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Nutritional and health benefits of heat - moisture treated maize starch and maize meal with stearic acid

By

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Submitted in partial fulfilment of the requirements for the degree

PhD (Food Science)

In the

Department of Consumer and Food Sciences
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DECLARATION

I declare that the thesis, which I hereby submit for the degree PhD (Food Science) at the University of Pretoria, is my own work and has not hitherto been submitted by me for a degree at this or any other tertiary institution.

Isaac Kwabena Asare

Date: …………………

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DEDICATION

To my wife Mrs. Fatima Obosu Asare and my late mother Faustina O. Asare;

For their patience and encouragement. I am grateful to have you both with me, but for my mother, it was a short time because you should have waited for me to finish school.

To my sustainer and strength.

For from Him, and through Him, and to Him are all things: to whom be the glory forever.

Amen - (Romans 11:36)

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ABSTRACT

Nutritional and health benefits of hydrothermally treated maize starch and maize meal with stearic acid

By

Isaac Kwabena Asare

Supervisor: Prof M.N Emmambux

Co-Supervisor Prof Bruce R. Hamaker

Diet-related non-communicable disease such as obesity and type – 2 diabetes is increasing especially in the Sub-Sahara African region, and this is related to the increased consumption of processed foods and the decline of high fibre foods. In Sub-Saharan Africa, maize meal stiff pap or soft porridge is a staple food, and it is presumed to be rapidly digestible and estimated as high glycaemic index food. Resistant starch is considered as dietary fibre and can also lower the glycaemic index of starchy foods. This study investigates the formation of resistant starch from maize through the use of heat moisture treatment alone, stearic acid addition alone and their combination on both maize starch and maize meal on upper gastro-intestinal tract (GIT) in vitro digestibility as well as the in vitro faecal fermentation to simulate the lower GIT. Stearic acid was added to both maize starch and maize meal at 1.5 % (w/w) alone, maize starch and maize meal were also treated with heat-moisture treatment at the moisture of 20 % at 110 °C for 16 h, and the combination of both stearic acid and heat – moisture treatment was done, as well as their control.

Addition of stearic acid moderately changed the peak, breakdown and final viscosities of the paste compared to untreated maize starch and maize meal. The lower peak viscosity was attributed to the coating of the granule surface by the stearic acid to reduce the intake of water that prevents swelling. Stearic acid treatment pastes were non-gelling resulting from complex formation which prevents the development of junction zones among starch molecules which is an integral part of gel formation. Heat moisture treatment without stearic acid more significantly reduced peak, breakdown and final viscosities and produced a stronger gel than the untreated maize starch and maize meal. The strong gel can be attributed to increased cross-linking between the starch polymer

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chains to form more junction zones. Also, addition of stearic acid followed by heat moisture treatment similarly reduced the peak, breakdown and final viscosities compared to untreated maize starch and maize meal. XRD and DSC of unpasted maize starch and maize meal with added stearic acid alone, heat moisture treatment alone and their combination increased gelatinization temperatures and crystallinity compared to the control. Following of pasting of maize starch and maize meal, there was the formation of amylose – lipid complex of type I and IIa and IIb with a corresponding increase in the degree of crystallinity. There was a decrease in the *in vitro* starch digestibility and lower estimated glycaemic index with addition of stearic acid alone, heat moisture treatment alone and in combination treatments for both maize starch and maize meal. This reduced digestibility is attributed to an increase in crystallinity due to formation of resistant starch as amylose-lipid complexes.

In vitro faecal fermentation of both maize starch and maize meal with added stearic acid, heat moisture treatment alone, and the combination saw increased production of short-chain fatty acids with the corresponding rise in gases and a decrease in the pH. Although fructooligosaccharides produced higher concentrations of the short-chain fatty acid, the combination treatment was seen having significantly higher concentrations in terms of the indigestible residue compared to its control. With regards to individual short-chain fatty acids produced, acetate and butyrate were in higher concentrations compared to propionate. DNA sequencing showed that the production of short-chain fatty acids was of these modified and unmodified starches was associated with certain of the microbes in the colon. Firmicutes was the dominate phylum followed by Bacteroidetes and then Actinobacteria. Comparably high butyrate levels were associated with Firmicutes, as it contains the major butyrogenic bacteria.

