind seed mucilage increases with increasing temperature (Alpizar-Reyes *et al*. 2017).

A significant functional property of hydrocolloids is its oil holding capacity, which also indicates oil absorption capacity (Ma *et al*. 2020). Mucilage may improve the texture of food products due to its high oil holding capacity. Because of the presence of mono-polar molecules, it has been suggested that fruit or seed extracted mucilage has a high oil binding capacity, allowing it to trap larger amounts of oil particles while also preventing oil and flavor loss in food systems. As a result, mucilage derived from fruits or seeds can be used as a functional ingredient in formulated foods (Kalegowda, Chauhan and Urs 2017).

Good foaming capacity of mucilage is closely related to its flexible structure, which can reduce surface tension. The foaming property is reliant on many factors, including its molecular weight, the presence of additional compounds in the hydrocolloid, protein structure and carbohydrates (Mahfoudhi *et al*. 2014). Various studies have investigated the foaming capacities of various plant derived mucilage, with okra (*Abelmoschus esculentus*) mucilage reported to have foaming capacity ranging between 50 and 62% and the foaming stability of *Plantago* mucilage was reported as 88.4% (Behbahani *et al*. 2017; Gemede *et al*. 2018). Plant mucilages are gaining

popularity in food and pharmaceutical industries due to their good emulsifying capabilities (Quinzio *et al*. 2018). Mucilage is utilized for emulsion stabilization, crystallization control, thickening, particulate suspension, film formation and encapsulation (Hayati, Ching and Rozaini 2016; Tavares *et al*. 2018). Mucilage has also been reported as having antibacterial and antifungal properties depending on the source it is derived from, that can assist in preventing or lowering the risk of food-borne disease, illness and food spoilage (Tantiwatcharothai and Prachayawarakorn 2020). It has also been noted to have antioxidant properties due to the presence of a variety of phenolic compounds, including polyphenols and flavonoids (Liguori *et al*. 2020).

Functional properties of mucilage also determine the extent to which it is broken down and used by gut microbiota (Cui *et al*. 2019). Solubility, viscosity, water and oil binding abilities are important determinants for fermentation of mucilage and other polysaccharides (Cui *et al*. 2019). For instance, solubility is described as how much a compound is able to go into solution (dissolve) (Lovegrove *et al*. 2017). Gibb *et al*. (2015) showed how psyllium fiber was able to increase glycaemic control in human subjects due to its relatively good solubility. According to Dikeman and Fahey Jr (2006), viscosity is the level of flow resistance and pertains to a fiber's tendency to thicken when hydrated. Viscous dietary fiber intake has been shown to influence transit time in the upper gut, in regulating small intestine transit and in lowering rate of gastric emptying (Müller, Canfora and Blaak 2018). The ability of a substance to interact with water under limited conditions can be defined as the water holding capacity (WHC) (Zhuang *et al*. 2020).

Water holding capacity is made up of associated water, water that is physically trapped and hydrodynamic water (Zhuang *et al*. 2020). Strong water hold capacity is one of the primary factors contributing to DF's effectiveness in enhancing human health. Dietary fibers are shown to have a lubricating effect after absorbing water, that can encourage intestinal motility and intestinal peristalsis in addition to expanding in size and volume creating a fuller feeling which can result in less food intake (He *et al*. 2022). The amount of oil that a sample can absorb per unit of weight is referred to as its oil holding capacity (Lam *et al*. 2018). According to Matsuhiro *et al*. (2006), dietary fiber can lower the body's calorie intake by its ability to absorb fat (oil) which can assist in the prevent obesity.

2.17.4 Uses of mucilage

Previously, plant mucilage was limited to just traditional uses, however recently, there has been much interest in modern applications of mucilage as pharmaceuticals, functional foods and in cosmetics (Dybka-Stępień *et al*. 2021). Mucilage is a biologically active compound that is sustainable and cost-effective. It is also non-toxic, biocompatible, biodegradable and environmental friendly (Iravani 2020). Mucilage have a variety of applications, such as gelling, structuring, texturing and film-forming agents in food and nutraceuticals, stabilizers in cosmetics and disintegrants and binders in drug delivery systems in pharmaceuticals as can be seen in Figure 2.11 (Liu *et al*. 2021b). Mucilages also have potential application in the paper and textile industry and can be utilized in paint production (Soukoulis, Gaiani and Hoffmann 2018). Knowledge on the chemical structure and physical properties of mucilage is critical for determining its health-promoting properties and potential function in specific applications (Soukoulis, Gaiani and Hoffmann 2018; Liu *et al.* 2021b). The molecular weight and distribution of polysaccharides present in mucilage determines the technological (emulsification and rheological) properties. High weight mucilages are utilized as structuring (gel-forming) and thickening agents in foods such as bread and pastas (gluten-free products) because they improve the flexibility and viscosity of continuous phase (Korus *et al*. 2015; Knez Hrnčič *et al*. 2019). Furthermore, it has been reported that chai seed mucilage has been applied as a fat substitute in bakery products such as bread and cakes and further application in dairy products such as cheese, ice cream and yogurt (Fernandes and de las Mercedes Salas-Mellado 2017; Ribes *et al*. 2021).

Since mucilage are nontoxic and biocompatible, they have been effectively employed in drug-delivery systems in the pharmaceutical industry. Mucilage act as a dis-integrant or binder during tablet formulation, allowing for specifically aimed and sustained drug release. Mucilage derived from *Cassia tora*, *Moringa oleifera* and *Aloe vera* have been successfully utilized in drug delivery systems (Choudhary and Pawar 2014; Amiri *et al*. 2021). Mucilage thickening properties prove to be beneficial in the cosmetics industry. It has been reported that mucilage derived from *aloe vera* and *quince* are employed as moisturizers in soaps, creams and lotions. Mucilage ability to entrap water (water holding capacity), combined with antimicrobial and moisturizing properties, makes them suitable for the production of wound and skin burn dressings, as well as the treatment of inflammation (Kawahara *et al*. 2017).

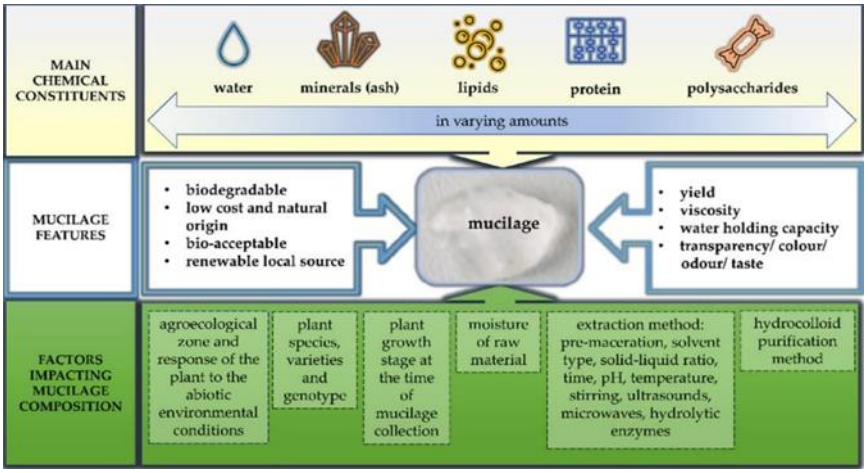

Figure 2. 11 Factors influencing the functional properties and nutritional value of mucilage derived from plants (Knez Hrnčič *et al*. 2019).

2.17.5 Amadumbe mucilage

Amadumbe is reported as being rich in mucilage (3-19%) (Njintang *et al*. 2014) and has been reported to be made up structurally of arabinose, galactose, xylose and mannose units in the presence of arabinogalactan protein, forming a highly branched structure (Andrade, Nunes and Pereira 2015). Previous studies have been conducted on the structure and composition of other varieties of taro mucilage for application as a functional ingredient in both the food and pharmaceutical industries (Tavares *et al*. 2011a; Nguimbou *et al*. 2014), however limited data has been generated on the structure-function relationship of Southern African varieties, thus, limiting its utilization for industrial application in food and non-food products.

Taro mucilage is notable for its use in the food, pharmaceutical and cosmetic industries as a thickener, binder, emulsifier and stabilizer (Singh and Kumar 2016; Mijinyawa, Durga and Mishra 2018). It can be utilized as a natural emulsifier in baking to provide sensory properties comparable to breads containing commercial synthetic emulsifiers. Contado *et al*. (2009) found in sensorial analysis that the addition of taro mucilage to loaves of bread resulted in a higher percentage of acceptability than breads made with a 1.0 percent addition of synthetic emulsifier. Bicalho *et al*. (2019) reported that the utilization of taro mucilage provided good palatal distribution in French-type bread, a preferable characteristic for the product. It was also observed that the emulsifying impact of taro mucilage added to the texture and as a result, improved the quality (increased volume and finer crumb texture) of the bread. Saxby (2020) demonstrated the capacity of taro mucilage in preventing colorectal cancer. Brown *et al*. (2005)

demonstrated the ability of Poi made from the ground taro corm, to activate lymphocytes that can lyse malignant cells and cause colonic adenocarcinoma cells to undergo apoptosis. The potential of taro mucilage as a prebiotic was previously hypothesized by Anwar *et al*. (2021).

2.17.6 Okra mucilage

According to various studies, okra mucilage is composed of D-galactose, L-rhamnose and L-galacturonic acid, with trace amounts of glucose, mannose, arabinose and xylose (Gbenga and Zulikha 2013). Okra is an anionic polysaccharide with the ability to form more stable gels due to the presence of the galacturonic acid residue (Xiong *et al*. 2021). In comparison to chai seed mucilage which is more popular due to its profound nutritional profile and functionality as a thickener (Mohammed *et al*. 2019). Okra mucilage has been reported to have good functional properties such as water holding capacity, emulsification and gelling abilities and has been applied in dough formulations, cookies, as fat replacers in chocolates and in drug formulations (Datsomor *et al*. 2019; Tufaro, Bassoli and Cappa 2022).

Noorlaila *et al*. (2015) found that okra mucilage incorporated into a coconut milk oil-water emulsion system improved the stability, texture and appearance of the overall product. Rindiani and Kumalasari (2021) on partial substitution of wheat flour with okra flour nutritionally enriched the product, found it to increase the amount of fiber. This is due to okra having a higher fiber content than wheat flour. Addition of okra mucilage in yogurt formulation was observed to increase its water holding capacity contributing to the elasticity and firmness of the overall product (Xu *et al*. 2019). Yuennan, Sajjaanantakul and Goff (2014) observed that inclusion of okra mucilage in ice cream formulation, increased the viscosity significantly, in addition to decreasing the growth of ice crystals in the mixture, both of which are important factors for a satisfactory sensory perception (Yuennan, Sajjaanantakul and Goff 2014). In most studies, okra mucilage has been applied in the microencapsulation of probiotic strains (Dantas, Alonso Buriti and Florentino 2021). It has also been demonstrated by Yuan *et al*. (2020) the extent to which okra mucilage may be digested.

The functional food and nutraceutical market is one of the fastest-growing food segments globally. This has created renewed interest in plant-based compounds with pro-health benefits, and functional properties (Szutowska 2020). Mucilage derived from plants has gained vast research interest due to its desirable properties (Haruna, Aliyu and Bala 2016). However, the incorporation of mucilage as a functional ingredient in various food and beverage products is

highly dependent on its source, extraction technique and structural composition which entail continuous scientific research (Tosif *et al*. 2021). Aside from its application as a functional ingredient, plant mucilage has great potential in the treatment of various metabolic diseases such as obesity, diabetes, polycystic ovarian syndrome and gut- related diseases (AIN*et al*. 2019; Elkhalifa *et al*. 2021). This leaves space for further pre-clinical and clinical trials to be carried out.

2.18 Conclusion

In conclusion, proposed modulation and sustainability of gut microbiota by prebiotics has been a stimulated approach in the prevention of various chronic illnesses such as obesity and gastrointestinal disorders. The inclusion of prebiotics in diets, can significantly contribute to the overall health in humans by inducing positive modulation of beneficial gut microbiota. In addition to their health benefits, the functional properties of prebiotics enable them to improve on sensory characteristics as well as textural properties in new food product applications or in the improvement of exiting products. Amadumbe and okra are good sources of mucilage and are presumed prebiotic, however due to limited scientific data, this prevents their utilization as prebiotic food ingredients or as targeted supplements. By understanding the structure-function relationship and assessing the prebiotic potential of amadumbe and okra mucilage, this can enable the design of targeted dietary supplements aimed at specific microbiota profiles for better health or the prevention of specific illnesses.

2.19 Hypothesis, aim and objectives

2.19.1 Hypothesis

It is expected that both amadumbe and okra mucilages can stimulate gut microbial profiles differently due to their differences in structure, composition and functional properties. Metzler-Zebeli *et al*. (2010) observed that the composition and diversity of gut microbiota are influenced by carbohydrate composition and that the addition of particularly non-starch polysaccharides to diet can alter the composition and diversity of gut microbiota based on gut microbiota's preferences for particular substrates.

2.19.2 Aim

To investigate the prebiotic effects of amadumbe and okra mucilage for potential application as functional foods supplements.

2.19.3 Objectives

- To determine composition and functional properties of extracted amadumbe and okra mucilage using various spectroscopy and chemical techniques.
- To investigate the prebiotic effect of amadumbe and okra mucilages through *in-vitro* fermentation using faecal samples.

CHAPTER THREE

Composition and functional properties of amadumbe and okra mucilages

Abstract

Amadumbe and okra are traditional mucilage rich crops. Mucilages are high molecular weight hydrocolloids that contribute to structure and texture of food and non-food systems due to their functional properties. Previous studies have probed the structure and composition of mucilage from amadumbe (*Colocasia esculenta*) and okra (*Abelmoschus esculentus* (L.) Moench) however, limited data has been generated on the structure and functional relationship of Southern African varieties. The objective of this study was to determine the chemical composition and functional properties of amadumbe and okra mucilages.

Mucilage was extracted from amadumbe and okra by cold water extraction. Purified mucilage was obtained by removal of lipids, protein and low molecular weight compounds. The chemical composition of mucilage samples were determined by spectroscopy and chemical analysis. Functional properties including water and oil holding capacity, swelling and solubility were investigated. Results revealed that glucose, mannose, arabinose and xylose were the predominant monosaccharides present in amadumbe mucilage. Galactose, rhamnose, arabinose and ribose were present in okra mucilage. Carboxylic and hydroxyl groups were the main functional groups identified in both mucilage samples. In comparison to okra mucilage, amadumbe mucilage showed low water holding capacity 5 g/ 100 g. The low water holding capcity of amadumbe mucilage can be attributed to its high solubility 61% and relatively low swelling power 1.26 g/ 100 g. These results highlight potential of Southern African varieties amadumbe and okra for application as functional food ingredients capable of competing with other commercial hydrocolloids.

Keywords: Polysaccharide, amadumbe, okra, functional foods

3.1 Introduction

Polysaccharides are among the most prevalent biomolecules naturally found in plants, algae, animals and microorganisms (Wijesinghe and Jeon 2012). Due to their biological and functional properties, there has been increased interest in obtaining polysaccharides from novel sources (Gawai, Mudgal and Prajapati 2017). It was anticipated, during the projection period (2020-2023) that the global market for polysaccharides grows just over 5% and reaches an estimate of US$ 22 billion (Ruales-Salcedo *et al*. 2022). Driving this growth is the demand for polysaccharides that are both beneficial to human health and can also improve on structure and functionality such as texture given their application (Bisulca, Odegaard and Zimmt 2016; Du Toit *et al*. 2019). A group of polysaccharides suggested to have the most promising potential is mucilage. This is due to differences in their structure, composition and functional properties in applications such as food, cosmetic and biomedical products (Albuquerque *et al*. 2016; Razavi 2019).

Mucilages are described as heterogeneous, complex and highly branched polysaccharides (Monrroy *et al*. 2017; Razavi 2019). They are of relatively high molecular weight, composed of many sugar units that are either acidic or neutral in nature, joined together by glyosidic linkages (Cui 2005; Naveed *et al*. 2017). Due to their heterogeneous nature, mucilages have been found to be associated with other compounds such as tannins, lipids and glycoproteins (Gebre-Mariam 2012; Naveed *et al*. 2017). In addition to their technological functions in foods, mucilage is regarded as soluble fiber with significant health benefits. In recent years, there has been increased research interest in mucilage from novel plant sources to cater for the growing demand for functional ingredients (Carnachan *et al*. 2019).

Amadumbe (*Colocasia esculenta*), is a species of the *Colocasia* genera belonging to the Aracaea family of plants (Lim 2015). It is a rooted vegetable crop native to South-East Asia, however, its cultivation has spread across most tropical and sub-tropical regions (Rashmi *et al*. 2018). In Africa, the crop is cultivated on a large scale in countries such as Cameroon and Ghana (Fonjong and Gyapong 2021) and is gaining increased importance in parts of the east, north and south of Africa (Grimaldi 2016). In South Africa, the crop is commonly known as amadumbe, and Taro in West Africa. Amadumbe is considered an underutilized traditional crop, grown in the province of Kwa-Zulu Natal (Shange 2004). Traditionally, amadumbe corms are consumed boiled, as porridge, in stews, fried as chips or processed into flour

(Plucknett 2019) and offer nutritional security to poorer communities (Mabhaudhi, Modi and Beletse 2014). Amadumbe is rich in mucilage (3–19%) (Njintang *et al*. 2014) and has been reported to be made up of arabinose, galactose, xylose and mannose units in the presence of arabinogalactan protein, forming a highly branched structure (Andrade, Nunes and Pereira 2015).

Previous studies have probed the structure and composition of mucilage from other varieties of taro i.e. *Cyrtosperma merkusi*, *Xanthosoma sagittifolium* and *Alocasia macrorriza* for application as a functional ingredient in both food and pharmaceutical industries (Lewis 2000; Englberger *et al*. 2008), however, limited data has been generated on the structural and functional relationship of Southern African varieties. This has limited the utilization of amadumbe of Southern African variety for industrial application in food and non-food products. Amadumbe mucilage can potentially be applied as an emulsifier in dough formulation and bread making (Bicalho *et al*. 2019), as a fortifying agent and hydrocolloid in pudding-like products (Hendek Ertop, Atasoy and Akın 2019) and as biopolymer for the encapsulation of pharmaceutical ingredients (Singh and Kumar 2016).

Okra (*Abelmoschus esculentus* (L.) Moench), is a flowering vegetable crop belonging to the Malvaceae family cultivated primarily for its edible pods (Badrie 2016). The okra crop is well established due to it high nutritional profile and is cultivated in most tropical and sub-tropical regions (Petropoulos *et al*. 2018; Agbenorhevi *et al*. 2020). Okra has been reported to be a multifunctional crop providing nutrition and serving as a functional ingredient in food, non-food and pharmaceutical products (Ghori *et al*. 2014; Kpodo *et al*. 2018). It is known to be rich in mucilage similar to amadumbe and is high in carbohydrates, neutral sugars, minerals and protein (Gemede *et al*. 2018). There have been many studies reporting on the functional properties of okra mucilage as well as its application (Etaware and Etaware 2019; Nampuak and Tongkhao 2020; Olawuyi *et al*. 2020).

Okra mucilage has been reported to be made up of D-galactose, L-rhamnose and L-galacturonic acid with some proportions of glucose, mannose, arabinose and xylose (Gbenga and Zulikha 2013). Okra is an anionic polysaccharide and the presence of the galacturonic acid residue increases its capability of forming more stable gels (Tai *et al*. 2019). Its mucilage has been reported to have good functional properties such as water holding capacity, emulsification and gelling abilities and has been applied in dough formulations, in cookies, as fat replacers in chocolates and in drug formulations (Alamri, Mohamed and Hussain 2012; Ghori *et al*. 2017),

in comparison to chai seed mucilage which is increasing in popularity due to its profound nutritional profile and its functionality as a thickener (Brütsch *et al*. 2019). As per literature, the structure, composition and functional properties of mucilage can be highly dependent on type of cultivar and species (Du Toit *et al*. 2019). Limited data has been generated on the molecular structure of Southern African amadumbe and okra mucilage which is important to understand its functionality for potential application in food and non-food products. Therefore, the aim of this study was to determine the chemical composition and functional properties of amadumbe and okra mucilage.

3.2 Material and methodology

3.2.1 Plant material

Okra pods and amadumbe corns were purchased from a local market in Durban, Kwa-Zulu Natal, South Africa. Both crops are of landrace variety and were cultivated and harvested in the Kwa-Zulu Natal province by local small scale farmers. Amadumbe corns and okra pods, were washed to remove dirts and debris and placed in a cool place to dry.