In conclusion, the results from this study demonstrated that modification of both maize starch and maize meal by HMT with added stearic acid produced resistant starch containing amylose-lipid complexes that has the potential to reduce estimated glycaemic index and to act as a potential prebiotic. The combination treatment produced a high amount of short-chain fatty acids, which has beneficial effects associated with diet-related non-communicable diseases like obesity and type-2 diabetes.

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INTRODUCTION

1.1 Problem statement

Maize meal is a traditional and staple food in Sub-Saharan Africa which is prepared from maize flour containing about 70 to 80% of starch (Reviewed by Arendt and Zannini, 2013). It is eaten as a porridge or stiff pap and it is rapidly digestible and estimated to have high glycaemic index (GI) (Mbanya *et al*., 2010) of about 70 or higher. The high glycemic index is caused by the increased rate of starch digestion, which results in quick glucose absorption and an increase in blood glucose levels. There are certain concerns associated with eating high GI meals since they cause hyper-postprandial glucose reactions, which can lead to type 2 diabetes (Benmoussa, *et al*., 2004).

The occurrence of type-2 diabetes in Sub-Saharan Africa is increasing rapidly and urbanization and lifestyle changes associated with economic development are the drivers of this disease (Holmes et al., 2010). Change in diet, physical activity, smoking, and adiposity are the lifestyle factors that must be taken care of (Mbanya et al., 2010).

In rural and traditionally living South Africans, type-2 diabetes is rare or unusual, although it is more common in metropolitan areas (Omar, Seedat, Motala, Dyer & Becker, 1992; Walker & Walker, 1991). A low-glycaemic-index diet has been associated to a lower risk of diabetes and other diet-related non-communicable diseases like cardiovascular disease, cancer, and obesity.

Modification of maize starch and maize meal can be used to change their physical properties to form resistant starch which has lower glycaemic index. Chemical modification can be done using chemicals such as propylene oxide, acetic anhydride (2.5%) and octenyl succinic anhydrides that are synthetically derived. However, the use of these chemicals elicits health concerns amongst consumers due to chemical residues in the final product (Reviewed by BeMiller, 2003; Bao & Bergman, 2004 and Lui 2005). Consumers' anxiety now is more in the direction of 'clean label' food and food ingredients (Arocas, Sanz, & Fiszman, 2009). As a result, the Food and Agriculture Organization (FAO) of the United Nations and the Food and Drug Administration (FDA) of the United States have laws governing the nature and level of chemical starch modifiers.

Stearic acid as a fatty acid has been used to modify starch into starch-stearic acid complexes especially through amylose-fatty acid interactions (Reviewed by Obiro *et al*, 2012). The stearic acid as a lipid result in inclusion compound with amylose through hydrocarbon portion of the lipid

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situated within the helical cavity of the amylose (Kaur *et al*, 2000). This starch-lipid interaction has an influence on starchy foods, where it reduces granule swelling and peak viscosity, increases final viscosity and decreases susceptibility to the starch digestibility by alpha-amylase digestion (Wokadala et al., 2012). Starch modified with fatty acids can produce resistant starch and is a consumer-friendly substitute than the use of chemicals.

Heat moisture treatment (HMT) is a physical alteration of starch molecules to intensify crystallinity and granule stability (Stute, 1992). Hoover & Manuel (1996) found that heat- moisture treatment increased the X-ray pattern strengths with improved crystallinity of the starch. Heat-moisture treatment of starch has been observed as a technique of increasing slowly digestible and resistant starch fractions due to the changed crystalline nature making the glycosidic bonds unapproachable to enzyme hydrolysis (Chung et al., 2009).

Resistant starch (RS) is considered a non-digestible carbohydrate and as a dietary fibre source by the joint FAO/WHO Office of Food Labelling (2010). They are starches that cannot be hydrolysed by enzymes in the small intestine but are rather fermented in the colon by the colonic bacteria (Birt et. al., 2013). Consumption of food that contains resistant starch has several health benefits including reduced glycaemic response (Hasjim et al., 2010), improved blood lipid profile (Dodevska et al., 2016) and potentially increased satiety response (Wanders et al., 2011; Clark and Slavin 2013). It also has prebiotic potential, promoting the growth of beneficial bacteria in the colon through the fermentation process (Lockyer and Nugent, 2017). The gut microbiota ferments dietary fiber in the colon, creating short-chain fatty acid metabolites. Humans acquire about ten percent of their daily energy needs via short-chain fatty acid absorption. The microbial populations and metabolites formed during the fermentation of dietary fiber in the colon have a significant impact on the health of consumers.