3.2.2 Sample preparation and mucilage extraction

3.2.2.1 Okra mucilage extraction

Okra pods were sliced and seeds removed. Thereafter, 1 kg of sliced okra was weighed out and placed in de-ionized water at 4 °C for a duration of 24 h (Ameena *et al*. 2010). Okra mucilage was extracted by homogenizing the soaked sliced pods using a commercial blender. The okra mixture was centrifuged at 5000 × g for 20 min at 4 °C using Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzip, Germany) and supernatant was collected. The obtained crude okra mucilage was lyophilized for 60 h in a freeze drier at -60 °C (Christ freeze- drier, Niedersachsen, Germany) (Du Toit, De Wit and Hugo 2018). The sample was thereafter milled into powder. Crude okra mucilage powder was then packed in an airtight container and stored.

3.2.2.2 Amadumbe mucilage extraction

Amadumbe mucilage extraction was conducted according to the method of Manhivi *et al*. (2018) with modification. Amadumbe corns were peeled and sliced into cubes. Thereafter, 150 g of the sliced corns were suspended in 300 ml of de-ionized water at 4 °C and left to soak over a duration of 24 h. Crude amadumbe mucilage was obtained by centrifugation at 5000 ×

g for 20 min at 4 °C using Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzip, Germany) and supernatant collected. Crude amadumbe mucilage was lyophilized for 60 h in a freeze drier at -60 °C (Christ freeze- drier, Niedersachsen, Germany) (Du Toit, De Wit and Hugo 2018) and was thereafter milled into powder. Crude amadumbe mucilage powder was then packed in an airtight container and stored.

3.2.3 Purification of mucilage

The purification of okra and amadumbe was conducted according to the method reported by Amid and Mirhosseini (2012b).

3.2.3.1 Removal of lipids

Lipids were removed from crude amadumbe and okra mucilage samples utilizing defatting process, using extraction solvents hexane and isopropanol (60:40, v/v). Precisely, 10 g of crude okra and amadumbe mucilage were weighed and 100 ml of solvent was added. The respective mucilage samples were then placed on a magnetic stirrer at room temperature for a duration of 4 h. The residue was removed by centrifugation at 1400 × g for 15 min using Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzip, Germany), with the supernatant discarded, and the pellet vacuum-dried.

3.2.3.2 Removal of proteins

Sevag solution was prepared utilizing butanol and chloroform (1:5, v/v). This solution (100 ml) was then added to the defatted okra and amadumbe mucilage. The respective mucilage mixtures were then centrifuged at 2500 × g for 10 min utilizing Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzip, Germany). The interphase layer was discarded as it contained denatured proteins (Wang *et al*. 2015). The defatted and de-protonated okra and amadumbe powder was re-dissolved in de-ionized water (1000 ml) and put into 10 kDa dialysis tubes. The buffer utilized was de-ionized water. Buffer was changed every 6 h for a duration of 36 h. Defatted, de-protonated and dialyzed okra and amadumbe mucilage was then further purified with 95% ethanol (1:2, v/v) for 30 min. The precipitate was then collected and washed twice with acetone at 4 °C. Okra and amadumbe mucilage was then oven dried at 40 °C overnight and milled into a powder.

3.2.4 Chemical characterization of mucilage

Determining the chemical composition of amadumbe and okra mucilage generates preliminary understanding of their structural and functional relationship. In order to determine the composition and nutritional profile of extracted amadumbe and okra mucilages, the following analysis were performed.

3.2.4.1 Proximate composition of mucilage

Crude and purified okra and amadumbe mucilage samples were analyzed for moisture, ash, fat and total nitrogen utilizing official method according to the American Association of Cereal Chemists (2000) standard methods 44 – 19, 08 – 01, 30 – 10 and 46- 10 respectively (Maktouf *et al*. 2016). The phenol sulfuric method was utilized to determine total sugar in crude and purified okra and amadumbe mucilage samples (Koh, Chou and Liu 2009).

In brief, the ash content was carried out by weighing 5 g of respective mucilage sample in a crucible. Samples were then place in a furnance and heated at 585 °C till constant weight. The samples were removed, cooled in a dessicator to room temperature and the remaining reside weighed. Moisture was carried out by weighing out 5 g of amadumbe and okra mucilage in crucibles, samples were then heated at 130 °C in an air oven for 6 min. Thereafter, samples were removed, cooled to room temperature and the remaining residue was recored. Fat content was carried out by weighing 5 g of amadumbe and okra mucilage samples, this was then extracted by Soxhlet utilizing petroleum ether. The solvent was evapourated and the residue was dried to constant weight. The remaining residue was weighed and recorded.

3.2.4.2 Determination of total starch content

The total starch content of crude and purified amadumbe and okra mucilage was conducted in accordance to method by Mccleary, Gibson and Mugford (1997), utilizing the Megazyme analysis kit. Precisely, 100 mg of amadumbe and okra mucilage samples were weighed out into centrifuge tubes. Added to the tubes was 0.2 ml of 80% of aqueous ethanol and the tubes were vortexed to aid in dispersion and dissolution of starch. Thereafter, 2 ml of cold 1.7 M sodium hydroxide solution was added to the samples and mixed for 15 s. Tubes were then further mixed for 15 min. Following mixing of the slurry, 8 ml of 600 mM sodium acetate buffer, pH 3.8 containing 5 mM calcium chloride was added to the tubes and vortexed, proceeding with the addition of 0.1 ml of undiluted thermostable α- amylase and 3.300 U/ ml of AMG. The blank sample consisted of 0.2 ml of sodium acetate buffer. The samples were vortex for 3 s and thereafter, tubes were incubated at 50 °C in water bath for 30 min. Samples were removed and

allowed to cool to room temperature. Once cooled, 2.0 ml of each sample solution were transferred into microfuge tubes and centrifuged at 13 000 rmp for 5 min using Biofuge pico Heraeus (Analytical Instrument Brokers, LLC). In separate tubes, 1.0 ml of the supernatant containing 4.0 ml of 100 mM sodium acetate buffer at pH 5 and were vortexed. GOPOD reagent was added to the samples and incubated at 50 °C for 20 min. The absorbance was read at 510 nm using UV Mini 1240 spectrophotometer (Shimadzu, USA) and percentage total starch calculated using the Megazyme program (Mega-Calc ™).

3.2.4.3 Determination of resistant starch content

The determination of resistant starch content in crude and purified amadumbe and okra mucilage was conducted in accordance with the procedure by McCleary and Monaghan (2002) which consisted of hydrolysis and solubilization of non-resistant starch followed by the measurement of resistant starch content in the respective samples. For the hydrolysis, 100 mg of respective mucilage sample was weighed out into centrifuge tubes. Approximately 4.0 ml of pancreatic α-amylose containing AMG (3 U/ml) was added to each sealed centrifuge tube and vortexed.

The centrifuge tubes were then incubated at 37 °C in a water bath with continuous shaking (200 strokes/min) for 16 h. The tubes were removed, and excess water dried off with a paper towel. To the tubes, 4.0 ml of 99% (v/v) of ethanol was added and the test tubes were vigorously stirred, thereafter centrifuged at 1500 x g for 10 min using accuSpin 1R centrifuge (Fisher Scientific, Germany). The supernatant was discarded, and the pellet re-suspended in 2 ml of 50% ethanol and vortexed. A further 6 ml of 50% ethanol was added, and the contents again centrifuged. The supernatant was then discarded, and the centrifuge tubes were inverted and placed on an absorbent paper towel to drain excess liquid. A magnetic stirrer bar was placed into each centrifuge tube and 2 ml of 2 M KOH was then added to each tube and the pellets were re-suspended for 20 min in a cold-water bath. Thereafter, 8 ml of 1.2 M sodium acetate buffer at pH 3.8 was added to each tube on a magnetic stirrer plate. Immediately after, 0.1 ml of AMG solution was added and mixed. This was incubated at 50 °C for 30 min. Proceeding the treatment, 0.1 ml aliquots supernatant was transferred into clean test tubes to which 3.0 ml of GOPOD reagent was added and incubated at 50 °C for 20 min. The absorbance was measured at 510 nm using a spectrophotometer. Percentage resistant starch was calculated using the Megazyme program (Mega-Calc ™).

3.2.4.4 Determination of β-glucan content in mucilage

The β-glucan content in respective mucilage samples was conducted in accordance with the procedure described by McCleary and Codd (1991), utilizing the analysis by Megazyme (2017). Approximately, 80 mg of the respective mucilage samples were weighed into centrifuge tubes. Samples were wet with 0.2 ml of 50% (v/v) aqueous ethanol to aid dispersion. Thereafter, 4.0 ml of 20 mM sodium phosphate buffer (pH 6.5) was added and tubes vortexed. The tubes were transferred to a water bath set at 100 °C and heated for 60 s. Samples were then taken out, vortexed and placed back into water bath for a further 2 min. Tubes containing the respective mucilage samples were taken out and thereafter incubated in a 50 °C water bath and allowed to equilibrate for 5 min. Thereafter, 0.2 ml of 10 U Lichenase was added to the tubes. The tubes were sealed and incubated for 1 h at 50 °C with regular intervals of mixing.

Thereafter, 5.0 ml of 200 mM sodium acetate buffer at pH 4 was added to the tubes, vortexed and tubes were allowed to equilibrate at room temperature with further centrifugation at 1000 x g for 10 min using accuSpin 1R centrifuge (Fisher Scientific, Germany). Following the treatment of the samples, 0.1 ml of treated mucilage samples were dispensed in three tubes, 0.1 of 0.2 U β- glucosidase were added to two of the tubes and 0.1 ml of acetate buffer into the tube. All tubes were incubated at 50 °C for a duration of 20 min. Tubes were removed and absorbance was measured at 510 nm using a spectrophotometer against blank that consisted of 0.1 ml DH2O, 0.1 ml sodium buffer and 3.0 GOPOD reagent. Calculation of β-glucan was conducted utilizing the Megazyme program (Mega-Calc ^{TM}) (Megazyme 2017).

3.2.4.5 Analysis of amino acid content

In order to determine the amino acid profile of amadumbe and okra mucilage, samples were firstly hydrolyzed using 0.5 M H_2SO_4 for 1 h in a water bath at 100 °C, followed by the addition of 1 M H_2SO_4 for 4 h proceeding with the addition of 2 M H_2SO_4 for 4 h in a water bath at 100 °C (El-Mahdy and El-Sebaiy 1984). Then, 100 mg of respective treated amadumbe and okra mucilage samples were weighed into sealable test tubes, followed by the addition of 5 ml of each of the respective acids for the specific time interval as mentioned above. The tubes were sealed and put into a preset water bath (100 °C) for the specific hydrolysis time intervals as stated above. Following each treatment, nitrogen gas was applied to each sample to remove excess oxygen. Once the 3-part hydrolysis was complete, samples were left at room temperature to cool, and then neutralized with barium hydroxide. The neutralized samples were filtered

through Whatman No. 1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator and was thereafter lyophilized. Crude and purified amadumbe and okra mucilage hydrolysates were prepared as 2 mg/ml, thereafter vortexed and centrifuged at 1 300 x g for 5 min. The supernatant was retained and filtered through 0.22 µm Whatman micro-filters for amino acid analysis using high pressure liquid chromatography (HPLC). Samples were analyzed on Thermo Scienifc Dionex AAA- direct amino acid analysis system (ICS – 5000 +). The column used was Dionex AminoPac PAIO anion exchange column, the volume injected was 25 µl, eluents were deionized water (18.2 megaohm); 250 mM sodium hydroxide and 1 M sodium acetate. Quantification of amino acids were obtained by comparing the peak areas of the respective treated mucilage hydrolysates with standard calibration curves.

3.2.5 Molecular characterization

The molecular structure of polysaccharides includes monosaccharides present, molecular weight and the presences of functional groups (Nie and Xie 2011). Determining the molecular structure of amadumbe and okra mucilage enables better understanding of their functional properties. Functional properties have been suggested to be influenced by their molecular structure (Guo *et al*. 2017). Since mucilage samples can potentially serve as functional food ingredients in food and beverage applications, they also become potential substrates for gut microbial fermentation. As proposed by Klassen *et al*. (2021), molecular structure of polysaccharides influences its extent of fermentation, therefore some analysis were carried out in order to determine the molecular structure of amadumbe and okra mucilage samples as subsequently described below.

3.2.5.1 FT-IR spectroscopy

The FT-IR spectra for crude and purified amadumbe and okra samples were collected using a Perkin Elmer Spectrum 400 FTIR spectrometer with a diamond crystal ATR (Waltham, MA, USA). The crystal area was cleaned, and the mucilage powder was applied to the small crystal area before positioning the pressure arm over the crystal area. The spectrum was collected after applying force to the sample and pushing it onto the diamond surface. The spectra of the mucilage powders were obtained with a resolution of 1 cm^{-1} from the scanning range 400-4000 cm^{-1}.

3.2.5.2 Molecular weight determination

The molecular weight distribution profile of crude and purified amadumbe and okra mucilage was conducted according to the method described by Jeddou *et al*. (2016). The molecular weight distribution of samples was obtained using high performance size exclusion chromatography (HPSEC) and Refractive Index Detector Shimadzu (RD – 10 A). Samples were prepared by diluting in distilled water and filtered through 0.22 μm Whatman microfilters.

3.2.5.3 Monosaccharide analysis

The monosaccharide profile of crude and purified amadumbe and okra mucilage samples was conducted using a 3-part hydrolysis (El-Mahdy and El-Sebaiy 1984). Following hydrolysis, samples were prepared for high-performance liquid chromatography (HPLC) according to Manhivi *et al*. (2018) method. The mucilage samples were weighed out (1 mg) and diluted in 1 ml of de-ionized water. Monosaccharide analysis was performed using the Shimadzu UHPLC system (Shimadzu, Kyoto, Japan) equipped with Biorad Aminex HPX-87H column (300 × 7.8 mm). Separation was achieved under the following conditions: temperature of 50 °C, Milli- Q water was used as the mobile phase, with an injection volume of 10 μl, at a flow rate of 0.5 ml/min for a duration 15 min. Quantification of monosaccharides was obtained by comparing the peak areas of the respective treated mucilage hydrolysates with standard calibration curves.

3.2.6 Analysis of functional properties

Investigating the functional properties of amadumbe and okra mucilage, generates an understanding for their potential processing and application in food and non-food systems (Wang *et al*. 2021). It can also contribute to understanding its potential health benefits.

3.2.6.1 Water holding capacity (WHC)

The water holding capacity of crude and purified okra and amadumbe mucilage samples were determined using the method of Thanatcha and Pranee (2011). Precisely, 0.25 g of the mucilage samples were weighed out into 50 ml centrifuge tubes and to this 25 ml of de- ionized water was added. The mucilage samples were shaken for 15 min and thereafter centrifuged at 10 000 × g for 3 min using Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzip, Germany). The supernatant was discarded, and the wet weight of the respective mucilage samples were recorded. The WHC of crude and purified okra and amadumbe mucilage was calculated using the equation.

Equation 3. 1 $$Water\ holding\ capacity = \frac{wet\ sample - dry\ sample}{weight\ of\ dry\ sample}$$

3.2.6.2 Oil holding capacity

Using a method modified from Thanatcha and Pranee (2011), the oil holding capacity was determined for amadumbe and okra mucilage samples. Crude and purified amadumbe and okra mucilage were accurately weighed (0.5 g) into centrifuge tubes, and 10 ml vegetable oil was added. The sample were mixed for 1 min using a vortex stirrer, and then kept at room temperature for 30 min. The samples were then centrifuged at 10000 × g for 30 min. The supernatant was removed and the tube was turned upside down for 1 min. Finally, the oil absorbed sample weight was weighed and the oil absorption calculated.

Equation 3. 2 $$Oil\ holding\ capacity = \frac{oil\ absorbed\ sample\ weight - dry\ sample\ weight}{dry\ sample\ weight}$$

3.2.6.3 Swelling power and solubility index

Swelling power [SP_f] and water solubility index [WSI_f] were analyzed using the method of Li and Zhu (2017). Crude and purified okra and amadumbe mucilage were weighed (0.25 g) into 15 ml centrifuge tubes [W_o]. Respective samples were then suspended in 10 ml deionized water. The tubes were shaken for a duration of 30 min. The samples were thereafter centrifuged at 3000 × g for 30 min using the Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzip, Germany). The supernatant was poured into a weighed aluminum pan prior to drying to a constant weight [W_i] at 105 °C. The remaining sediment (pellet) in the centrifuge tube was weighted [Ws].

Equation 3. 3 $$Swelling\ power\ [SPf] = \frac{Ws}{Wo \times (1 - WSIf)}$$

Equation 3. 4 $$Water\ solubility\ index\ [WSIf] = \frac{Ws}{Wo \times (1 - SPf)}$$

3.2.7 Statistical analysis

The means of replicate data (n=3) were subjected to analysis of variance (ANOVA) for determination of significant differences among treatment groups ($p < 0.05$) using IBM SPSS

software (IBM Corporation, New York, USA).

3.3 Results and Discussion

3.3.1 Proximate composition

Summarised in Table 3.1 is the results of proximate composition of crude and purified amadumbe and okra mucilage. There was significant difference observed in the proximate composition between crude mucilage and purified mucilage samples ($p < 0.05$). Purified amadumbe and okra mucilage showed a reduction in protein, fat and ash content in comparison to crude mucilage samples. For amadumbe mucilage, values for protein content were significantly higher (10.08 and 7.54 g) than crude and purified okra mucilage (8.54 and 5.87 g) ($p< 0.0001$). These results were higher than those previously reported by Manhivi *et al*. (2018). In terms of fat and ash content, okra mucilage displayed higher values than amadumbe mucilage. Crude okra mucilage had high fat content of 3.97 g/g of sample in comparison to crude and purified amadumbe mucilage (2.86 and 1.36 g/g). The ash content of crude and purified okra mucilage were 9.34 g/g and 5.47 g/g. Previous studies have reported that the amount of ash could influence some functional properties of non-starch polysaccharides (Amid and Mirhosseini 2012a). There was no significant difference in ash content between purified amadumbe mucilage and purified okra mucilage (4.63-5.47 g), however a significance difference was noted between crude amadumbe mucilage and crude okra mucilage (7.15 - 9.34 g) ($p= 0.0006$). In the present study, purified amadumbe and okra mucilage had significantly higher carbohydrate content (86.88 – 72.20 g) than that of crude amadumbe and okra mucilage (72.18 – 60.09 g) ($p< 0.05$). Dietary carbohydrates are reported as being the main source from which gut microbiota derive their energy from (Flint *et al.* 2012). Both amadumbe and okra mucilage samples were found to be rich in dietary carbohydrates and can potentially be fermented by gut microbiota. The reduction seen in protein, fat and ash content of purified mucilage samples, suggests that the purification processes were successful in eliminating impurities that might have been present in the crude mucilage such as unconjugated proteins, free lipids and low molecular weight molecules (Ren *et al*. 2019). The high protein content found in amadumbe mucilage is possibly as a result of the suggested presence of arabinogalactan-protein as Andrade, Nunes and Pereira (2015) observed taro mucilage to be rich in protein. Dietary protein in relation to metabolism by gut microbiota has been extensively reported (Wu *et al.* 2022). Several studies have reported that plants rich in protein such as rice,

soy and wheat were capable of improving the composition and diversity of gut microbiota (Zhao *et al.* 2019). Amadumbe due to its high protein content can be potentially beneficial in stimulating gut microbial composition and diversity. The high fat content of okra mucilage can be attributed to the presence of hydrophobic constituents. Okra mucilage has been suggested as being highly amphipathic (having both hydrophilic and hydrophobic components) (Jideani and Bello 2009). The higher carbohydrate content seen in purified mucilage samples is possibly due to accumulation of relatively water-soluble monosaccharides such as glucose during the purification process and the separation of low molecular weight carbohydrates (Brummer, Cui and Wang 2003).