Many researchers have worked on separate modification of starch with stearic acid (S.A) and heat moisture treatment (HMT) to improve the properties for food industries. The combination of the two treatments (i.e., addition of S.A followed by HMT) in terms of the metabolite production or changes to the gut microbiota has not been thoroughly explored. This research seeks to establish the properties of both maize starch and maize meal modified with stearic acid followed by heat moisture treatment. Also, the research will identify the gut microbial fermentation outcomes.

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2.0 Literature Review

This chapter reviews nutritional and health benefits of HMT maize meal and maize starch with S.A. Maize meal and maize starch are generally characterized as being rapidly digestible starch. The effects of hydrothermal treatment alone, stearic acid addition alone and the combination treatment will be discussed. The relative effect of the resistant starch formed from the afore-mentioned treatment to maize meal and maize starch will be reviewed as well.

2.1 Maize crop, consumption, and product

Maize (Zea mays) is a staple nourishment for an enormous segment of the black population in sub-Saharan Africa but most especially in Southern or South Africa (Breitenbach and Fényes, 2000). Yellow maize is mainly utilized as animal feed, while white maize for humans. Maize meal is the primary dry milling item and starch is the principal wet milling ingredient from maize. Maize meal is produced through dry processing of the maize crop, where the maize is de-germed, dehulled and mostly endosperm is coarse milled to produce maize meal (Delcour & Hoseney, 2010). Maize meal is consumed as a stiff pap or porridge (Taylor and Emmambux 2008), and it is made up of 70 – 75% starch.

2.2 Maize Starch

Maize starch contains 25 to 30% (w/w starch) amylose; however, there are other maize starch types that are referred to as low amylose starch (0.5% w/w starch), also known as waxy starch and high amylose starch may contain about 71% amylose (w/w of the starch) (Damodaran et al., 2007). Amylose molecules are essentially linear polymer of alpha (1→4) related to D-glucosyl entities. Amylopectin molecules are primarily comprised of small linear chains of alpha (1→4) connected to D- glucopyranosyl chains and are highly diverged through alpha (1→6) bonds (Arendt and Zannini, 2013). Starch from maize is used as a thickener, gelling agent, bulking agent, and water retaining agent as ingredients in foods (Singh, Singh, Kaur, Sodhi, & Gill, 2003)

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2.2.1 Starch structure and granules

Starch occurs as granules in plants. The morphology and nature of the granulated form is subject to the basis of the plant (Jane et al. 1994). Starch granules exhibit several forms, such as circular, polygonal, oval, kidney and extended forms and scale range from submicron to over hundred microns (Jane et al. 1999). There are even several shapes within one type of starch, for example maize starch can be angular and spherical (Figure 2.1). Unmodified starch granules observed under polarized light microscopy exhibit a Maltese cross and a semi-crystalline structure (Hibi et al. 1993).