Table 3. 1 Proximate composition of crude and purified amadumbe an okra mucilage

Parameters (g /100 g)	**Amadumbe mucilage**		**Okra mucilage**	
	Crude	Pure	Crude	Pure
Moisture	13.08 ± 0.18^{c}	6.93 ± 0.45^{a}	14.74 ± 0.77^{c}	10.80 ± 0.12^{b}
Ash	7.15 ± 0.14^{b}	4.63 ± 0.77^{a}	9.34 ± 0.09^{c}	5.47 ± 0.05^{a}
Fat	2.86 ± 0.13^{ab}	1.36 ± 0.57^{a}	3.97 ± 0.62^{b}	1.25 ± 0.13^{a}
Protein	10.08 ± 0.06^{d}	8.54 ± 0.52^{c}	7.54 ± 0.59^{b}	5.87 ± 0.19^{a}
Carbohydrates	72.18 ± 4.68^{ab}	86.88 ± 4.48^{c}	60.09 ± 4.51^{a}	72.20 ± 4.48^{ab}

Means ± SD; n=3; values with different letters within the same row differ significantly ($p<0.05$).

3.2.2 Total starch and resistant starch content

Amadumbe mucilage showed significant levels of starch in comparison to okra mucilage ($p< 0.05$) as seen in Figure 3.1. Crude mucilage samples were observed to have double the content of starch present in comparison to purified mucilage samples. Amadumbe crude mucilage was observed to have the highest content of starch present 4.80 g/100 g in comparison to purified amadumbe 2.48 g/100 g, crude okra mucilage 0.12 g/100 g and purified okra mucilage 0.06 g/100 g. The high content of starch found in crude mucilage samples can possibly be attributed to starch being present as an impurity as suggested by Zhao, Qiao and Wu (2017). In purified mucilage samples, the purification process was observed to be effective in reducing the starch content. The high starch content found in amadumbe mucilage was comparable to those previously reported in other studies (Tavares *et al*. 2011b; Andrade, Nunes and Pereira 2015).

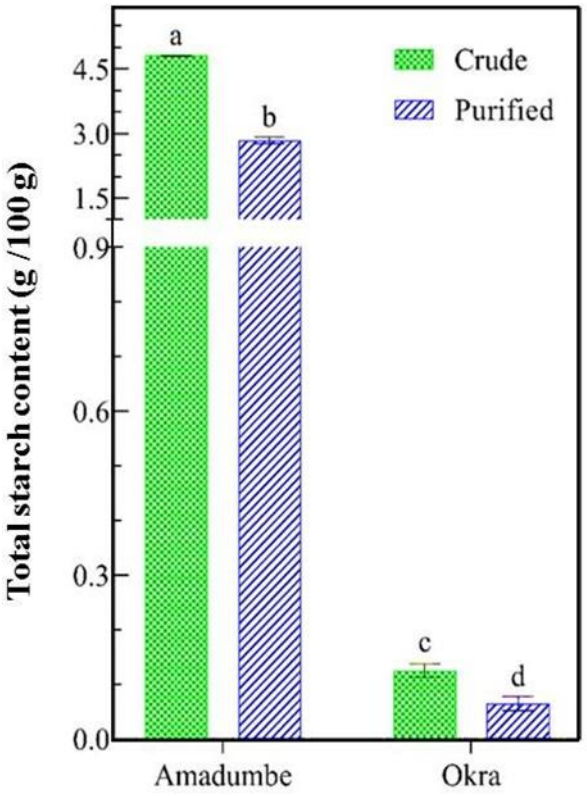

Figure 3. 1 Total starch content in crude and purified amadumbe and okra mucilage. Values with different subscript letters are significantly different ($p < 0.05$). Data denotes mean ± standard deviation ($n = 2$).

A significant difference in resistant starch content was noted between all mucilage samples ($p< 0.05$). The presence of resistant starch was found to be higher in amadumbe mucilage samples than in okra mucilage samples (Figure 3.2). Amadumbe crude mucilage had the highest content of resistant starch 4.72 g/100 g in comparison to the rest of the mucilage samples. The resistant starch content was found to be lower in purified mucilage samples. The content of resistant starch present in purified amadumbe mucilage was 2.63 g/100 g. Crude okra mucilage was found to have a resistant starch content of 0.72 g/100 g and in purified okra mucilage the resistant starch content was 0.13 g/100 g.

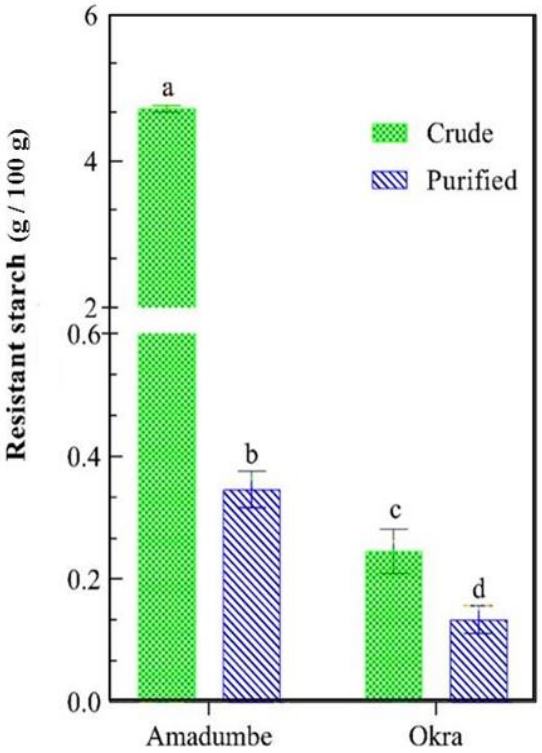

Figure 3. 2 Resistant starch content in crude and purified amadumbe and okra mucilage. Values with different subscript letters are significantly different ($p < 0.05$). Data denotes mean ± standard deviation ($n = 2$).

In the present study, the resistant starch present in both crude and purified amadumbe mucilage and ranged between 4.72 and 2.63 g/100 g. This was lower than 52 g/100 g previously reported by Naidoo, Amonsou and Oyeyinka (2015). As seen in Table 3.1, amadumbe mucilage contains significant levels of carbohydrates, most likely present in the form of starch. Total starch content accounts for both the digestible and resistant fractions of starch (Birt *et al*. 2013). The high resistant starch content in amadumbe mucilage suggest that most of its fraction is resistant to digestion (Tattiyakul, Asavasaksakul and Pradipasena 2006). It also suggests its potential to be used in the formulation and development of low glycaemic foods. Resistant starch is known to have numerous positive health claims for instance, regulation of glucose levels, lowering cholesterol and triglycerides and improving mineral absorption (Tattiyakul, Asavasaksakul and Pradipasena 2006). Resistant starch or its presence as polysaccharides have also been reported to stimulate gut microbiome and contribute to the production of metabolites essential in human health (Simsek and El 2012; Ho Do, Seo and Park 2021) and therefore its high presences in amadumbe mucilage can be considered advantageous.

3.3.3 β-glucan content

β-bonded polysaccharides are biopolymer compounds utilized as functional ingredients with nutritional and health benefits. They are derived from a variety of sources, including higher plants, yeasts and fungi (Danielson *et al*. 2010). Applications of β-bonded polysaccharides provide numerous advantages which include novel sensory properties, texture, less fat use and increased digestive health (Ahmad *et al*. 2012). Amadumbe mucilage has been previously reported to comprise of linear chains of β-D-glucopyranosyl units that are linked by (1→3) and (1→4) linkages. This structural composition is suggested to contribute to its hydration properties (Du *et al*. 2019).

In the present study, there was a significant difference recorded in the β-glucan content of all the mucilage samples ($p < 0.05$). The presence of β-glucan was found to be higher in crude and purified amadumbe mucilage compared to crude and purified okra mucilage (Figure 3.3). Purified mucilage samples were found to be richer in β-glucan. Purified amadumbe mucilage had the highest content of β-glucan 0.24 g/100 g when compared to crude amadumbe mucilage 0.20 g/100 g, crude okra mucilage 0.07 g/100 g and purified okra mucilage 0.04 g/100 g.

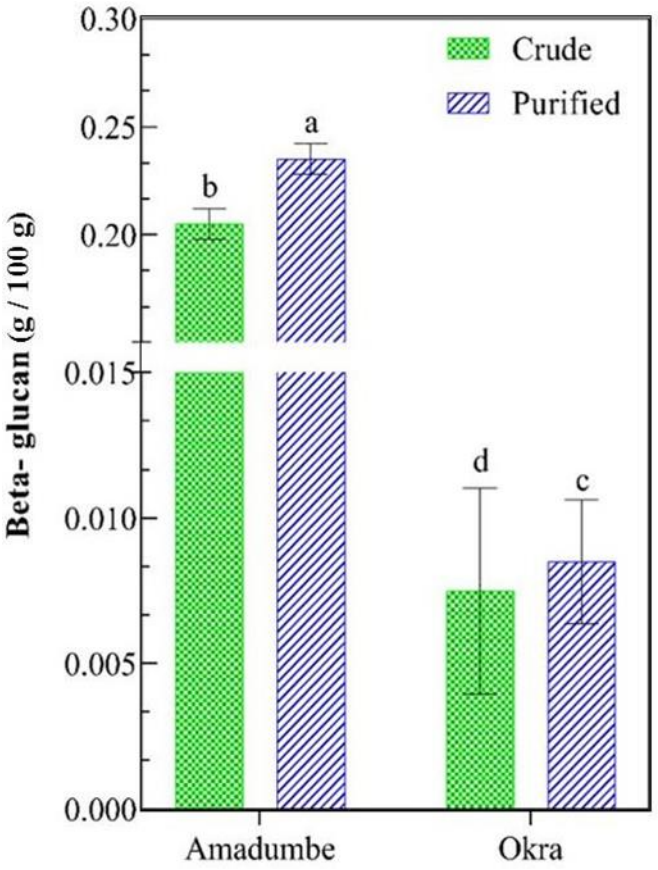

Figure 3. 3 Beta – glucan content in crude and purified amadumbe and okra mucilage. Values with different subscript letters are significantly different ($p < 0.05$). Data denotes mean ± standard deviation (n = 2).

Purification was shown to increase the exposure of beta-linkages present in amadumbe mucilage samples as also observed by Muhidinov *et al*. (2020). The presence of beta linkages in polysaccharides has been found to contribute to fat substitution in processed foods such as cheese and ice cream (Aydinol and Ozcan 2018). β-bonded polysaccharides have also been applied in the production of high-fiber products such as non-caloric stabilizing and thickening components (Dobson *et al*. 2019). Since amadumbe mucilage is rich in beta linkages it can potentially be applied in bread and dough formulations as a functional ingredient (Nagata, Andrade and Pereira 2015). The presence of beta linkages in amadumbe mucilage could possiblly influence its hydration properties (water holding and swelling capability) as well. Beta-linkages has also been suggested to contribute to thickening properties when incorporated into food systems (Ghotra, Vasanthan and Temelli 2008). Furthermore, the high presence of beta-glucan seen in amadumbe mucilage could potentially contribute to its utilization by gut microbiota. Beta-glucan have been reported as being very beneficial in encouraging the

proliferation of *Bacteroidete* species (Tamura *et al*. 2017).

3.3.4 Amino acid composition

Amino acids present in amadumbe and okra mucilage samples are summarized in Table 3.2. A notable reduction was observed in the amino acid content of crude and purified mucilage samples. Asparagine, proline, glutamine and threonine were the common amino acids found in both mucilage samples. In comparison to okra mucilage, the amino acid content of amadumbe mucilage was slightly higher and was in accordance to those previously reported in the literature (Njintang *et al*. 2014; Andrade *et al*. 2020). In crude and purified amadumbe mucilage, the presence of arabinogalactan protein is evident by the presence of proline (10.40 and 6.34%), serine (0.55 and 0.50%), alanine (1.42 and 1.22%) and threonine (1.37%) (Manhivi *et al*. 2018). Amadumbe mucilage was found to have appreciable amounts of essential amino acids phenylalanine (1.25%), valine (1.00%), threonine (1.37%), methionine (0.66%), leucine (0.58%), isoleucine (0.37%), lysine (1.60%) and histidine (0.26%), in comparison to okra mucilage. The presence of hydrophobic amino acids was found to be higher in crude okra and amadumbe mucilage 14.07 and 13.99% than in the purified okra and amadumbe mucilage 12.62 and 11.87%. The presences of acidic amino acids were shown to be high in crude and purified okra mucilage 3.8 and 3.28%.

The reduction in amino acids present in purified mucilage samples is possibly due to the purification process, as purification is suggested to eliminate low molecular weight molecules (Huppertz 2010). Asparagine was present in all mucilage samples and in significant amounts. This is can be considered advantageous as asparagine (non-essential amino acid) is utilized by the body in the central nervous system, where it aids in the production of proteins that are utilized by the brain to develop neurons and aid in signal transmission (Noorlaila *et al*. 2015). Asparagine is also popular as a nutritional supplements as it is suggested to improve athletic efficiency, focus, concentration and attention. Both crude and purified amadumbe mucilage had appreciable amounts of tryptophan, isoleucine and leucine, which has been suggested to contribute to its potential emulsifying property (Karami and Akbari-Adergani 2019). Crude okra and amadumbe mucilage were higher in hydrophobic amino acids (14.07 and 13.99 %) than in comparison to purified okra and amadumbe mucilage (12.62 and 11.87 %) as seen in Table 3.3.

Proteins are known to have both hydrophobic and hydrophilic amino acids present, making them ideal emulsifiers (Nicolai and Durand 2013). As reported, the presences of more hydrophobic amino acids, the better the emulsifying property and improved solubility of the protein (Aryee, Agyei and Udenigwe 2018). Okra mucilage samples had a slightly higher hydrophilic amino acid composition as presented in Table 3.3. The hydrophilic content of amino acids is reported to react with air within food systems forming flexible, coherent, film creating stable foams (Karami and Akbari-Adergani 2019). The term "branched-chain amino acids" (BCAAs), describes the chemical structure of amino acids, mostly present in foods of animal origin like eggs and meat products (Karami and Akbari-Adergani 2019). From Table 3.3, it can be seen that crude amadumbe and okra mucilage have substantial branched amino acid content. This indicates that plant sources can be used as alternatives to animal protein. Increasing data suggests that amino acids which make up dietary proteins can influence gut microbial composition and species diversity (Chen *et al.* 2020). According to Zhao *et al.* (2019), gut microbiota metabolise amino acids into secondary metabolites such as SCFAs, hydrogen sulfate and indole which are beneficial to host health. For instance, Yin *et al*. (2018) observed that lysine increased aubance of *Bacteroides*, *Bacillus* and *Faecalibacterium*, where as Ren *et al.* (2021) found that restriction of methionine in diet decreased inflammation causing bacteria and promoted SCFAs producing bacteria. Okra mucilage has been utilized in food systems as emulsifiers and stabilizers (Noorlaila *et al*. 2015). Amadumbe mucilage due to its high protein content and amino acid profile has potential to be utilized in beverage and food products as emulsifiers. Both okra and amadumbe due to its amino acid profile depict potential in bringing about changes to gut microbiota composition.

Table 3. 2 Comparison of percentage amino acid content in mucilage samples

	Amadumbe		Okra	
	Crude	Pure	Crude	Pure
Essential Amino Acids (EAA) (a)				
Lysine	1.50 ± 0.05^{a}	1.43 ± 0.01^{b}	0.34 ± 0.06^{c}	0.30 ± 0.05^{d}
Valine	0.90 ± 0.11^{a}	0.48 ± 0.05^{b}	0.66 ± 0.06^{bc}	0.26 ± 0.03^{d}
Threonine	0.37 ± 0.16^{c}	N/A	1.38 ± 0.02^{a}	1.34 ± 0.01^{b}
Cystine	0.03 ± 0.05^{a}	0.01 ± 0.04^{c}	0.02 ± 0.09^{b}	0.02 ± 1.70^{b}
Tyrosine	0.81 ± 0.08^{a}	0.32 ± 0.04^{b}	0.27 ± 0.01^{c}	0.27 ± 0.01^{c}
Isoleucine	0.37 ± 0.03^{a}	0.25 ± 0.01^{b}	0.05 ± 0.01^{c}	0.04 ± 0.01^{c}
Leucine	0.48 ± 0.11^{c}	0.25 ± 0.02^{d}	1.11 ± 0.02^{a}	0.85 ± 0.02^{b}
Methionine	0.66 ± 0.04^{a}	0.24 ± 0.02^{b}	0.19 ± 0.20^{c}	0.05 ± 0.03^{d}
Histidine	0.26 ± 0.05^{a}	0.23 ± 0.03^{bc}	0.24 ± 0.05^{a}	0.02 ± 0.03^{c}
Phenylalanine	1.25 ± 0.06^{a}	1.16 ± 0.01^{ab}	0.42 ± 0.06^{c}	0.03 ± 0.01^{d}
Non – Essential Amino Acids (NEAA) (a)				
Proline	8.00 ± 1.19	6.34 ± 0.18	7.25 ± 0.48	7.02 ± 0.02
Serine	0.55 ± 0.06^{a}	0.50 ± 0.01^{b}	0.45 ± 0.02^{b}	0.29 ± 0.01^{c}
Glutamate	0.91 ± 0.13^{c}	0.41 ± 0.02^{d}	3.15 ± 0.18^{a}	2.79 ± 0.06^{b}
Aspartate	1.14 ± 0.09^{a}	1.07 ± 0.01^{a}	0.63 ± 0.10^{b}	0.25 ± 0.03^{c}
Glutamine	1.27 ± 0.92	1.19 ± 0.73	2.68 ± 0.20	2.35 ± 0.07
Alanine	1.42 ± 0.06^{a}	1.22 ± 0.02^{a}	0.97 ± 0.03^{b}	0.64 ± 0.17^{c}
Asparagine	80.06 ± 1.34^{a}	72.06 ± 2.63^{d}	78.25 ± 2.7^{ab}	76.06 ± 0.57^{abcd}

Means ± SD; n=3; values with different letters within the same row differ significantly ($p<0.05$).
N/A - not applicable

Table 3. 3 Percentage nutritional quality amino acids in amadumbe and okra mucilage

% Nutritional Qualities	Amadumbe		Okra	
	Crude	Pure	Crude	Pure
Total EAA (b)	6.63	5.01	4.77	3.43
Hydrophobic (c)	13.99	11.87	14.07	12.62
Hydrophilic (d)	1.76	0.95	2.16	2.07
Acidic (e)	2.05	1.69	3.85	3.28
Basic (f)	1.76	1.90	0.59	0.35
Aromatic (g)	2.06	1.70	0.70	0.32
Branched –chain (h)	1.75	1.12	1.86	1.24

(a) Each amino acid content/total amino acids × 100, (b) EAA/total amino acids × 100, (c) hydrophobic amino acids/total amino acids × 100, (d) hydrophilic/total amino acids × 100, (e) acidic amino acid/total amino acids × 100, (f) basic amino acids/total amino acids × 100, (g) aromatic amino acids/total amino acids × 100, (h) branched amino acids/total amino acids × 100.

3.3.5 FT-IR spectrum of amadumbe and okra mucilage

Previous research has shown peptides, sugars and polysaccharides contribute to the structure of amadumbe and okra mucilage (Xu, Guo and Du 2017; Chukwuma, Islam and Amonsou 2018). These components were also found to be present in the FT-IR spectra of the studied mucilage samples. The spectrum curve for both crude and purified amadumbe and okra mucilage are presented in Figure 3.4. Purified amadumbe and okra mucilage samples showed the same characteristic peaks as crude mucilage samples but at a lower intensity, this suggests that purification had contributed to a more stable and uniform structure (Ren and Liu 2020).

The broad absorption peaks at 3341 and 3362 cm^{-1} seen in amadumbe mucilage (Figure 3.4a) and okra mucilage (Figure 3.4b) respectively, correspond to vibrational stretches related to free inter and intramolecular bound hydroxyl groups, which are characteristically found in polysaccharides, confirming the existence of carbohydrates (Selek *et al*. 2007; Chukwuma, Islam and Amonsou 2018). The weak peak at 2928 cm^{-1} observed in okra mucilage samples is

attributed to potential C–H vibrational stretching of CH_2 groups (Hua *et al*. 2014; Li *et al*. 2017). Peaks observed at 1634 cm^{-1} (Figure 3.4a) and 1637 cm^{-1} (Figure 3.4b) are suggested C=O vibrational stretching of peptide linkages, also indicating the presence of proteins (Dimopoulou, Ritzoulis and Panayiotou 2015). Peaks in the regions of 1413 cm^{-1} (Figure 3.4a) and 1415 cm^{-1} (Figure 3.4b) likely correspond to symmetrical and asymmetrical vibrational stretching of C=O (carbonyl group) and of free carboxyl groups respectively, indicating that uronic acid is present in amadumbe and okra mucilage (Wang *et al*. 2018b). The stretching of the C- O-C group is represented by peaks in the region of 1047 and 1037 cm^{-1} (Freitas *et al*. 2015). The FT-IR spectra obtained in this study were comparable to those previously mentioned (Chukwuma, Islam and Amonsou 2018). The presence of hydroxyl groups have been suggested to allow for hydrogen bonding thereby influencing hydration properties of polysaccharides including their water holding capacity as well as their swelling ability (Guo *et al*. 2017). Carboxylic groups have been suggested to contribute to the solubility of polysaccharides (Lebrilla *et al*. 2022). Hydroxyl and carboxylic groups were seen to be present in amadumbe and okra mucilage and can possibly influence their hydration and solubility properties.

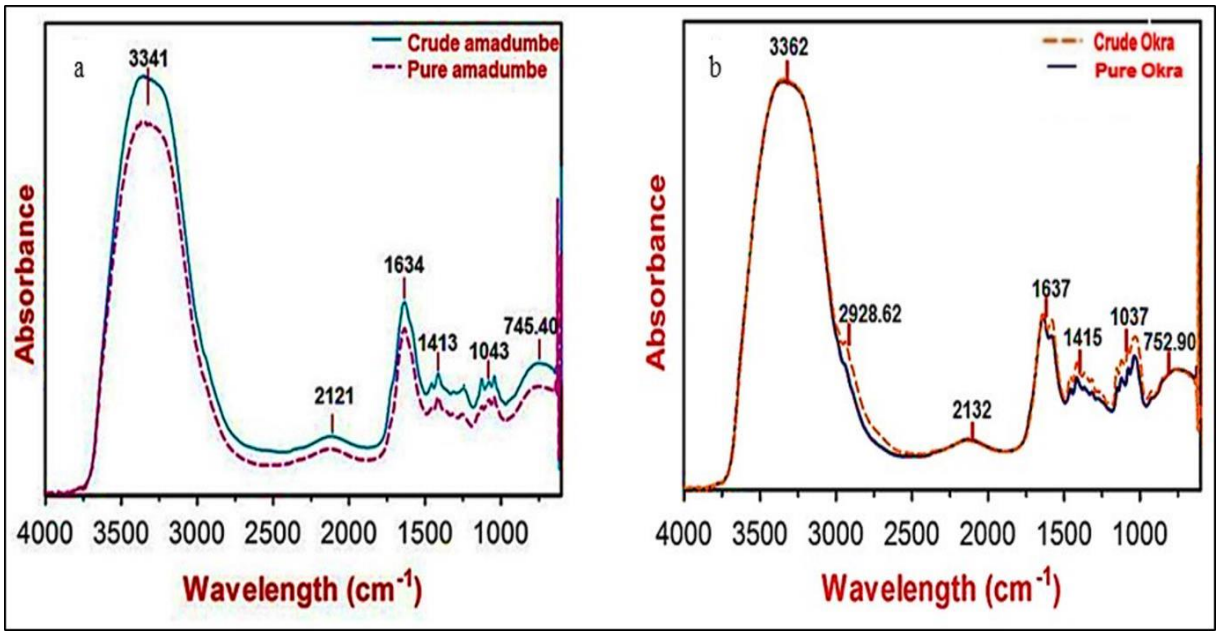

Figure 3. 4 Compares the FT-IR absorption spectra of amadumbe and okra mucilage samples between the regions of 1000 - 4000 cm^{-1} (3.4 a) amadumbe mucilage and (3.4 b) okra mucilage.

3.3.6 Molecular weight composition

The molecular weights of amadumbe and okra mucilage varied to a certain extent, as seen in Table 8. Purified mucilage samples seemed to have a slightly higher molecular weight than

crude mucilage samples. In the present study, okra mucilage samples had a higher molecular weight than amadumbe mucilage samples. The molecular weight of crude amadumbe and purified amadumbe ranged between 219.87 and 220.22 kDa. This was in accordance to that previously reported by Lin and Huang (1993), who observed the molecular weight of taro mucilage to be > 1000 kDa. The molecular weight of crude and purified okra ranged between 224.45 and 244.08 kDa. This was lower than 3265 kDa, previously reported values by Xu, Guo and Du (2017), but higher than 170 kDa reported by Georgiadis *et al*. (2011). The increase in molecular weight seen in purified mucilage samples can possibly be due to the purification process. Purification processes have been suggested to eliminate impurities, such as low molecular weight compounds, free proteins, sugars and lipids (Karimi *et al*. 2018). The differences in molecular weight of amadumbe and okra mucilage can be attributed to factors such as cultivar type, environmental growth conditions and plant developmental state (Werner *et al*. 2022). As reported by Chen *et al.* (2020), there is strong correlations between molecular weight of polysaccharides and gut microbial compositiona and diversity. Li *et al.* (2022) reported that lower molecular weight polysaccharides are more rapidly fermented, with higher carbohydrate consumption rates and gas production, making them undesirable. Since amadumbe and okra are of high molecular weight, both can potentially be slower fermented by gut microbiota with less gas production.

Table 3. 4 Molecular weight distribution of amadumbe and okra mucilage samples.

Mucilage substrate	Molecular weight (Mw) kDa
Crude amadumbe	219.87 ± 0.45^{c}
Pure amadumbe	220.22 ± 0.16^{c}
Crude Okra	224.45 ± 0.29^{b}
Pure okra	244.08 ± 0.09^{a}

Means ± SD; n=2; values with different letters differ significantly ($p<0.05$).

3.3.7 Monosaccharide profile of amadumbe and okra mucilage

Table 3.5, presents data on the monosaccharide profiling of amadumbe and okra mucilages. Both mucilage samples were observed to have comparable qualitative sugar profile as those previously reported in literature (Manhivi *et al*. 2018; Dantas, Alonso Buriti and Florentino 2021). Amadumbe mucilage was found to have a neutral sugar profile with glucose and

mannose being the significant monosaccharides. The main monosaccharides present in okra mucilage were galactose and glucose (Figure 3.5). In the present study, the monosaccharides present in amadumbe mucilage were found to be lower 74.6% in comparison to 91.8% reported by Manhivi *et al*. (2018). Okra mucilage was lower in galactose 15% in comparison to 33% reported and in glucose 11% than 14% reported by Chen *et al.* (2016). Such variations in monosaccharide composition may occur due to extraction methods as well as cultivar type. According to Al-Shawi *et al*. (2021), different extraction methods can influence the structure and composition of mucilage. Liu *et al*. (2018b) found that hot water extraction yielded arabinose, galactose and rhamnose in okra mucilage, in comparison to ultrasound extraction which yielded glucose, mannose, fructose, rhamnose, galactose, arabinose and xylose in okra mucilage (Wang *et al*. 2018a). As suggested by Nazari *et al*. (2020), cultivar type, genotype and biological origin are factors which influence structural composition of mucilage. Basiony *et al*. (2022) observed that Egyptian cultivated amadumbe mucilage was found to be made up of glucose, fructose, rhamnose, mannose and galactose, in comparison to Chukwuma, Islam and Amonsou (2018), who reported galactose, mannose and arabinose as predominant monosaccharides present in amadumbe mucilage. Monosaccharide composition of dietary polysaccharides can significantly influence their utilization by gut microbiota as suggested by Payling *et al.* (2020). Larke *et al.* (2023) observed that monosaccharides are selectively fermented by gut species, for instance, arabinose, xylose and GalA are highly fermented and utilized by *Lachnospira* and *Ruminiclostridum* E and to a lesser extent utilized by *Blacitia* and *Faecalialea*. Hou *et al.* (2022) reported that neutral monosaccharides are more easily degrade by gut microbiota. Since amadumbe and okra are composed of different monosaccharides, it is most likely that these mucilage samples would be selectively fermented by gut microbiota.

Table 3. 5 Percentage monosaccharide composition of amadumbe and okra mucilage.

*Monosaccharides	Amadumbe mucilage	Okra mucilage
Glucose (%)	31.37 ± 0.1^{a}	11.02 ± 1.5^{b}
Galactose (%)	0.0	15.52 ± 2.6^{a}
Arabinose (%)	7.92 ± 0.4^{a}	7.93 ± 0.2^{a}
Rhamnose (%)	0.0	4.38 ± 0.6^{a}
Xylose (%)	6.31 ± 0.007^{a}	0.0
Mannose (%)	29 ± 0.5^{a}	0.0
Ribose (%)	0.0	5.61 ± 0.01^{a}

Means ± SD; n=2; values with different letters within the same row differ significantly ($p<0.05$).

*Monosaccharide compositions are expressed as % of the total sugars.

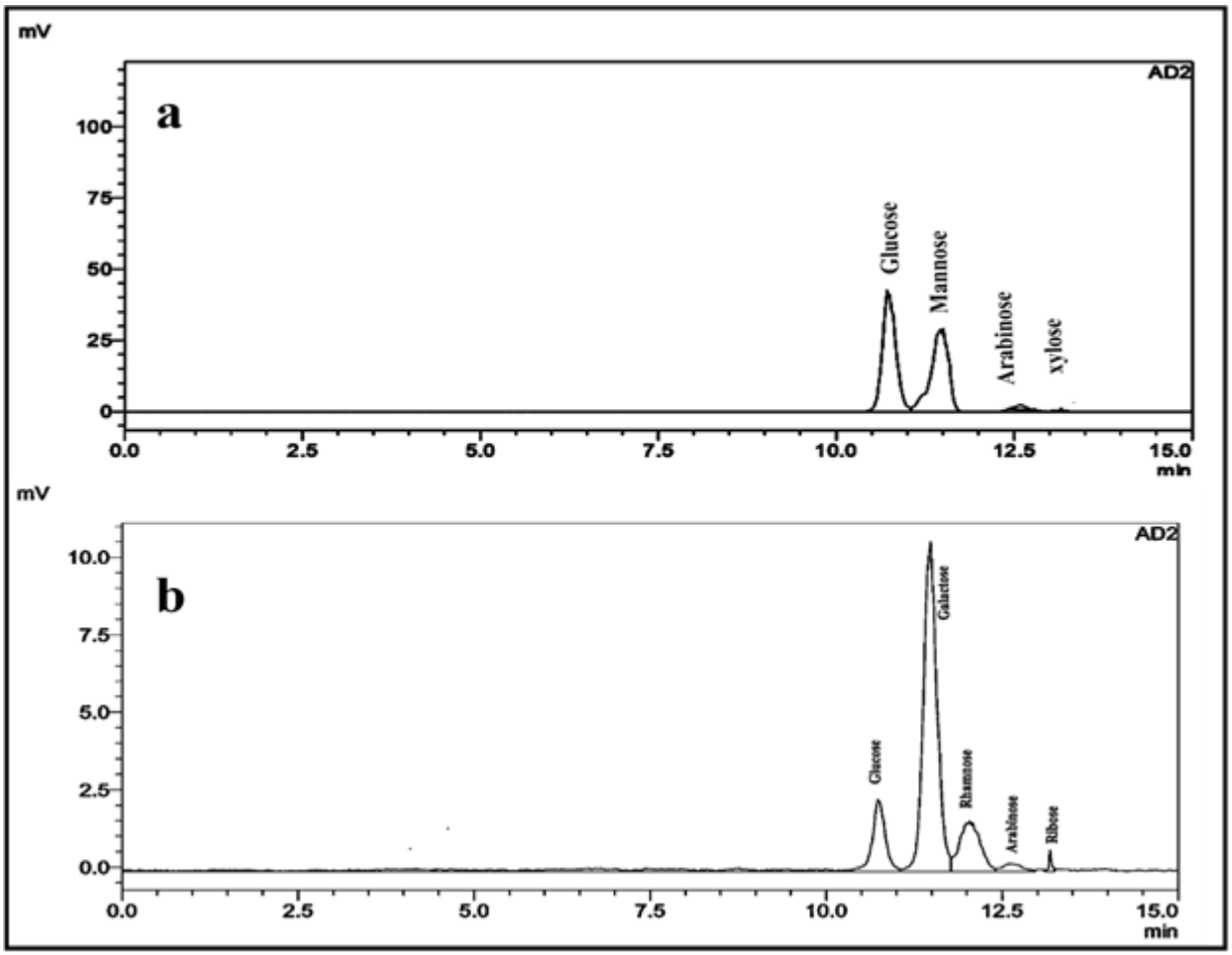

Figure 3. 5 (a) Chromatogram of mucilage present in amadumbe mucilage **(b)** and okra mucilage.

3.3.8 Functional properties of amadumbe and okra mucilage

3.3.8.1 Water holding capacity (WHC)

In the present study, the water holding capacities of purified mucilage samples were found to be significantly higher than that of crude mucilage samples ($p < 0.05$) as seen in Figure 3.6. Okra mucilage was found to have better water-holding capacity in comparison to amadumbe mucilage. Amadumbe crude mucilage had a WHC of 5.92 g/100 g which was lower than in okra crude mucilage 9.30 g/100 g. Purified amadumbe mucilage was observed to have a WHC of 9.89 g/100g, which was lower in comparison to purified okra mucilage 12.89 g/100 g but higher than that of amadumbe crude mucilage.

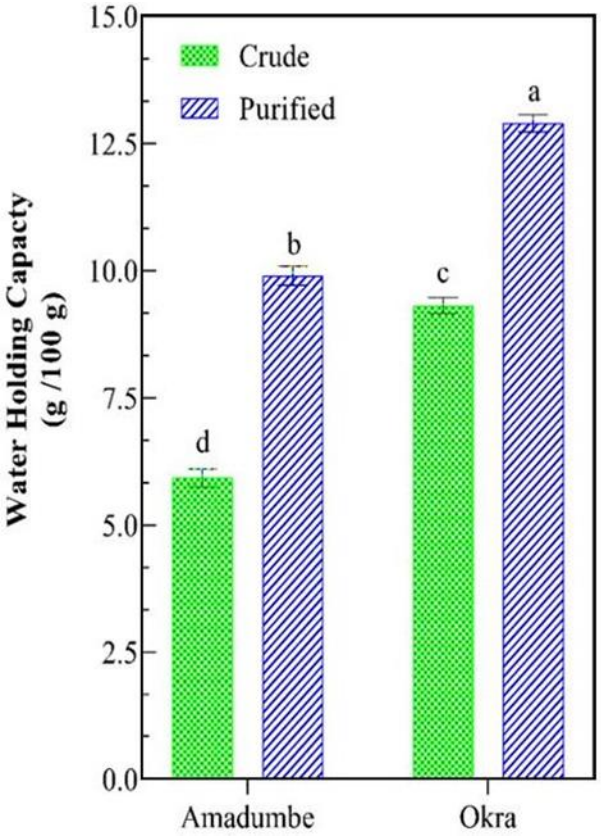

Figure 3. 6 Water holding capacity of crude and purified amadumbe and okra mucilage. Data denotes mean ± standard deviation (n = 3). Values with different subscript letters are significantly different ($p < 0.05$).

The ability of a substance to interact with water under limited conditions can be defined as the water holding capacity (WHC) (Zhuang *et al*. 2020). Water holding capacity is made up of associated water, water that is physically trapped and hydrodynamic water (Zhuang *et al*. 2020). Findings show that the purification method effectively enhanced purified mucilage samples ability to absorb and retain water. As suggested by Amid and Mirhosseini (2012), efficient purification techniques are able to reduce impurities thus improving the functional properties of the polysaccharide. According to Sciarini *et al*. (2009), purification can induce the unfolding of constituent polysaccharides, leading to a higher ratio of ramification that impacts water absorption capacity. As a result, after purification, water interacts more easily with the polysaccharide (Sciarini *et al*. 2009).

The high WHC seen in okra mucilage samples possibly is attributed to the presence of monosaccharides such as galactose, glucose and rhamnose (Table 3.5) in the mucilage as reported by Gao *et al*. (2018). Nep and Conway (2010) suggested that a polysaccharides high affinity for water could be due to polymer absorption sites, which increases its ability to form hydrogen bonds with water molecules. The WHC of okra mucilage was higher than 2.67 g/100 g, previously reported by Gemede *et al*. (2015) but lower than 157.25 g/100 g, reported by

Noorlaila *et al*. (2015). The water holding capacity of amadumbe mucilage samples was lower than 23.48 g/100 g, previously reported by Hozifa, El-Desouky and Salem (2020). According to Ren, He and Li (2021), the origin, environment, harvesting age and cultivar type highly influences the mucilage structure and functional properties. Studies have reported on taro mucilage and its functional properties, particularly hydration properties (Basiony *et al*. 2022). Hydration properties of amadumbe mucilage are influenced by numerous free hydroxyl groups and fiber-rich fractions that can bind with water molecules (Hozifa, El-Desouky and Salem 2020). As explained by Singh, Sethi and Tiwari (2009), the water holding capacity relies on the ability of hydroxyl groups on the branched structure of galactomannan, which affects the polysaccharides water retention site (Singh, Sethi and Tiwari 2009). As seen in the FT-IT spectrum (Figure 3.4), amadumbe mucilage contains free carboxylic and hydroxyl groups that allow for hydrogen bonding. Furthermore, water holding properties of polysaccharides is reliant not only on the functional group of carbohydrates but also on the presence of proteins, as they comprise of amino acids capable of increasing water binding properties (Thanatcha and Pranee 2011).

As shown in (Table 3.1), the protein content of amadumbe mucilage samples ranged between 10.0 and 8.54%. As suggested by Ghosh and Bandyopadhyay (2012), the charges on the protein molecules result in the strong correlation of protein hydration with polar constituents, as well as the hydrophilic interaction via hydrogen bonding thus contributing to its water holding capacity. Hydration properties such as water holding capacity of polysaccharides such as mucilage are reported to affect gut tansit time and absorption of minerals, in addition to influencing bulking, laxation and body weight (Tan *et al.* 2017). Wanders *et al.* (2013) reported how polysaccharides with high water holding capabilities contribute to stool bulking which leads to easier evacuation, resulting in less chances of constipation, reduced inflammation and enhanced excreation of free radicals.

3.3.8.2 Oil holding capacity

The oil holding capacity of crude mucilage samples were observed to be significantly higher in comparison to purified mucilage samples ($p < 0.05$) as seen in Figure 3.7. Crude amadumbe and crude okra mucilage had a higher oil holding capacity of 11.73 and 10.33 g/100 g respectively. Purified amadumbe and okra mucilage had an oil holding capacity of 7.99 and 5.94 g/100 g.

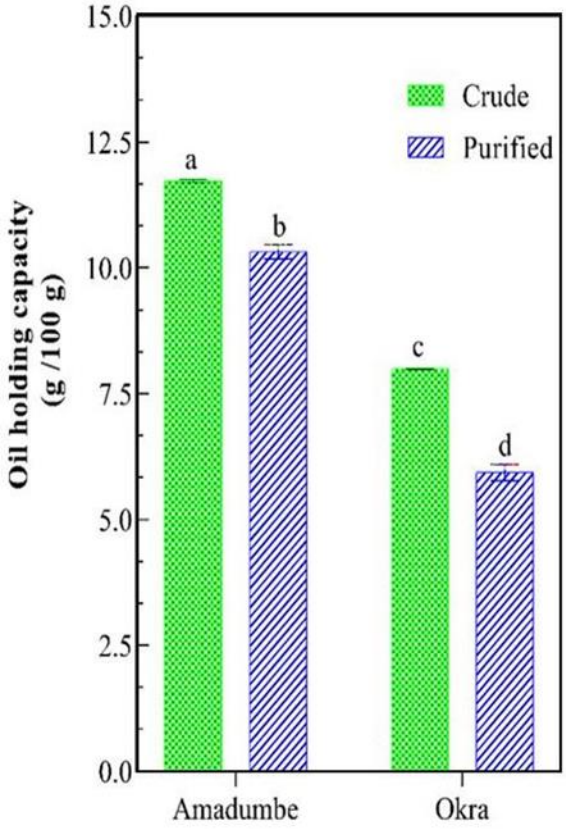

Figure 3. 7 Oil holding capacity (OHC), of crude and purified amadumbe and okra mucilage. Data denotes mean ± standard deviation (n = 3). Values with different subscript letters are significantly different ($p < 0.05$).