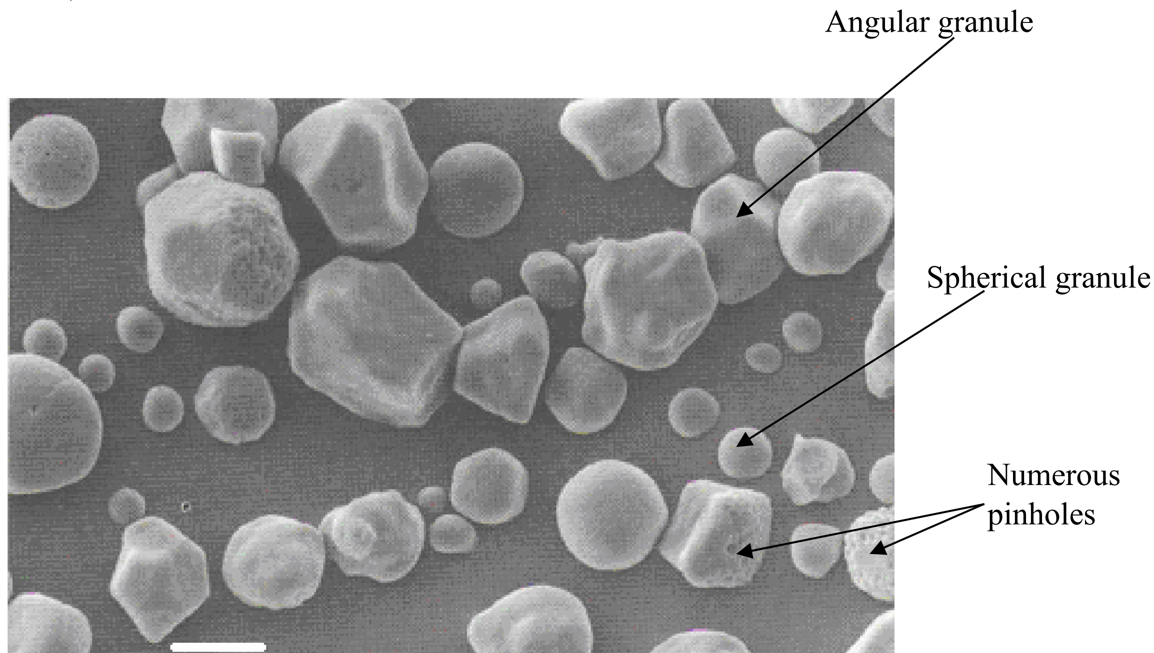

Figure 2.1: Maize starch granule micrograph with spherical and angular granule with numerous pinholes (Whistler and BeMiller, 1997a). Scale bar is 10 micrometers

2.2.2 Starch molecules

Molecular composition of starches are amylose and amylopectin as well as inconsequential constituents like proteins and fats found in it. Molecular weight of amylose is between 10^5 and 10^6 g/mol with degree of polymerization of 1000 – 10,000 (Jane 2004), and amylopectin differs from 10^7 and 10^8 g/mol for variety of starches with degree of polymerization exceeding one million (Yoo and Jane 2002). . Amylopectin comprises 70-80 percent of starch whereas amylose contains

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about 20-30 percent. Waxy starch is mainly amylopectin with amylose making up 0-8 percent. High-amylose starch have more than 40 percent amylose molecule.

Amylose may form a left-handed single helix or parallel left-handed double helical junction zones (Chung & Liu, 2009). The core of amylose single helices are hydrophobic due to abundance of CH-groups, while outside the amylose helix are hydrophilic. due to hydroxyl groups. The hydrogen bonds stabilize the amylose helical structures. Amylose can form a gel when cooled to room temperature after wet heat processing. This is because of the re-association or retrogradation of the solubilized starch polymers after being cooked, enabled by hydrogen bonding to form double helical chains. There is a molecular entanglement of the free amylose and amylopectin chains and re-association of the amylose chains forming junction zones to bring about gelling (Chung and Liu, 2009).

Amylopectin is an extremely branched polymer of alpha D-glucopyranosyl units mainly connected by (1-4) bonds with branches following from (1-6) links. Amylopectin molecules are radially organized in the starch granule and, as the radius rises, so does the number of branches needed to fill the space with consequent creation of concentric region of alternating amorphous and crystalline structure (Parker and Ring, 2001). Hanashiro et al. (1996) reported that amylopectin branch chain dimension is connected to the crystalline starch structure. Amylopectin molecules within the starch granules are arranged in the form of crystalline structures with specific crystalline patterns as determined by X-ray diffraction. These crystalline arrangements are type A or B or C. The A chains are not branched and have a weight average chain length (in degree of polymerization) between 19 – 28, the B chains are branched and have greater long chain fractions and a weight average chain length between 29 – 31 and the C chains have a reducing end and have equally very long and very short chains with a weight average chain length between 25 – 27. Amylopectin may partly crystallize gels by the process of double helical structure interaction by exterior chains of neighboring molecules. Amylose and amylopectin chain interaction gives rise to the functional properties of the starch (Bertoft et al., 2016).