The amount of oil that a sample can absorb per unit of weight is referred to as its oil holding capacity (Lam *et al*. 2018). Oil-holding capacity (OHC) is among the most prominent functional properties of a hydrocolloid (Phimolsiripol, Siripatrawan and Cleland 2011). Many polysaccharides are suggested to impart fat attributes by binding large amounts of fat, causing plasticity, lubricity and melting sensation (Rincón *et al*. 2009). The purification processes in the present study resulted in a significant ($p < 0.05$) decrease in the oil holding capacity of the respective mucilage samples when compared to the crude mucilage samples. The high oil capacity observed in crude mucilage samples can possibly be due to the presence of non-polar side chains and hydrophobic fractions (protein and fat), that are suggested to bind to the hydrocarbon units of oil (Sathe and Salunkhe 1981). Purification methods were seen to reduce the presence of hydrophobic impurities, hence lowering the oil holding capacities of purified mucilage samples. Iwe, Obaje and Akpapunam (2004) reported that mucilage and gums that have low oil holding capacities, are more suitable for oil-in-water emulsion applications. The

oil holding capacity of okra mucilage is suggested to be attributed to the presence of non- polar side chains as well as hydrophobic fractions such as fat and protein (Table 3.1) which bind to oil hydrocarbon units hence resulting in good oil holding capacity (Thanatcha and Pranee 2011). The high oil holding capacity of crude amadumbe mucilage is most likely due to the presence of proteins and non-starchy carbohydrates (polysaccharide). The polysaccharide accounts for the hydrophilic fraction, while proteins with amino acids (non- polar) or weakly polar radicals account for the hydrophobic fraction. Amadumbe mucilage was also seen to be composed of high level of starch (Figure 3.1). As suggested by Andrade *et al*. (2020), starch present in amadumbe mucilage can be advantageous as it contributes to the oil holding capacity of the mucilage.

3.3.8.3 Swelling Power

As shown in the Figure 3.8, the swelling power of purified mucilage samples were significantly higher than in comparison to crude mucilage samples ($p<0.05$). Purified amadumbe and okra mucilage samples were observed to have high swelling power 2.72 and 6.69 g/100 g, in comparison to crude amadumbe and okra mucilage 1.26 and 4.49 g/100 g.

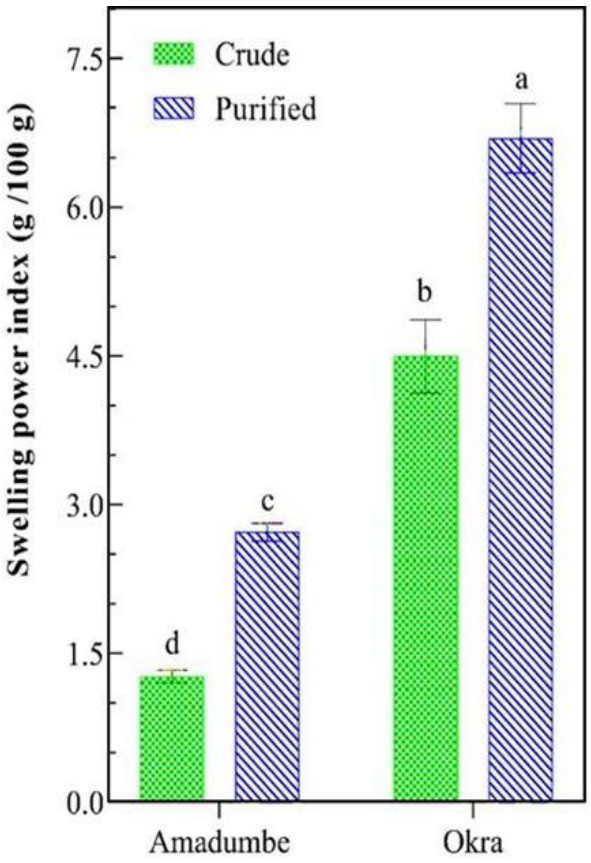

Figure 3. 8 Describes the swelling power of crude and purified amadumbe and okra mucilage. Data denotes mean ± standard deviation (n = 3). Values with different subscript letters are significantly different ($p < 0.05$).

The swelling power of okra mucilage was found to be higher than that of amadumbe mucilage. Swelling power can be defined as the proportion of the sediment gels weight to its dry weight (Li and Yeh 2001). The low swelling power of crude mucilage samples can possibly be attributed to impurities present in the mucilage, which prevent hydrogen bonding. Purified mucilage samples showed increased swelling power and this can be as result of the purification process which reduces and eliminates impurities such as low molecular weight components, protein, ash and water-soluble sugars (Amid and Mirhosseini 2012a). The high swelling power of okra mucilage can be linked to its high water holding capacity as shown in Figure 3.6. It has also been suggested that the carbohydrates present in okra mucilage contribute to its hydration properties and increased carboxyl group ionization (George, Joseph and Josekumar 2017). The low swelling power of amadumbe mucilage can been attributed to a higher degree of intermolecular association and high starch content (Figure 3.1). It can also be linked to the inability of amadumbe mucilage to retain water, the low water holding capacity (Figure 3.6).

3.3.8.4 Solubility index

In the present study, the solubility of purified mucilage samples was significantly higher than in comparison to crude mucilage samples ($p < 0.05$). In Figure 3.9, it can be seen that the percentage solubility for crude and purified amadumbe mucilage ranged from 61.33 - 73.33%. The percentage solubility between okra mucilage crude and purified ranged between 41.38 and 53.33%. Amadumbe mucilage samples displayed high solubility when compare to okra mucilage samples. The purification was shown to improve the solubility of purified mucilage samples.

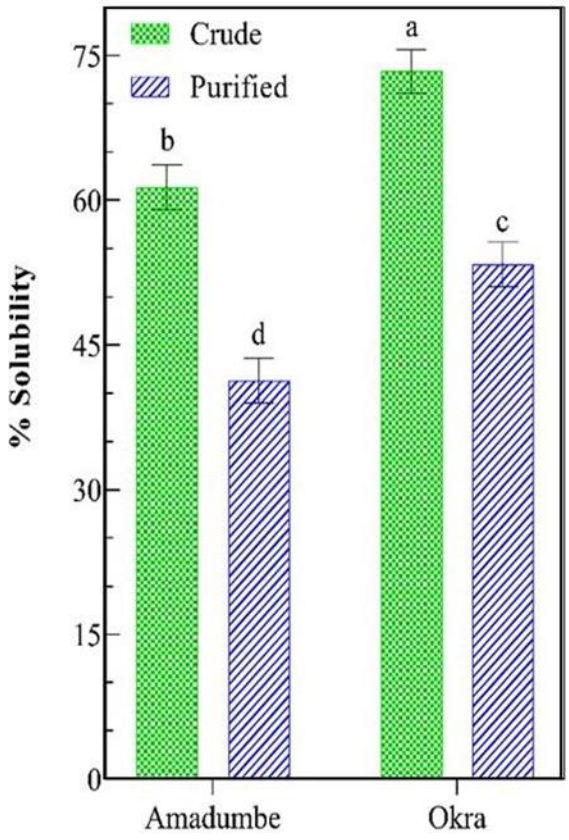

Figure 3. 9 Percentage solubility of crude and purified amadumbe and okra mucilage. Data denotes mean ± standard deviation (n = 3). Values with different subscript letters are significantly different ($p < 0.05$).

As explained, by Amid and Mirhosseini (2012a), the solubility of hydrocolloids is determined by the ratio of soluble to insoluble matter. As a result, the increased solubility in purified mucilage samples is possibly attributed to a decrease in insoluble matter during the purification process. In the present study, the noticeable increase in solubility could be attributed to the

decrease of protein content during the purification process (Table 3.1). The purification process also led to reduced ash (impurities) and fat (hydrophobic) contents, thereby enhancing the solubility (Amid and Mirhosseini 2012a). Crude mucilage samples showed low solubility, as seen in Figure 3.9, this possibly due to the presence of impurities, high molecular weight or large molecules, and insoluble matter. The low solubility shown by okra mucilage samples can be as a result of the dominant intramolecular associations between polymer segments, which has been suggested to lead to aggregation, swelling and eventually gelation. As seen in Figure 3.6, okra mucilage showed a high water holding capacity, this indicates the ability of okra mucilage to entrap water, as it increases in viscosity (Brax, Schaumann and Diehl 2019). Amadumbe mucilage samples showed higher solubility, this possibly due to its ability to dissolution rather than to entrap water. As explained by Guo *et al*. (2017), the interactions between polysaccharide molecules and water molecules are favorable, when the polymer chain dissociates, keeping the polysaccharide molecules apart.

3.4 Conclusion

In the present study, the purification process was shown to improve the chemical composition as well as the functional properties of mucilage samples. Amadumbe mucilage was found to be higher in protein and carbohydrate than in comparison to okra mucilage. Amadumbe mucilage was also seen to be rich β- glucan and resistant starch. Glucose and arabinose were the common monosaccharides seen in both mucilage samples. Carboxylic and hydroxyl groups were the main functional groups identified in amadumbe and okra mucilage. In comparison to okra mucilage, amadumbe showed low water holding capacity. This behaviour can be attributed to its high solubility and relatively low swelling power. Composition was observed to have influenced functional properties. The functional properties of amadumbe mucilage as noted suggest it can be employed in bread and dough formulations, as a fat replacer, as a fortifying agent and as a source of dietary fiber in emulsion based beverages. These results illustrate the potential of mucilage derived from southern variety amadumbe as a functional food ingredient which enable it to compete with other commercial hydrocolloids.

Variation seen in the composition and functional properties of amadumbe and okra mucilage could potentially influence its fermentation and utilization by gut microbiota. This was investigated in the next chapter.

CHAPTER FOUR

The prebiotic effects of amadumbe and okra mucilage on faecal microbiota

Abstract

Prebiotics have quickly emerged as a consequence of growing consumer knowledge between gut microbiota, human health and diseases. Southern African amadumbe and okra varieties are traditional mucilage rich crops. Although, amadumbe and okra mucilages are presumable prebiotics, there is lack of scientific evidence supporting their prebiotic potential. Therefore, the aim of this study was to investigate the prebiotic potential of amadumbe and okra mucilages and compare their fermentability to inulin (commercialized prebiotic) for potential applications as prebiotic dietary supplements.

In-vitro fermentation was carried out using faecal sample. Total gas generation was carried out using ANKOM GPM v11.4 software. Changes in microbial species diversity and composition were determined using DNA extraction and Bioinformatics processing. Inulin, amadumbe and okra mucilages were observed to decrease pH of the fermentation medium. Inulin showed maximum gas production of 233.19 ml, followed by amadumbe mucilage 158.98 ml and okra mucilage 113.98 ml. Gut microbiota analysis at phylum level showed that amadumbe mucilage encouraged the proliferation of Actinobacteria and reduced the presence of Firmicutes in comparison with okra mucilage. Results showed the potential of amadumbe and okra mucilage as an emerging prebiotic that could be used as a dietary supplement.

Keywords: Prebiotics, mucilage, okra, amadumbe, gut microbiota

4.1 Introduction

Scientific research on the modification and manipulation of gut microbiota in human health has increased (Quigley and Gajula 2020). Numerous clinical research has shown gut microbiota to influence many areas of human health, including metabolic, immunological and neurobehavioral characteristics (Levy *et al*. 2017; Valdes *et al*. 2018). Various studies have observed low gut species diversity to be linked to obesity and various autoimmune diseases such as cardiovascular disease, multiple sclerosis, diabetes and various cancers (Berer *et al*. 2017; Shi and Mu 2017; Garrett 2019). Inclusion of prebiotics in diet has been suggested as effective means of altering and positively shaping gut microbiota (Singh *et al*. 2017). This phenomenon has sparked a number of scientific investigations as well as industrial interest in prebiotics (Guarino *et al*. 2020). According to Global Market Insights, Inc. (Delaware, USA), the prebiotic market is currently growing and is expected to exceed USD 8.5 billion by 2024 (Fonteles and Rodrigues 2018). Furthermore, the COVID-19 pandemic has boosted total prebiotic component growth since 2020 (Olaimat *et al.* 2020).

Prebiotics are plant derived carbohydrates that serve as a source of energy for the growth and activity of beneficial gut microbial taxa, which results in a particular or selective alteration that confers health benefits to the host (Carlson and Slavin 2016). Many diet related studies have shown that within days of adjusting diet to incorporate more plant based foods, noticeable positive changes occur in gut microbiota composition and diversity (O'Keefe *et al*. 2015a; Willis and Slavin 2020). Prebiotics include gums, pectins and mucilages (Siva *et al*. 2019). Although prebiotic carbohydrates are derived from plants, their prebiotic potential varies based on their biological source, cultivar type, structure, composition (sugar composition, chain lengths, linkage patterns, composition of branch chains) and functional properties (water solubility, swelling and rheological behaviour). Therefore, it becomes crucial to assess the prebiotic potential of presumed prebiotic carbohydrates (Zhang *et al*. 2018; Payling *et al*. 2020).

Amadumbe (*Colocasia esculenta*), also known as taro, are edible corms cultivated in subtropical and tropical climates (Naidoo, Amonsou and Oyeyinka 2015). In South Africa, amadumbe was initially cultivated by subsistent farmers, however, over the years, its cultivation and consumption have increased, attainting it the status of a staple food (Mapumulo 2022). Okra (*Abelmoschus esculentus* L. Moench), also known as Lady's finger is cultivated in tropical and subtropical regions. In South Africa, okra is grown by small scale farmers and has become a vastly commercialized vegetable (Murovhi, Phophi and Mafongoya 2020). Southern African

amadumbe and okra varieties are traditionally mucilage rich crops (Gemede *et al*. 2015; Manhivi *et al*. 2018). Mucilage is a soluble polysaccharide which varies in structure, composition and functional properties (Fernandes, Filipini and de las Mercedes Salas-Mellado 2021). Recent studies have shown polysaccharides such as mucilage to be potentially prebiotic. Mucilage from nopal (*Opuntia* spp.) showed a hypoglycemic effect on diabetic-induced rats of various ages following consumption (Nuñez-López, Paredes-López and Reynoso-Camacho 2013). The ability of mucilage to alter gut microbiota in rats have also been demonstrated (Sánchez- Tapia *et al*. 2017).

Previous studies have hypothesized that structure, composition and functional properties of prebiotic polysaccharides are important factors that could influence gut microbiota composition and species diversity (Moreno *et al*. 2017). For instance, a clinical intervention study compared the consumption of wheat bran and resistant starch by obese individuals. Structurally, the fiber components of wheat bran are hemicelluloses and β-glucans (Wcislo and Szarlej-Wcislo 2014). Resistant starch is structurally that portion of starch, that cannot be hydrolyzed by enzymes in the small intestine and is passed into the large intestine (Jiang *et al*. 2020). Consumption of wheat-bran was found to increase faecal microbial species diversity (richness) in comparison to a diet supplemented with resistant starch. These findings suggest that heterogeneous polysaccharides encourage and supports greater microbiota species diversity (Salonen *et al*. 2014).

Functional properties of prebiotic polysaccharides are also considered a crucial factor. They determine their interactions with other components within the gut through abilities such as water holding capacity, gel forming properties and its ability to bind to organic compounds (Poutanen *et al*. 2017). Logan, Wright and Goff (2015) demonstrated that functional properties such as water holding capacity and gel forming ability of pectin increased stool bulk thus improving satiety in individuals. As suggested by Tamura *et al*. (2017), non-digestible food components primarily support human gut microbiota. Structurally, amadumbe mucilage composes of mainly glucose, mannose and arabinose, while okra mucilage contains galactose, glucose, rhamnose and arabinose (Anwar, McConnell and Bekhit 2021; Dantas, Alonso Buriti and Florentino 2021). According to López-Palacios *et al*. (2012), the structure, composition and functional properties of mucilage vary and are highly dependent on cultivar type, crop age and developmental stage at which it is harvested.

There have been studies that have shown the ability of okra mucilage to support glucose metabolism and to prevent harmful bacteria (Daliu *et al*. 2020). Mucilage derived from various

taro varieties in the Pacific has been shown to act as a dietary prebiotic (Saxby *et al*. 2020). Although, Southern African varieties amadumbe and okra mucilage are presumable to be prebiotics, there is lack of scientific evidence supporting their prebiotic potential. It can be hypothesized that since amadumbe and okra mucilage vary in structure and composition, it can be expected that they will stimulate microbial species diversity differently. To the best of our knowledge, there is limited publications that discussed the structure, composition and functional properties of amadumbe and okra mucilage and their prebiotic potential in stimulating gut microbiota Therefore, the aim of this study was to investigate the prebiotic potential of amadumbe and okra mucilage and compare its fermentability to inulin (commercialized prebiotic) by means of *in-vitro* fermentation.

4.2 Material and methodology

Materials Chemicals required for the preparation of buffer and mineral solutions were purchased from Sigma-Aldrich Corporation (St. Louis, USA). Additional reagents utilized in this study were all of analytical quality.

4.2.1 Extraction and preparation of amadumbe and okra mucilage

The substrates for *in-vitro* fermentation were crude and purified amadumbe and okra mucilage, with inulin (commercial prebiotic) being the control. Amadumbe mucilage extraction was conducted according to the method of Manhivi *et al*. (2018) with modification. Okra mucilage was obtained in accordance with method by Ameena *et al*. (2010). Crude amadumbe and okra supernatants were precipitated with 95% ethanol in the ratio 1:3. The obtained amadumbe and okra precipitates were lyophilized for 60 h in a freeze drier at -60 °C (Christ freeze- drier, Niedersachsen, Germany) (Du Toit, De Wit and Hugo 2018). Purified amadumbe and okra mucilages were obtained by de-protonation of crude mucilage using Sevag solution and defatted using hexane and isopropanol (60:40, v/v) (Amid and Mirhosseini 2012a). The defatted and de-protonated okra and amadumbe powders were redissolved with de-ionized water and put into 10 kDa dialysis tubes. De-ionized water was used as buffer and was changed every 6 h for a duration of 36 h. Defatted, de-protonated and dialyzed okra and amadumbe mucilages were then further purified with 95% ethanol (1:2, v/v) for 30 min. The precipitate was then collected and washed twice with acetone at 4 °C. Okra and amadumbe mucilages were then lyophilized and milled into a powder.

4.2.2 Collection of human faecal inoculum for *in-vitro* fermentation

Faecal sample were taken from a healthy, non-smoker donor who had not received any antibiotic treatment prior to the experiment, with no history of irritable bowel disorder. Donor maintained a non-specific western diet which did not include any health or dietary supplements. The faecal sample (200 g), was collected from the donor (Medline Specimen Collection Kit, Medline, Inc., Rogers, MN, USA) and immediately deposited into a sterile bag (Whirl-Pak bag, Nasco, Fort Atkinson, WI), while preventing as much exposure to oxygen as possible. Within 30 min after collection, faecal samples were brought to the laboratory and processed. The faeces were mixed by hand whilst in the bag to allowed for uniform mixture (Agbenorhevi *et al*. 2020).

4.2.3 *In-vitro* fermentation using human faecal inoculum

Accurately, 0.2 g of each sample was weighted and placed into sterilized 310 ml serum bottles. The inoculum was prepared by diluting blended faeces with buffer solution (0.5 g feces/ ml inoculum) made of 474 ml/L distilled water, 237 ml/L of macro-mineral solution (6.2 g/L of KH_2PO_4, 0.583 g/L of $MgSO_4 \cdot 7H_2O$, 5.7 g/L of Na_2HPO_4, and 2.22 g/L of NaCl), 0.12 ml/L trace mineral solution (100 g/L of $MnCl_3 \cdot 4H_2O$, 10 g/L of $CoCl_2 \cdot 6H_2O$, 132 g/L of $CaCl_2$, and 80 g/L of $FeCl_3 \cdot 6H_2O$), 237 ml/L of *in -vitro* buffer solution (made of 35 g/L of $NaHCO_3$ and 4.0 g/L of NH_4HCO_3) and resazurin (blue dye, 0.1% wt/ vol solution; 1.22 ml/L) and mixed with reducing solution (47.5 ml of distilled water, 335 mg $Na_2S \cdot 9H_2O$, and 2 ml 1M NaOH). Bottles were flushed with carbon dioxide, sealed with recording modules, and placed in a 39 °C water bath (Zhu *et al*. 2017). The experiment was performed in triplicates. Inulin was used as positive control and a blank containing no carbon source was used as negative control. At turning points 0, 6, 12 and 24 h of the fermentation, aliquots were collected using 2 ml sterile tubes for microbiome, gas production and pH determination. An additional sample was collected at 0 h for baseline reference.

4.2.4 pH determination

After collection, at each time point 0, 6, 12, and 24 h of the fermentation, pH of faecal slurry was measured using a pH meter (Accumet Basic AP61; Fisher Scientific, Fair Lawn, NJ).

4.2.5 Determination of gas production (Ankom automatic gas production recording system)

Each fermentation vessel (glass bottle), was sealed tightly with an attached recording module.

The battery pack was connected to the corresponding component of the circuit board and locked. ANKOM GPM v11.4 software (Ankom Technology, Macedon NY, USA), was set up to monitor and record (every 5 min) the rate of gas production for a duration of 24 h, with the battery being changed every 6-8 h (Jang *et al*. 2019).

4.2.6 Extraction of DNA and bioinformatics processing

DNA extraction was performed in accordance with method of Omontese *et al*. (2022), with modification. Respective, individual samples were thawed on ice before being homogenized in a blender for 2 min. This was to ensure the mixing of the liquid and solid fractions. The mixture was then centrifuged at 10,000 g for 20 min (Thermo Sorvall ST16R Refrigerated). Proceeding centrifugation, the supernatant was removed and the pellet was then vortexed before being dissolved in four parts extraction buffer (10 mM ethylenediaminetetraacetic acid [EDTA], 100 mM Tris/HCl, 0.15 M NaCl pH 8.0). The eluates were then incubated for 1 h at 4 °C. This procedure was intended to optimize the release of particle-associated microbes. After the incubation period, the samples were centrifuged at 500 g for 15 min. The supernatant was transferred to a new tube and centrifuged for 25 min at 4 °C at 10,000 g. The supernatant was removed and the final pellet was used to extract DNA. Repeated bead-beating was utilized to extract DNA, which was then precipitated, eluted and purified using columns from the QIAamp® DNA PowerSoil Kit (Germantown, MD) following manufacturer's instructions. Following DNA integrity testing, high quality DNA from samples was used for bacterial community profiling utilizing the Illumina MiSeq sequencing platform at the University of Minnesota Genomic Center (UMGC). Raw reads were then trimmed to remove primers with cutadapt and filtered (fastx toolkit) to remove low quality reads (less than Q = 30). High-quality reads were selected for downstream analysis using the DADA2 plugin within qiime2 (Bolyen *et al*. 2019), which performs paired-end read merging, denoising and chimeric sequence removal to generate unique amplicon sequence variants (ASVs).

4.2.7 Determination of prebiotic index (PI)

To assess the selectivity of fermentation of substrates, the prebiotic index was utilized as a quantitative measure. This was done in accordance to Chamidah (2018), which measures the changes in favourable gut microbiota to those harmful microbes with the primary concentration calculated using Equation 4.1

Equation 4. 1 $$\left[\frac{(Bif\ {}^{a}/_{b})}{(Total\ {}^{a}/_{b})}\right] + \left[\frac{(Lac\ {}^{a}/_{b})}{(Total\ {}^{a}/_{b})}\right] - \left[\frac{(Bac\ {}^{a}/_{b})}{(Total\ {}^{a}/_{b})}\right] - \left[\frac{(Clos\ {}^{a}/_{b})}{(Total\ {}^{a}/_{b})}\right]$$

Where:

- *Bif: the number of Bifidobacteria (at sample time) / number at the time of inoculation.*
- *E. coli: the number of E. coli (at sample time) / number at the time of inoculation.*
- *Lac: the number of Lactobacilli (at sample time) / number at the time of inoculation.*
- *Clos: the number of Clostridia (at sample time) / number at the time of inoculation.*

4.2.8 Statistical analysis

The R statistical interface was used for all microbial community ecology analyses (Omontese *et al*. 2022). The R vegan package as per Oksanen *et al*. (2013) was used to calculate alpha diversity (Shannon, Observed, and Simpson distances) and beta diversity (Bray Curtis distances). The phyloseq package by McMurdie and Holmes (2013) was used to compute weighted and unweighted UniFRac distances. Graphs were created with the vegan, stats and ggplots R packages (Wickham and Chang 2008).

4.3 Results and discussion

4.3.1 pH determination

As seen in Figure 4.1 the pH of the blank sample remained constant, in comparison to the other samples. This is possibly due to the absence of a carbon source. Gut microbiota ferment and break down the carbon source using it for energy and as a result, produce secondary metabolites (SCFA and gases) (Rowland *et al*. 2018). Inulin, amadumbe and okra mucilages were seen to have decreased the pH of the fermentation medium, which is due to its break down via fermentation by gut microbiota (Liu *et al*. 2021a). In inulin the pH rapidly decreased from 7.01 to 6.64 within 6 h of fermentation. This was significantly different from mucilage samples ($p<0.05$). A notable difference in pH was observed in the fermentation of amadumbe and okra mucilage. The pH of crude okra mucilage dropped from 7.02 to 6.78 after 24 h fermentation, lower than that of crude amadumbe mucilage which decreased from 7.01 to 6.77 during 24 h fermentation, no significance was noted ($p = 0.7613$). From Figure 4.1, it can also be seen that crude mucilage samples, both amadumbe and okra, decreased the pH of the fermentation medium slight lower than that of purified mucilage samples. The pH of purified okra mucilage

decreased from 7.01 to 6.77 which was lower than in comparison to purified amadumbe mucilage which decreased from 7.02 to 6.80 during 24 h fermentation. During the fermentation of polysaccharides, an indication of short chain fatty acid production was observed by a decrease in pH of the fermentation medium (Fernández *et al*. 2016). Hence, the fermentation of mucilage and inulin (commercial prebiotic sample) was compared. Inulin was observed to have decreased the pH of the fermentation medium significantly, probably due to its structure and composition which enable it to be easily and faster fermented (Slavin and Feirtag 2011). Amadumbe and okra mucilage are high molecular weight polysaccharides. High molecular weight polysaccharides are reported as slow fermenting (Goñi, Martín and Saura-Calixto 2005). At the end of fermentation, okra mucilage was seen to have a lower pH than amadumbe mucilage, possibly due to okra mucilage being an acidic hetero-polysaccharides (Olawuyi *et al*. 2020) in comparison to amadumbe mucilage a neutral polysaccharide (Andrade *et al*. 2020).

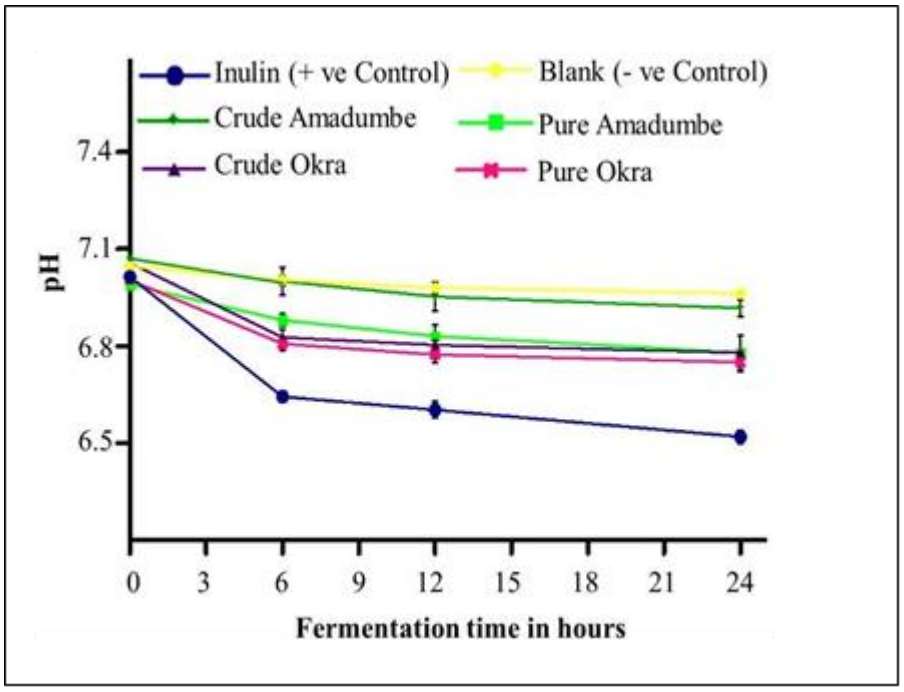

Figure 4. 1 Describes the shift in pH during *in-vitro* fermentation of amadumbe and okra mucilage. Error bars represent standard deviations from biological replicates.

4.3.2 Gas production during *in-vitro* fermentation

In the present study, gas accumulation rapidly reached one-fourth of the maximum accumulation in 2 h (Table 4.1) and parameter L was very close to 0, which resulted in the model failing to converge. Therefore, L(h) data were removed from the final model. The

constants b (h^{-1}) and c ($h^{-1/2}$) determine the fractional rate of degradation of the substrate μ (h^{-1}), which is postulated to vary with time as follows: $\mu = b+c/(2\sqrt{t})$, if $t \geq L$, Kinetics parameters (Gf, t=T/2, and μ at T/2) were compared in the statistical analysis, with T/2 representing the time to half- asymptote when G=Gf/2. The kinetics of gas production parameters were modeled using PROC NLIN of SAS 9.4 (SAS Inc., Cary, NC) and fitted gas production kinetic parameters were analyzed using PROC GLM of SAS 9.4 (SAS Inc., Cary, NC).

Table 4. 1 Fitted kinetics parameters of gas accumulation during 24 h fermentation

		Crude		Pure		
	Inulin	**Amadumbe**	**Okra**	**Amadumbe**	**Okra**	**P-value**
G_f	245.67^{a}	155.10^{b}	120.36^{b}	179.77^{ab}	110.82^{b}	<0.001
T/2	6.55^{a}	2.97^{b}	5.88^{ab}	3.99^{ab}	6.79^{a}	0.02
b	0.24	0.32	0.20	0.22	0.24	0.14
c	-0.33	-0.14	-0.18	-0.08	-0.34	0.03
μ	0.18^{ab}	0.28^{a}	0.16^{b}	0.19^{ab}	0.17^{ab}	0.04

Note: Gas accumulation curves recorded during the 24 h of fermentation were modeled according to France *et al*. (1993): G (mL/g dry matter) =0, if $0<t<L$; G (ml/g dry matter) = Gf (1-exp (-[b(t-L) +c ($\sqrt{t}-\sqrt{L}$)])), if $t \geq L$; where G denotes the gas accumulation at a specific time (t), Gf (ml/g dry matter)was the maximum gas volume for $t = \infty$, and L (h) represents the lag time before the fermentation began. Values with different subscript letters are significantly different ($p<0.05$).

Figure 4.2, shows the gas production curves for inulin, amadumbe and okra mucilage. The curves of amadumbe and okra mucilage excluding inulin had general comparable form. Inulin produced more gas at a maximum rate (Gf) and took less time to reach half of the gas accumulation as seen in Table 4.1. Inulin exhibited a lag face, followed by a rapid increase in gas production. There was no significant difference in gas production between crude mucilage substrates and purified mucilage substrates. Gas production in amadumbe was slightly higher compared to okra mucilage. At the end of 24 h fermentation, inulin had maximum gas production of 233.19 ml, followed by amadumbe mucilage 158.98 ml and okra mucilage 113.98 ml. As by-products of their metabolic processes, bacteria generate gases. Gut microbes mostly obtain energy they need through the fermentation of non-digested carbohydrates, which results in the generation of SCFAs and a few gas such as methane (CH_4), carbon dioxide (CO_2) and hydrogen (H_2) (Hopper *et al*. 2020). Factors influencing gas generation include gut microbiota

composition and the structure and composition of the fermentation substrates (Jensen and Jørgensen 1994). Structurally, most inulin has been reported to have DPs which range from 10 to 60 monomers (Ritsema and Smeekens 2003). Amadumbe and okra mucilage when compared to inulin are long chain compounds with DPs > 60 monomers (BeMiller 2018). This particular structural characteristic makes amadumbe and okra mucilage possibly less susceptible to enzymes in comparison to inulin which is most susceptible to enzymes and is easily fermented. This possibly shows why inulin generated the greatest amount of gas. Its rate of fermentation is much quicker than in comparison to long chained polysaccharides. Most polysaccharides such as amadumbe and okra mucilage which are reported as high molecular weight compounds are suggested to be slowly fermented by gut microbiota and hence have lower gas production rates (Li *et al*. 2022).

The low gas generation shown by okra mucilage could be attributed to its complex structure. As previously reported type I partly methylated and/or acetylated rhamnogalacturonnans, which have relatively short galactosyl-residue side branches, are the main polysaccharides of okra mucilage (Sengkhamparn *et al*. 2009). This particular characteristic of methylation possibly results in slower fermentation and less gas production rates (Tian *et al*. 2016). It is interesting to note that the pH decreases did not entirely coincide with the increases in gas production, indicating that differences between mucilage substrates and the commercial prebiotic may not only result from variations in how well they are utilized, but also from the involvement of various microorganisms or catabolic pathways.

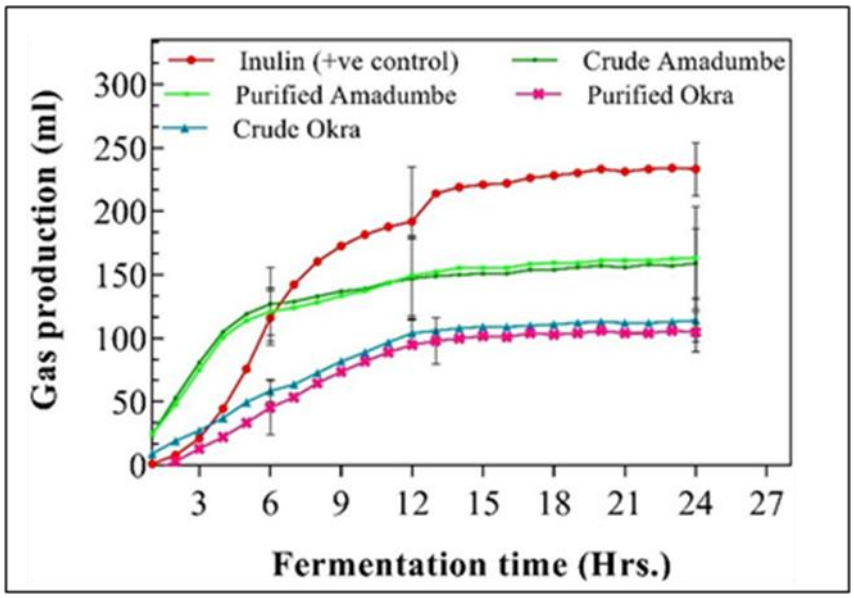

Figure 4. 2 Gas generation by gut microbiota in relation to *in-vitro* fermentation with amadumbe and okra mucilage. Error bars represent standard deviations from biological replicates.

4.3.4 Effect of mucilage and inulin on gut microbiota composition and diversity

4.3.4.1 Determining β– diversity

The principal component analysis (PCOA) and cluster analysis at the operational taxonomic unit (OTUs) level were employed to show β-diversity and assess variations in species complexity from various treatments. In contrast to Unifrac distances, which include branch length when looking for differences between sample groups, Bray-Curtis distances are a beta-diversity metric that separates two or more samples based on abundance (Chamidah 2018). In contrast to Unifrac distances, which include branch length when looking for differences between sample groups, the error ellipses can be used to illustrate a 2D confidence interval and represent an iso- contour of the Gaussian distribution (Vrese and Schrezenmeir 2008).

For a collection of 2D normally distributed data samples, each ellipse displays an ellipse with a 95% confidence level. The area covered by this confidence ellipse represents where 95% of the samples that can be taken from the underlying Gaussian distribution fall (J Hall *et al*. 2011). To ascertain the quality of the tests, features are randomly selected within the feature table and those features are plotted using the distance matrix and shows how dissimilar the randomly selected features relative to the features plotted. This gives a non-bias conclusion of the integrity of the analyzed beta diversity (Schmitz *et al*. 2013). The results of the PCOA in

Figure 4.3, illustrates that at 0 h, there was a statistical separation among amadumbe and okra mucilage features in relation to inulin (control). Crude and purified amadumbe mucilage showed a core closely related diversity and are closely related and clustered mostly inside the eclipse, thus fermentation influence bacterial communities change and there were displacements in the diversity shifts in the fermented samples compared to the control or blank. The same trend was also noted in both the crude and purified okra mucilage. At 6 h, similar features were observed in samples for crude and purified amadumbe mucilage relative to the control, indicating similarity in beta-diversity. At the end of 24 h fermentation, sample groups may have been the most dissimilar than in the other time groups, because randomly selected features from crude and purified amadumbe samples at 24 h are outside the 95% CI ellipse and only appear as outliers.

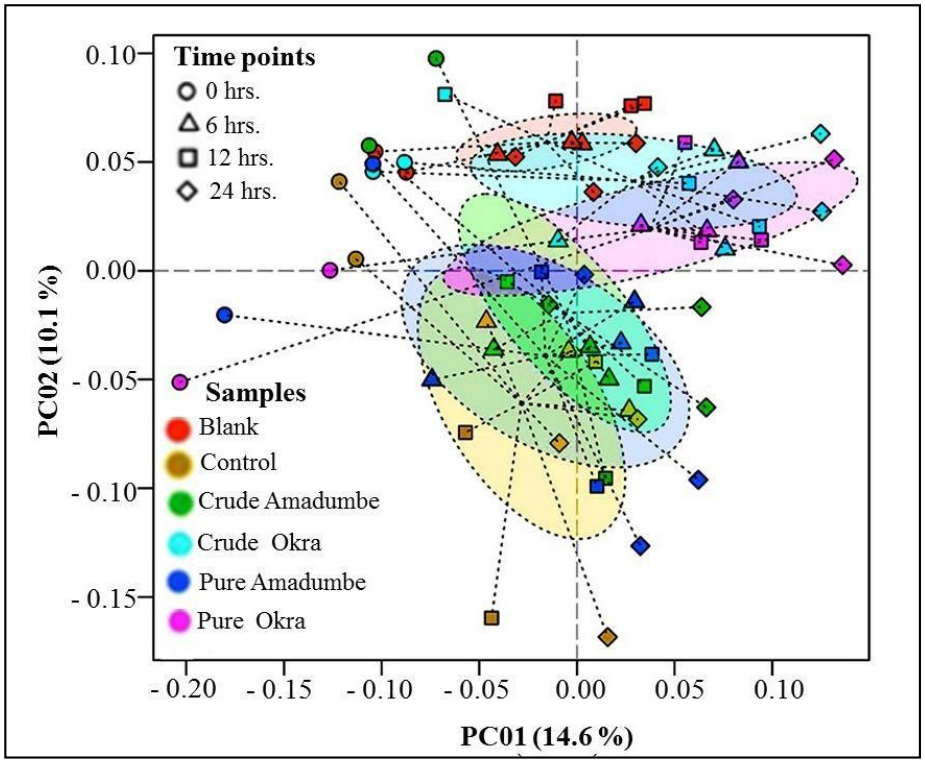

Figure 4. 3 Beta-diversity illustration using the Bray Curtis dissimilarity matrix which is based on abundance of features. The ordination shows the 95% CI where conclusions may or may not be made on closely or distant features in the data.

4.3.4.2 Determining alpha-diversity

To assess gut community diversity and richness, the α-diversity indices Shannon and Simpsons were calculated. These finds are shown in Figures 4.4-4.6. Simpsons index, which calculates

the likelihood that two randomly chosen individuals in a given sample will belong to the same species, is a weighted arithmetic means of proportionate abundance (Roswell, Dushoff and Winfree 2021). The value D has small values in data sets with great diversity and big values in data sets with low diversity, this is because the mean of the proportional abundance of the species increases with the decreasing species numbers and rising abundance of the most abundant species (Willis 2019). In Figure 4.4, the highest richness for the respective sample groups at different treatment times were observed at 0 h. Relative to this, overall richness can be concluded after 24 h fermentation time (Figure 4.4). Five of the six of the alpha diversity plots showed that at 0 h, greatest richness and evenness were observed among mucilage substrates and inulin. Throughout fermentation both crude and purified okra mucilage may be correlated to higher alpha diversity in comparison to crude and purified amadumbe mucilage. After 24 h fermentation mucilage substrates were observed to decrease in richness and evenness in someof the metrics when compared to the starting alpha diversity at 0 h. But in comparison to inulin (control) (Figure 4.4 c), crude okra mucilage may have higher richness and evenness. Amadumbe and okra mucilage were observed to have higher Simpson index (Figure 4.5) at the end of 24 h of fermentation in comparison to inulin (control) indicating greater relative abundance in species (Figure 4.5 c).