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2.2.3 Starch Gelatinization

When starch is wet heat processed with excess water, the granules of the starch will swell, and will transform the crystalline structure into amorphous mass. This process is called gelatinization and is characterized by irreversible property changes including granular swelling, starch crystallite melting, birefringence loss and starch solubilization. Parker & Ring (2001), reported that, the process of gelatinization includes the weakening of the hydrogen bonds and the hydration of amylopectin as the starch granules imbibe water. The van der Waals forces that hold the crystalline region are overpowered with more hydration. The outcome of this continuous process is the collapse of molecular order inside the starch granules. After this development the amorphous starch readily absorbs water and forms a paste which can develop into a gel during cooling. The gelatinisation temperature of maize starch was about 64°C - 81°C (Ai & Jane, 2014); BeMiller and Whistler (2009). The commencement of the melting endotherm marks the onset temperature (To), followed by the gelatinization peak temperature (Tp) and the end of the endotherm gives the conclusion temperature (Tc) (Figure 2.2). The heat energy required to melt the starch crystallites is known as enthalpy of gelatinization (ΔH).

2.2.4 Pasting and Gelling

After heating starch above gelatinization temperature, the crystalline structure of the starch granules is lost and absorbs water and swells, some disperse and develop substantial viscosity (BeMiller & Whistler, 2009), this condition is called pasting. Starch viscosity is a key in the application as thickening agents. Upon cooling native maize starch pastes can form a gel of a defined shape without fluidity, displaying a viscoelastic property. These rheological properties of starch paste, and gels hang on the starch concentration, amylose/amylopectin ratio, pasting temperature, pasting shear rate, storage temperature and time (Ai & Jane, 2014).

2.3 Starch Modification

Starches in their native form have restriction in their usage under various food processing conditions. Native starch tends to retrograde, and this may be undesirable in some foods like bread

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where it leads to staling (Wang et al., 2015). Native starch has poor thermal stability, and this is a limitation in food systems that require high thermal processing. Inconsistent viscosity of raw or unmodified starch is a limitation in the application of starch as a thickening agent. In order to promote utilization of native starches and widen their application, there is the need to improve the native starch by modifying it to achieve the highest desired functional attributes for certain industrial applications.

Chemical modification and physical modification are employed to improve these continuous release properties of starch, such as substitution and crosslinking (Onofre & Wang, 2010). Some of the processes of starch modification are summarized in Table 2.1. Chemical modification of starches include esterification, etherification, or oxidation of the available hydroxyl groups on the β-D-glucopyranosyl units that forms the starch polymers (Chiu & Solarek, 2009). Physical modification such as retrogradation, pre-gelatinization and hydrothermal treatment are usually responsible for changing the granular structure of the starch (Xie et al., 2005).

Table 2.1: Starch modification processes and products attributes

Category of modification	**Type of modification**	**Properties enhanced**
Chemical	Crosslinking by using difunctional chemicals such as orthophosphoric acid, $POCl_3$ (Chiu & Solarek, 2009)	Improve the integrity of the granule and prevent loss of viscosity and resistance to shear and high temperatures (Jane, 1995)
	Substitution (esterification and etherification) using chemicals such as propylene hydroxide, acetic anhydride, and vinyl acetate (BeMiller & Whistler, 2009)	Promote non-gelling tendencies, and improved the process tolerance of starch, that is, increased thermal, shear and freeze thaw stability

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Physical	Pre-gelatinized (BeMiller & Whistler, 2009)	Improved the texture of the starch yielding smooth slurries due to instant hydration and swelling of starch granules
	Hydrothermal treatment (Stute, 1992)	Improved the integrity of the granule and provides resistance to shear and prevented loss of viscosity.

Chemical modification of starch has been alleged to leave chemical residues in the starch after modification and this has made the chemically modified starches undesirable to consumers (Chiu & Solarek, 2009). Clean label starches are starches that do not have synthetic chemicals or been modified through natural and organic ways. The food industry is keen to develop a variety of clean label starches linked to the health and wellness trend and sustainability. The modification of starch using fatty acids involves the interaction of amylose and the fatty acids to form amylose-lipid complexes (ALC) (Arik et al., 2014). These ALCs were found to enhance the functional properties of starch. Hydrothermal treatment (annealing and heat-moisture treatment) which is a classification of physical treatments and 'chemicals' of natural source (such as fatty acids) have been used to produce clean 'label' starches. (Stute, 1992).