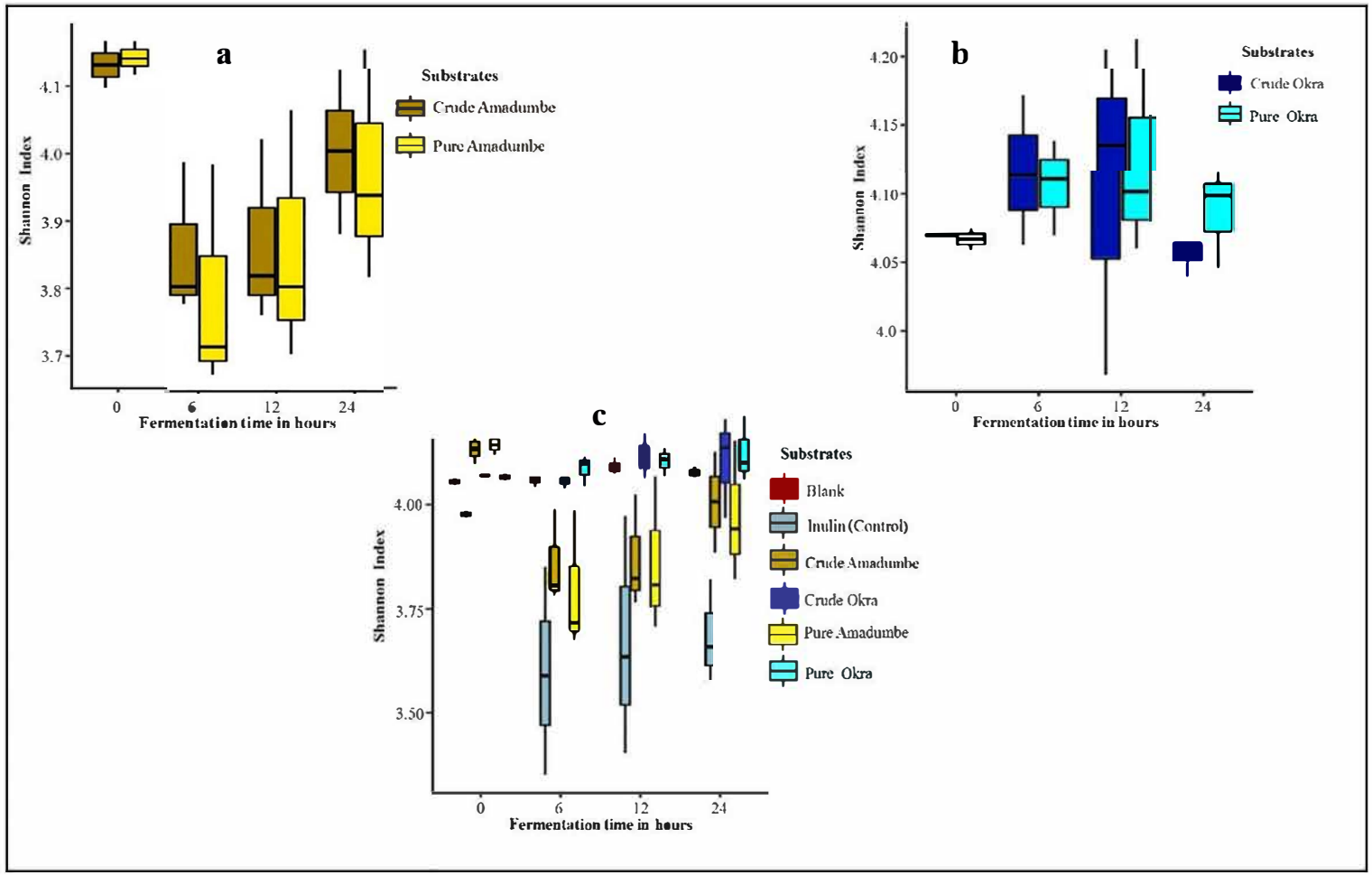

Figure 4. 4 The richness and evenness was measured for each treatment group. Using common alpha diversity metric, we can ascertain points in time where richness and abundance where at the maximum and overall trend of the reads curve (a) Shannon index of amadumbe mucilage (b) okra mucilage, (c) total samples

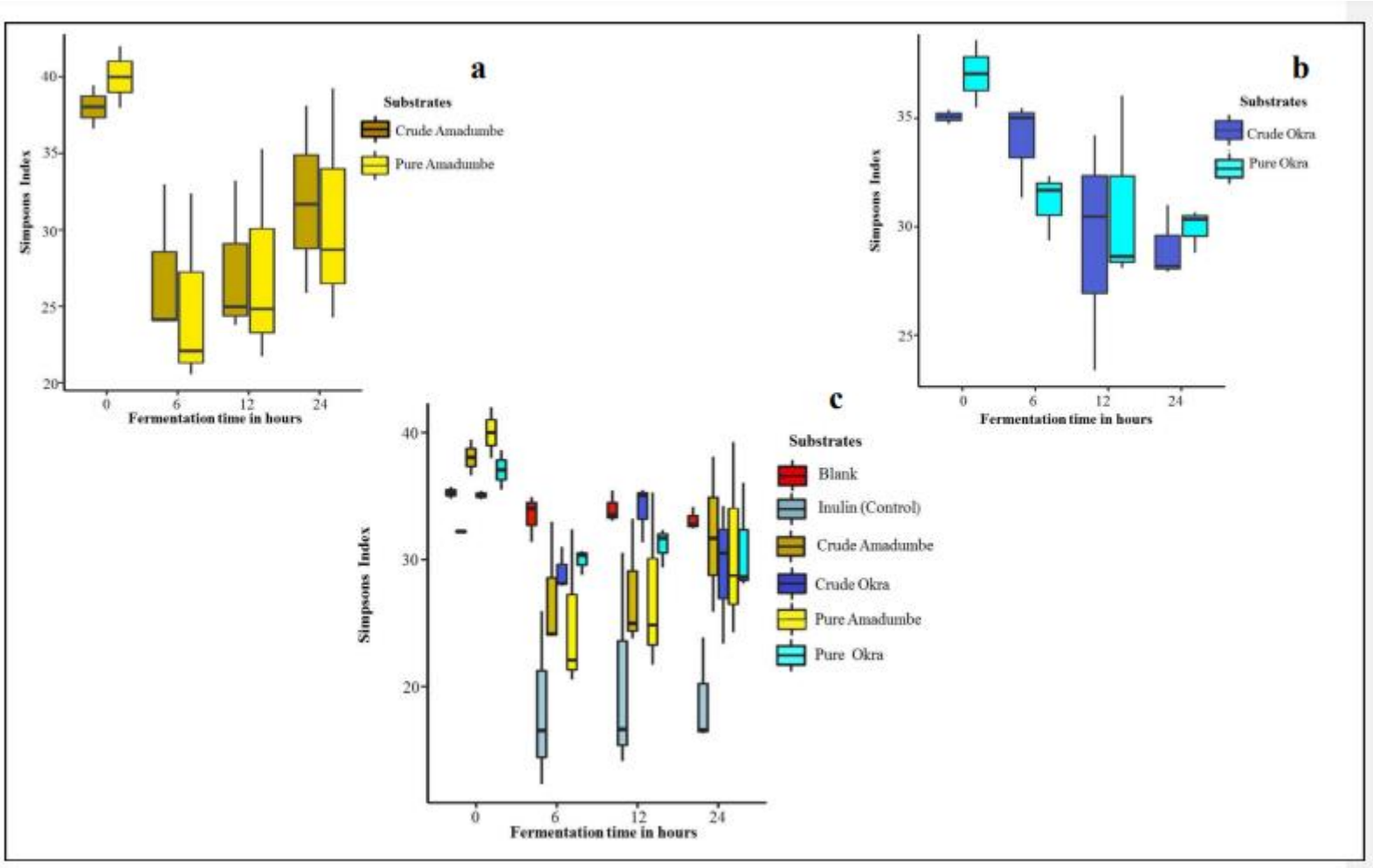

Figure 4. 5 The richness and evenness was measured for each treatment group. Using common alpha diversity metric, we can ascertain points in time where richness and abundance where at the maximum and overall trend of the reads curve. Simpson index of (a) amadumbe mucilage, (b) okra mucilage and (c) total samples

Based on strictly counts of certain features (OTU's or ASV's) within the rarefied table used for downstream analysis, Figure 4.6 is the plot of alpha diversity that negates different co- founding variables that tend to retard the quality of any analysis in the long run. For a procedure that can be said to be similar to a screening stage of an *in situ* study, the counts as a parameter for alpha diversities gives a rough idea about the amount of different features one has in an individual sample (Tuomisto 2010). Usually not just sample richness but evenness is attributed to many biological processes especially those that are of industrial importance (Ju *et al*. 2014). Simpson and Shannon give a well indication of sample richness and evenness but for strictly evenness more downstream analysis of food samples like the inclusion of phylogenetic richness which is well observed by faiths phylogenetic relationship metric for alpha diversity (Bruford *et al*. 2017).

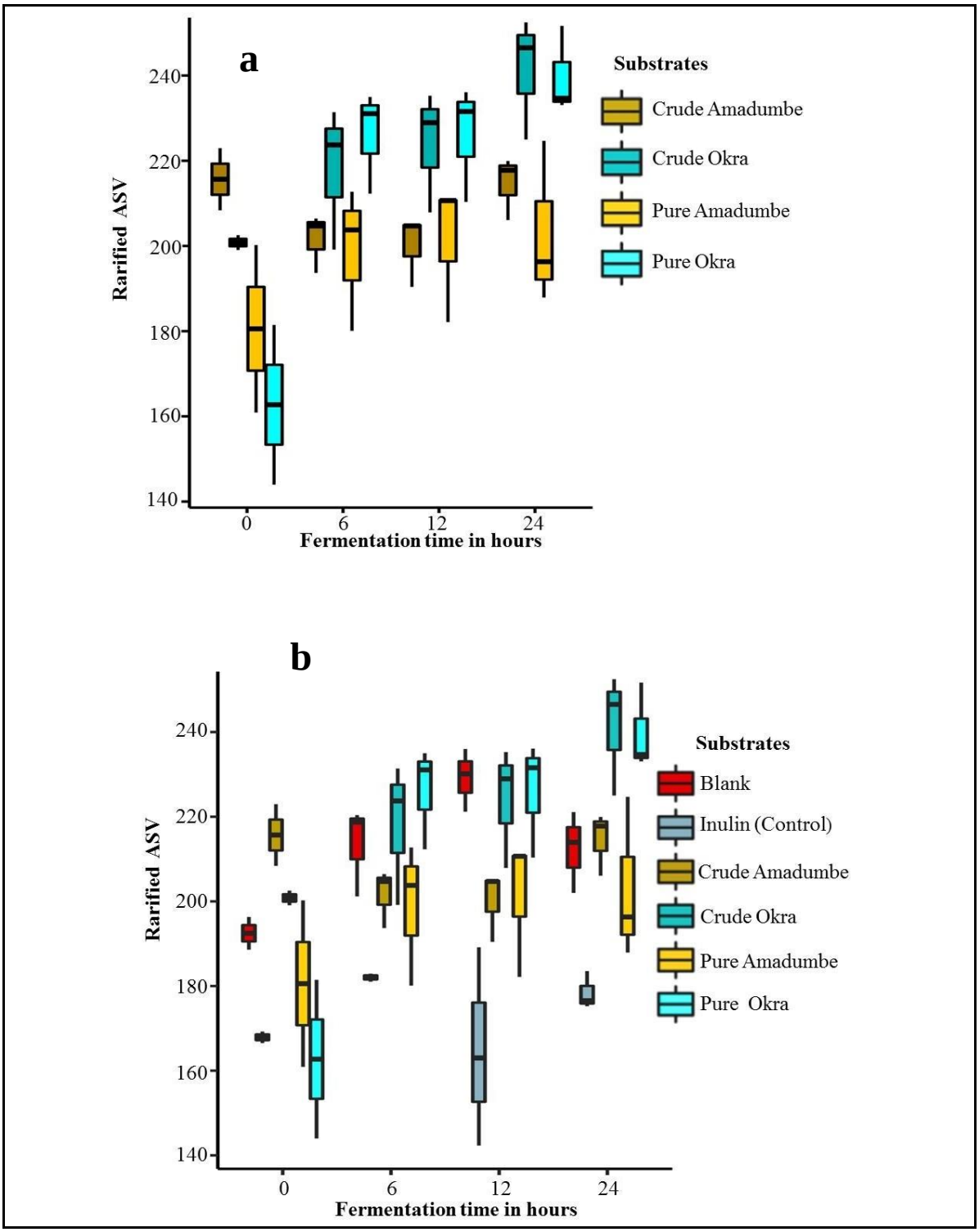

Figure 4. 6 Alpha rarefication of sample treatment groups that is pure based on ASV counts and can also be OTU counts. The maximum counts seem to be just over 240 reads and the median allows us to approximate average sample richness per sample group

4.3.5 Relative abundance in gut microbial composition and diversity

Investigation of gut microbiota composition of mucilage samples and inulin at the phylum level are displayed in Figure 4.7. Similar to a previous publication (Zhou *et al*. 2018), the gut microbiota was primarily composed of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, with the exception of Verrucomicrobia. The fermentation of amadumbe and okra mucilage was found to increase the relative abundances of Bacteriodetes and Proteobacteria and decreased the relative abundance of Firmicutes in comparison to inulin (control). Therefore, the ratio of Firmicutes to Bacteroidetes (F/B) was decreased. The ratio of Firmicutes to Bacteroidetes (F/B) has been linked to maintaining of homeostasis and variations in this ratio can result in a number of diseases (Stojanov, Berlec and Štrukelj 2020). Changes in F/B ratios were discovered to be a very important risk factor for obesity and irritable bowel syndrome (IBD) (Manichanh *et al*. 2006). Therefore, it is important that balance be maintained between these phyla. From Figure 4.7, it was also observed that crude amadumbe mucilage elevated Bacteriodetes 8.62% in comparison to crude okra mucilage 6.28%. Crude amadumbe and purified amadumbe mucilage were found to decreased Firmicutes by 11.87% and 16.55% respectively. There is emerging evidence linking the lowering of the F/B ratio and the preservation of intestinal barrier integrity (Manichanh *et al*. 2006; Ke *et al*. 2021).

Therefore, amadumbe mucilage has potential to be utilized as a functional food or as a nutraceutical for risk reduction in obesity and metabolic syndrome. Crude and purified amadumbe mucilage was also shown to elevate the presence of Actinobacteria by 6.12% and 6.84%, in comparison to crude and purified okra mucilage which decreased by 2.16% and 2.51%. This observation reiterates findings from previous studies, stating that gut microbiota are selective fermenters (Li *et al*. 2015). Structurally, amadumbe mucilage has been found to be associated with high levels of protein (Manhivi *et al*. 2018), which could have possibly stimulated the enrichment of Actinobacteria (Liu *et al*. 2014). The abundance of Proteobacteria was seen to be higher in okra mucilage than in inulin (control) and amadumbe mucilage.

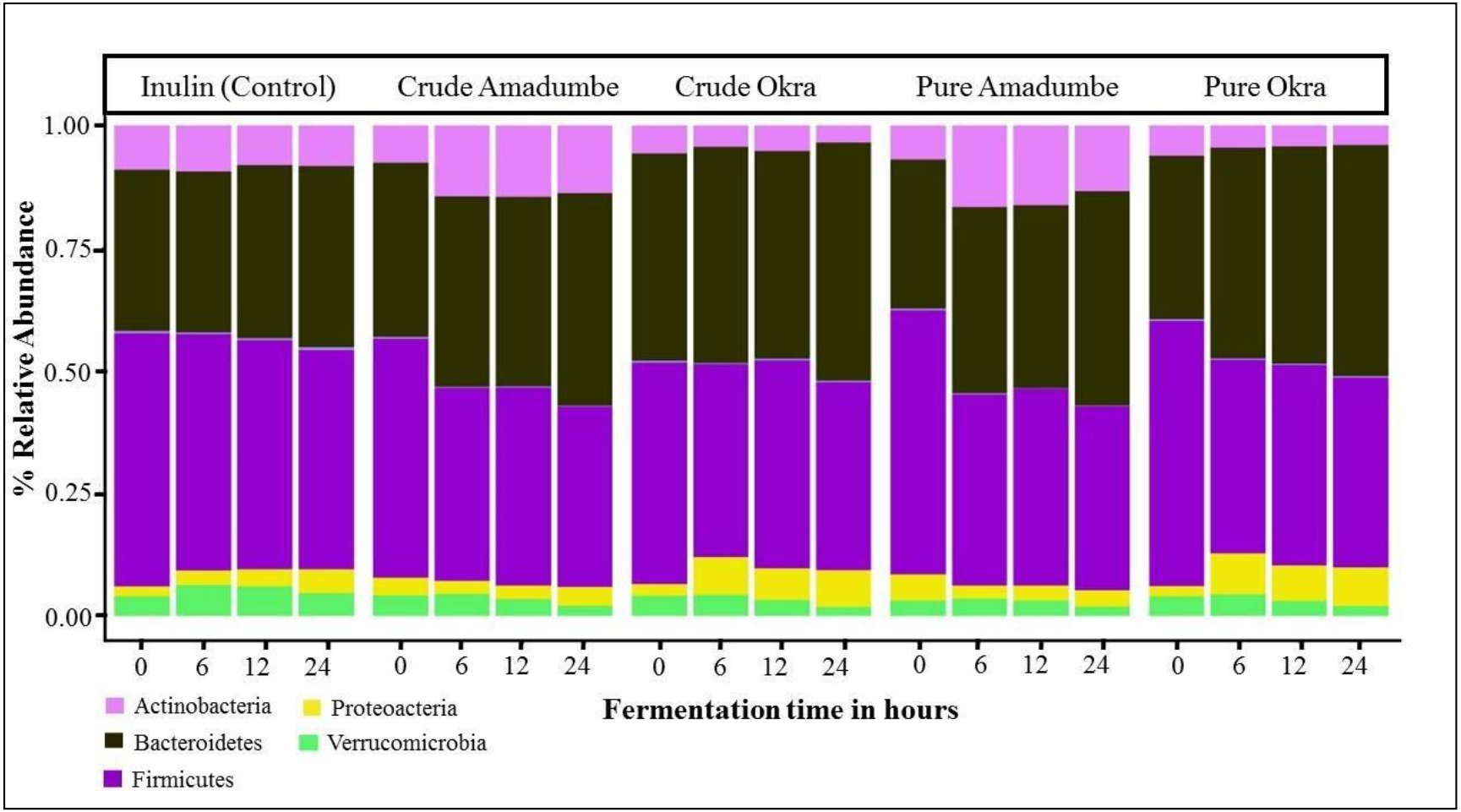

Figure 4. 7 Taxonomy bar plot for the relative abundance at phylum level for mucilage samples and inulin.

As seen in Figure 4.8, the initial gut composition was composed mainly of *Bacteriodaceae Bacteroides* (species unclassified) (9.71%), *Bacteroides Uniformis* (3.88%), *Faecalibacterium prausndzii* (3.88%), *Akkermansia muciniphilla* (4.86%) and *Alcaligenaceae Sutterella* (4.86%), some of which have previously been linked to gut dysbiosis and disease (Whittaker 2010; Hwang *et al*. 2017). The addition and fermentation of amadumbe and okra mucilage was shown to elevate beneficial gut microbial species of the initial microbiome. In comparison with inulin which is an establish prebiotic, amadumbe and okra mucilage showed potentially better stimulation of beneficial species.

After 24 h fermentation, amadumbe mucilage was found to have increased species *Bifidobacterium adolescentis* (7.69%), *Bacteroidaceae bacteroides* (1.58%), *Bacteroides uniformis* (6.7%) and *Porphyromonadaceae parabacteroides* (1.92%). Many of these species have been reported to be beneficial to health, for instance, *B. adolescentis* contributes to immunomodulatory activity (Jung *et al*. 2019) and *B. uniformis* has been reported to promotes the synthesis of thymic stromal lymphopoietin (TSLP), a cytokine identified to be regulated by intestinal bacteria and necessary for T-regulatory cells (Treg) development (Mosconi *et al*. 2013). Okra mucilage was found to promote the growth of species *Bacteroidaceae bacteroidetes* (14.43%), *Bacteroides ovatus* (4.81%) and *Bacteroides uniformis* (11.54%). Studies have previously reported on the ability of *Bacteroides ovatus* to induce systemic antibody response to inflammatory bowel disease (Saitoh *et al*. 2002).