2.3.1 Heat-moisture treatment (HMT)

HMT is a physical treatment that has been reported to alter the physico-chemical properties of starch whereas the granule structure is not terminated (Stute, 1992). HMT uses limited moisture contents (<35% W/W), and usually exposes it to a temperature beyond glass transition temperature (Tg) but less than the onset temperature (To) of gelatinization for a specified time (Jacobs & Delcour, 1998). The temperatures that have been used for heat-moisture treatment by many researchers is between 90 °C – 130 °C depending on the moisture content of the sample used (Jacobs & Delcour, 1998). The temperature – moisture conditions for HMT have frequently been selected without considering the precise gelatinization temperature of the starch at that specific

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moisture level. Therefore, the results of HMT can be influenced by partial gelatinisation (Eerlingen et al., 1997). HMT facilitates the partial opening of the amylopectin chains, and this promotes molecules that make up amylose in amorphous region to interrelate with the branched sections of amylopectin in the crystalline regions (Hoover, Swamidas, & Vasanthan, 1993). The improved chain interaction caused modification in the structural organization of starch chains inside the amorphous and crystalline domains (Chung et al., 2009).

Eerlingen et al. (1997), anticipated that the transformation of the amorphous amylose chains and the interaction between crystalline and amorphous matrix during the HMT was responsible for increasing the gelatinization temperatures of HMT of starch. HMT was found to limit the swelling of the starch granules by increasing the intermolecular bonding between amylose and the branched segments of the amylopectin side chains, which held the starch molecules together. Heat-moisture treatment has been found to decrease the initial pasting peak viscosity (Figure 2.3) and to reduce the relative crystallinity of maize starch (Figure 2.4). However, other researchers such as Hoover & Manuel (1996a), found out that HMT increased the X-ray arrangement intensities of maize starch. These increased X-ray arrangement intensities suggested that HMT increased the crystallinity of the starch.

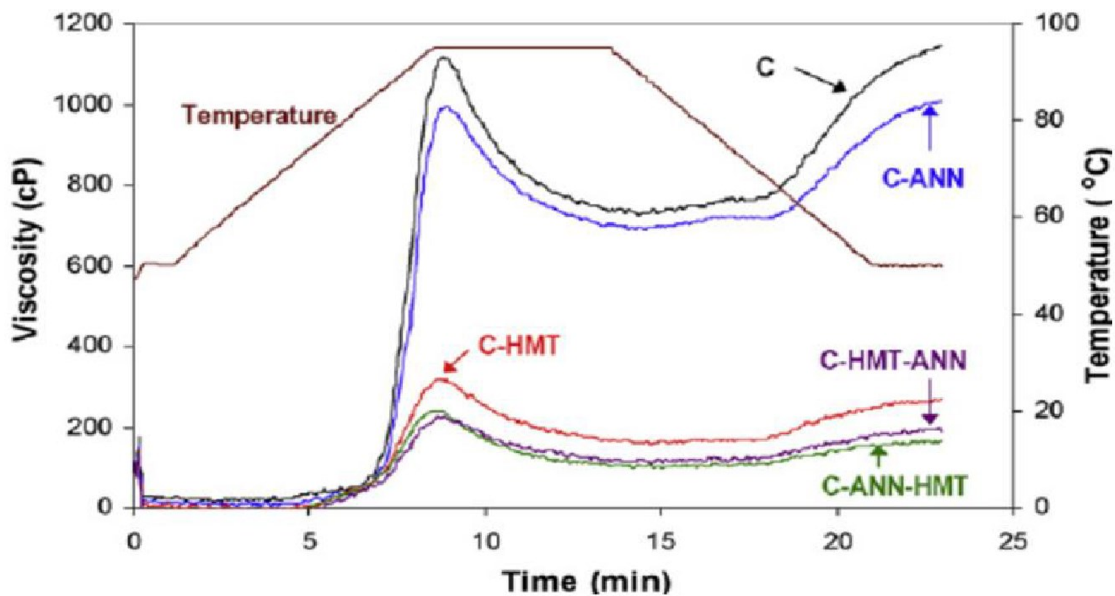

Figure 2.3: RVA pasting curves of native and modified maize starches. C, native maize starch; C-ANN, annealed maize starch; C-HMT, heat-moisture treated maize starch; C-ANN- HMT,