Akkermansia muciniphila, *Bacteriodaetes* (unclassified at species level), *Bifidobacterium adolescentis* and *Bacteriodes ovatus* were the only species with a noticeable abundance change throughout the treatment time. In addition to stimulating the growth of beneficial bacteria, it was also observed that certain pathogentic species were capable of fermenting okra mucilage such as *Alistipes putredinis* (5.03%), which is reported in patients most likely suffering from appendicitis, abdominal and rectal abscess (Parker *et al.* 2020). Okra mucilage was also found to stimulate *Sutterella* (4.87%), which are frequently reported associated with inflammatory bowel disease (IBD), however, the interactions of these species with host still remains unclear despite their prevalence (Hiippala *et al.* 2016). The taxa bar plot can be some what of a gamma-diversity plot that includes sample metadata and abundance data before results are represented (Whittaker 2010). Noticeably the sequencing strategy may have been designed to elucidate more than prokaryotes in a specifically sample set, but downstream dependent on the interest can be used for much more. In the case of specifically focusing on what would be the output of 16S NGS output the bacterial abundance, phylogenetic relationship and richness/evenness, the

output of the taxa bar plot on Figure 4.8, is completely relative to other groups identified in the conversion of what would be qiime2's rep seqs folder. What is notable is that the final value is relative to OTU's that are associated with Archea and bacteria instead of fungal and viruses (which would best be shown by a shotgun sequence strategy or ITS based plan (Regalado *et al*. 2020). The mucilage groups and inulin shown in the figures for alpha diversity are strictly based on identified OTU or ASV groups and abundance in- terms of count.

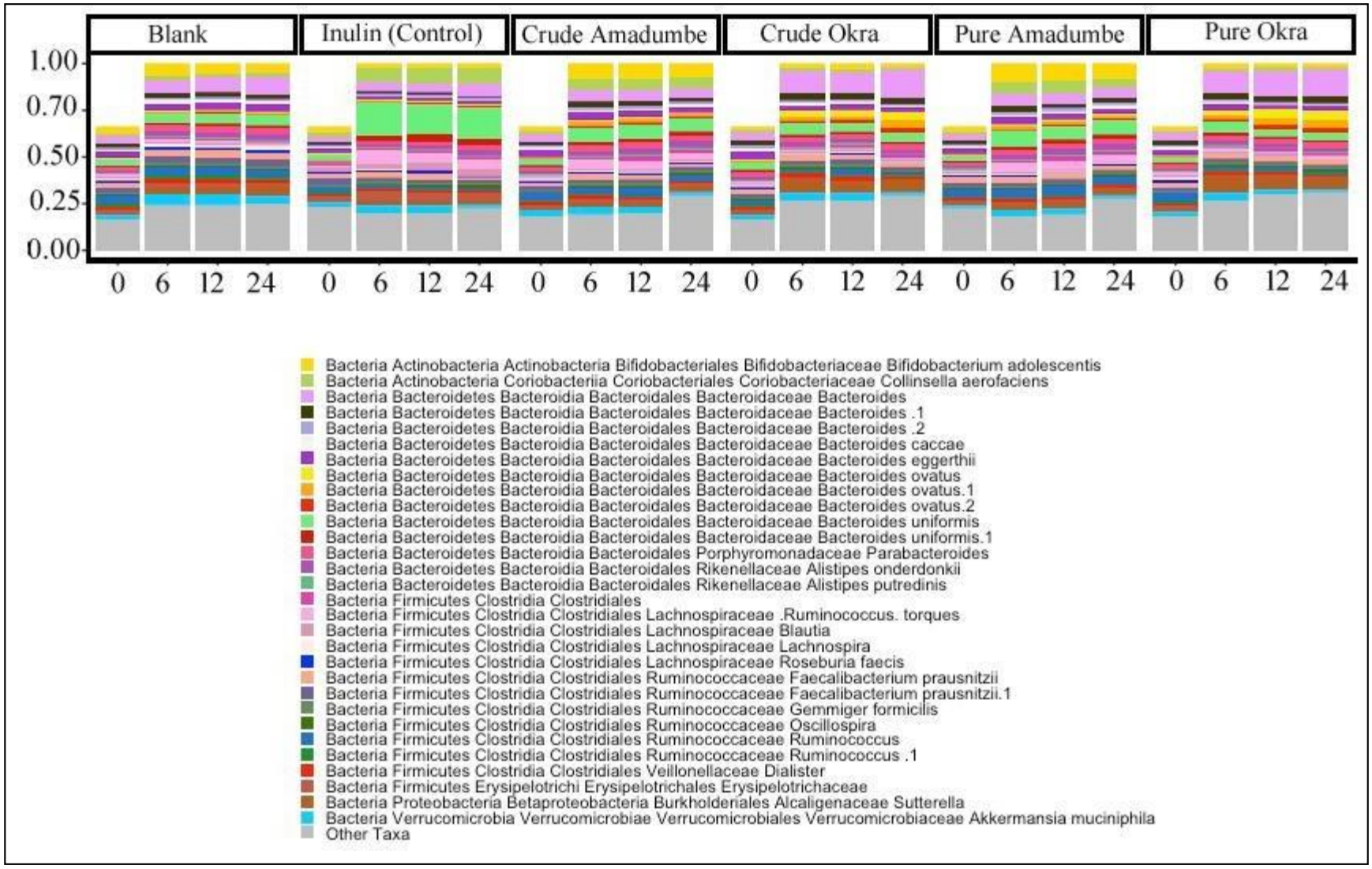

Figure 4. 8 Taxonomy bar plot for the relative abundance at genus level for different time treatment groups.

4.3.6 Prebiotic index of mucilage samples

The link between changes in beneficial gut microbiota and undesirable gut microbiota as related with their beginning levels is described by the prebiotic index (Chamidah, 2018). According to the Palframan equation, growth in *Bifidobacteria* or *Lactobacilli* numbers is considered to have a favorable effect, whilst an increase in *Bacteroides* and *Clostridia* (sub grouphistolyticum) numbers is considered to have a negative impact (Chamidah 2018). Table 4.2 displays the relative abundances for the specific samples during 0 and 24 h, after each treatment. From Table 4.2, it can be seen that crude and purified amadumbe stimulated an increase in *Bifidobacterium*. Crude and purified okra were observed to have decreased in *Bifidobacterium* between time 0 and 24 h. The same trend was observed for inulin. This implies that *Bifidobacterium* was capable of breaking down and utilizing amadumbe mucilage to a greater extent than other samples. Most gut microbiota are known as selective fermenters (capable of breaking specific carbohydrates), however, previous studies have demonstrated the ability of *Bifidobacterium* to ferment a wide variety of carbohydrates (Vrese and Schrezenmeir 2008). Recent studies have also reported on the vast degrading enzymes present in *Bifidobacterium* species that enable it to ferment complex carbohydrates (Fujita, Sasaki and Kitahara 2019). Crude and purified amadumbe was also seen to favor the growth of *Bacteriodes*, this was also observed for crude and purified okra mucilage. At the end of fermentation, amadumbe and okra mucilage showed similar reduction in *Clostrium* compared to the control (inulin).

Table 4. 2 Relative abundances for the specific samples during 0 and 24 h after each treatment.

Bacterial	Substrate	Relative abundance of taxonomy 0 h	24 h
Bifidobacterium	Inulin	2.80 ± 1.04	1.25 ± 0.21
	Crude Amadumbe	2.55 ± 0.10	4.20 ± 0.95
	Pure Amadumbe	2.66 ± 0.12	5.08 ± 1.08
	Crude Okra	1.92 ± 0.13	1.36 ± 0.31
	Pure Okra	2.36 ± 0.08	1.22 ± 0.09
Bacteriodes	Inulin	1.27 ± 1.28	1.25 ± 0.02
	Crude Amadumbe	1.78 ± 0.03	4.20 ± 0.01
	Pure Amadumbe	1.56 ± 0.33	5.08 ± 0.001
	Crude Okra	2.13 ± 0.11	2.34 ± 0.13
	Pure Okra	1.70 ± 0.52	2.70 ± 0.11
Lactobacillus	Inulin	0.010 ± 0.01	0.22 ± 0.30
	Crude Amadumbe	0.03 ± 0.04	0.03 ± 0.0
	Pure Amadumbe	0 ± 0	0.01 ± 0.0
	Crude Okra	0.004 ± 0.01	0.04 ± 0.04
	Pure Okra	0.005 ± 0.07	0.06 ± 0.01
Clostridium	Inulin	0.20 ± 0.04	0.13 ± 0.02
	Crude Amadumbe	0.15 ± 0.15	0.11 ± 0.09
	Pure Amadumbe	0.17 ± 0.03	0.13 ± 0.03
	Crude Okra	0.15 ± 0.01	0.14 ± 0.02
	Pure Okra	0.18 ± 0.04	0.12 ± 0.01

Means ± SD; n=3.

The index value of mucilage prebiotic effect is presented in Table 4.3. Quantitative calculation was used to facilitate prebiotic fermentative analysis. The PI values of crude amadumbe (2.17) and purified amadumbe mucilage (3.07) illustrates that amadumbe mucilage has potential as a prebiotic, in comparison to inulin (1.40) and okra mucilage. Although okra mucilage was shown to have increased the evenness and richness in gut microbial diversity (as shown in Figure 4.4), it did not stimulate the growth of *Bifidobacteria* or *Lactobacilli*. This is probably attributed to selective colonic fermentation characteristic which causes actual alteration in specific microbiota that are able to breakdown and utilize okra. Purified mucilage samples

were found to have better prebiotic potential in comparison with crude mucilage substrate. Crude polysaccharides derived from plants and other natural sources are reported to contain various impurities such as proteins, lipids, pigments and low molecular weight molecules. Purification lead to a reduction and elimination of such impurities resulting in polysaccharide fraction only (Huang *et al*. 2022). The high prebiotic potential shown by amadumbe mucilage can also be attributed to its chemical composition. In comparison to okra mucilage, amadumbe has a higher resistant starch content and β-glucan content. Both of which have been reported to encourage proliferation of *Bifidobacteria* (Zhao and Cheung 2011; Tiwari, Singh and Jha 2019).

Table 4. 3 The prebiotic index and prebiotic scores for each of the dietary substrates at the end of fermentation (24 h).

Substrate	Prebiotic Index (PI)
Inulin (Control)	1.40 ± 2.30^{c}
Crude amadumbe mucilage	2.17 ± 1.03^{b}
Crude okra mucilage	$- 1.57 \pm 0.80^{d}$
Purified amadumbe mucilage	3.07 ± 1.06^{a}
Purified okra mucilage	$- 1.55 \pm 0.46^{d}$

Means ± SD; n = 3; Values with different subscript letters are significantly different ($p<0.05$).

4.4 Conclusion

The study investigated to what extent gut bacteria ferment amadumbe and okra, and if consumption of amadumbe and okra has a potential prebiotic effect in stimulating beneficial gut bacteria. The fermentation of amadumbe and okra mucilage were found to be slow and progressive when compare to inulin (rapidly fermented). Amadumbe and okra mucilage were shown to have contributed to positive compositional changes to the initial gut microbiome. Overall, okra mucilage showed highest richness and evenness in stimulating gut microbiota diversity in comparison to inulin. Moreover, amadumbe and okra mucilage were able to be broken down and fermented by gut microbiota, thus contributing to the reduction of pH. The relative abundance in gut microbiota, showed amadumbe and okra mucilage as substrates that are fermented by selective gut bacteria. Amadumbe mucilage showed better prebiotic potential in comparison with okra mucilage. Data illustrated the ability of both amadumbe and okra

mucilage to be explored as an emerging prebiotic with potential to compete with other commercially establish prebiotics.

CHAPTER FIVE

General discussion, conclusion and future recommendations

5.1 General discussion

The aim of this research was to investigate the composition, functional properties and the presumed prebiotic potential of mucilage derived from Southern African amadumbe and okra varieties. The first part of this dissertation discussed the relationship between composition and functional properties of amadumbe and okra mucilage (Chapter 3). The second aspect focused on the *in-vitro* fermentation of amadumbe and okra mucilage and its potential prebiotic effect (Chapter 4).

In the present study, purification processes were found to reduced impurities resulting in a higher polysaccharide fraction. Both amadumbe and okra mucilage were found to be heterogeneous in nature. The polysaccharide fraction was found to be associated with proteins. The chemical composition of amadumbe mucilage showed it to be high in protein, carbohydrates, moisture and low in fat. Okra mucilage was found to have a lower protein and carbohydrate content but a higher ash and fat content when compared with amadumbe mucilage. The amino acid profile of amadumbe mucilage was comparable to that of its flour and consisted of both hydrophobic and hydrophilic amino acids (Chapter 3). This potentially makes amadumbe suitable for applications such as dough formulations, cookies and in bread making. Further compositional analysis also showed amadumbe to be high in β-glucan, starch and resistant starch enabling it to serve as a potential functional ingredient in gluten-free product formulations. Since amadumbe mucilage was found to be composed of both hydrophilic (polysaccharide and protein) and hydrophobic (protein) components, this compositional characteristic could enable it to serve as a potential emulsifier (Chapter 3).

The majority of hydrocolloids currently used in the food industry are primarily viscosity modifiers. Amadumbe mucilage has good water holding capacity, is highly soluble, but has limited swelling capacity. This demonstrates that it cannot gel. In the food industry, to stabilize flavoring oils in beverages and stop them from coalescing, a hydrocolloid with low viscosity and emulsifying capabilities is required. Therefore, amadumbe mucilage may serve as a potential hydrocolloid in these situations. Oil holding capacity is a predominant functionality required in the food industry, the high oil holding capacity in crude amadumbe mucilage is

most likely due to the presence of proteins and non-starchy carbohydrates (polysaccharide). Amadumbe mucilage also has a high level of starch, which can be advantageous as it contributes to its oil holding capacity of the mucilage as noted in this study and elsewhere (Tosif *et al.* 2021). This potentially enables amadumbe to be utilized as a fat replace in the formulation of low fat meat and dairy products as well as in improving their sensorial quality. Composition of mucilage was proven to influence its functional properties. The functional properties of amadumbe and okra mucilage suggest it can be employed as functional food ingredients with the potential to compete with other commercial hydrocolloids.

Diet has a significant impact on the gut microbiota and is a key factor in maintaining human health (Greenhalgh *et al*. 2016). It has been shown, that Western diets can disrupt the gut microbiota's balance, leading to dysbiosis, which has been associated with various metabolic illnesses (Martinez, Leone and Chang 2017). Therefore, incorporating traditional, wholesome foods into diets could change the trajectory of chronic diseases. In addition to its techno-functional properties in potential food applications, mucilage derived from amadumbe and okra are suggested to have numerous health benefits (Kundu *et al*. 2012; Wahyuningsih *et al*. 2018). This study went on to further investigate the presumed prebiotic potential of amadumbe and okra mucilage.

In-vitro fermentation profiles of amadumbe and okra mucilage were found to differ in terms of pH, gas production and gut microbial composition. This can be potentially attributed to their chemical composition and functional properties. Results showed that both mucilage samples decreased the pH of fermentation medium suggesting the production of short chain fatty acids. When compared to amadumbe mucilage, okra mucilage was found to have a lower pH. This finding may be explained by its monosaccharide composition. Amadumbe mucilage is composed mainly of glucose, mannose, arabinose and xylose and okra mucilage composed mainly of galactose, rhamnose, arabinose, ribose and glucose (Olawuyi *et al*. 2020). Changes in microbial composition showed, crude and purified amadumbe mucilage to have closely related diversities at the start of fermentation, the same was noted for crude and purified okra mucilage. At the end of 24 h fermentation, there was visible dissimilarities in beta-diversity between crude and purified amadumbe and okra mucilage. Alpha diversity measures the diversity of a particular ecosystem, by expressing the species richness and evenness (Fedor and Zvaríková 2019). Throughout 24 h fermentation, crude amadumbe and okra mucilage were found to be associated with higher alpha diversity than compared with purified amadumbe and okra mucilage. This suggested that crude mucilage stimulated richer diversity. Okra mucilage

both crude and purified were observed to encourage greater richness and evenness among species, according to Shannon index when compared with inulin. Crude and purified amadumbe mucilages were found to have higher Simpson index and showed greater relative abundance in species when compared with inulin. Relative abundance at phylum level showed amadumbe mucilages to have increased growth of beneficial phyla such as Actinobacteria and Bacteriodetes and reduce phyla such as Firmicutes and Proteobacteria, which have been associated with obesity and other metabolic diseases. Okra mucilages were found to have increased growth of Bacteroidetes, members within this phylum are associated with short chain fatty acids production. Okra mucilage was also observed to be easily fermented by Proteobacteria. Relative abundance at species level showed the initial gut composition to be composed mainly of *Bacteriodaceae Bacteroides* (species unclassified), *Bacteroides Uniformis*, *Faecalibacterium prausndzii*, *Akkermansia muciniphilla* and *Alcaligenaceae Sutterella*, some of which have previously been linked to gut dysbiosis and diseases.

In comparison with inulin which is an establish prebiotic, amadumbe and okra mucilages showed potentially better stimulation of beneficial species and to have elevated beneficial gut microbial species of the initial microbiome. Amadumbe mucilage was found to have increased species *Bifidobacterium adolescentis*, *Bacteroidaceae bacteroides*, *Bacteroides uniformis* and *Porphyromonadaceae parabacteroides*. Many of these species have been reported to be beneficial to health. Okra mucilage was found to promote the growth of species such as *Bacteroidaceae bacteroidetes*, *Bacteroides ovatus* and *Bacteroides uniformis*, many which are associated in short chain fatty acid production.

In terms of evaluating their prebiotic index, crude and purified amadumbe stimulated an increase in *Bifidobacterium*. Crude and purified okra were observed to have decreased in *Bifidobacterium* at the end of 24 h fermentation. The same trend was observed for inulin. This implies that *Bifidobacterium* was capable of breaking down and fermenting amadumbe mucilages to a greater extent than okra mucilages and inulin. The high prebiotic potential shown by amadumbe mucilages can be attributed to its chemical composition. In comparison with okra mucilage, amadumbe mucilage has higher resistant starch content and β-glucan content. Both of which have been reported to encourage proliferation of *Bifidobacteria*. The overall fermentation showed prebiotic activity of amadumbe and okra mucilage to be species specific, as these two mucilage samples were broken down and fermented varyingly by different gut species.

The results of this dissertation provide evidence which contributed to the knowledge gap

existing between structure, composition and mucilage functionality. It also provides data relating to the nutritional, structural and functional properties of mucilage derived from Southern African amadumbe and okra varieties, which was found to hold prebiotic potential after *in-vitro* fermentation. The study also motivated the potential of amadumbe and okra mucilages as emerging prebiotic functional ingredients or as dietary supplements.

5.2 General conclusion

This study investigated to what extent composition influences functional properties of amadumbe and okra mucilage and the potential prebiotic effect of amadumbe and okra mucilage on gut microbiota composition and diversity.

Amadumbe and okra mucilage were shown to have improved composition and functional qualities after purification. When compared to okra mucilage, crude and purified amadumbe mucilage were associated with more protein and carbohydrates. Amadumbe showed lower water holding capacity and higher solubility, this possibly attributed to its poor swelling power. Glucose, mannose, arabinose and xylose were the main monosaccharides present in amadumbe mucilage. Galactose, rhamnose, arabinose and ribose were present in okra mucilage. The primary functional groups were carboxylic and hydroxyl in both amadumbe and okra mucilage.

The fermentation of amadumbe and okra mucilage were found to be slow and progressive in comparison to inulin (rapidly fermented). Okra mucilage showed highest richness and evenness in stimulating gut microbiota diversity in comparison to inulin. Amadumbe and okra mucilage were able to be broken down and fermented by gut microbiota, thus contributing to the reduction of pH. Amadumbe mucilage showed better prebiotic potential in comparison to okra mucilage. Amadumbe and okra mucilage may be utilized to improve human health and wellbeing by positively modulating gut microbiota composition and diversity. Results showed that both are potential candidates for development as emerging prebiotics with potential to compete with other commercially establish prebiotics.

5.3 Recommendations

Future research could focus on further investigating the influence of functional properties of amadumbe and okra mucilage on fermentation. Further investigations are required to probe the possibility of amadumbe and okra mucilage being developed into targeted nutraceuticals for particular metabolic diseases by altering specific gut bacteria.

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