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annealed maize starch subjected to heat-moisture treatment; C-HMT-ANN, heat-moisture treated maize starch subjected to annealing (Chung et al., 2009)

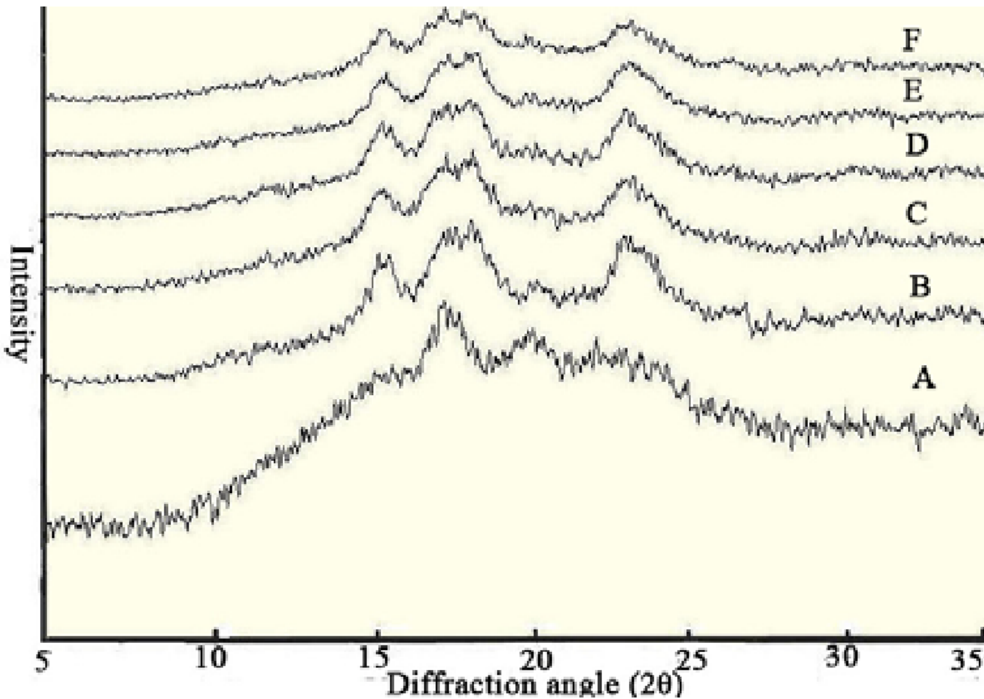

Figure 2.4. X-ray diffraction pattern of native and heat-moisture treated mung bean starch. (A) native starch; (B) HMT-15; (C) HMT-20; (D) HMT-25; (E) HMT-30; (F) HMT-35.

HMT allows the amylose and amylopectin portions to undertake a rubbery state, permitting and interrelating to create a double helix which will intensify the complete granule stability (Chung et al., 2009), causing an improved resistant starch (RS). When waxy potato starch (amylose content near zero percent) was subjected to heat moisture treatment at 20 to 25 percent moisture for 1, 5, and 9 hr with a temperature of 110°C, 130°C and 150 °C, the condition of 110 °C and 20 percent moisture for 5 h gave the optimum levels of resistant starch (66.8 percent). Starches that came treated with temperatures > 130 °C or 30 percent moisture content showed a decreased resistant starch (Lee et al., 2012). Once heat moisture treatment was done at 120 °C for two hr at moisture of 30 percent on starch from corn, pea, and lentil, the resistant starch levels improved by 7.7 percent, 11.2 percent, and 10.4 percent on gelatinized samples (Chung et al. 2009). HMT could increase RS levels in starch without the interruption to granular structure. Brumovsky and Thompson (2001) also established that high-amylose corn starch subjected to HMT witnessed 43.9

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percent RS related to 18.4 percent of unmodified starch. Table 2.2 summarizes the impact of HMT of starch and its useful properties.

Table 2.2: Summary of the impact of HMT on starch functional properties

Starch properties	**Influence by HMT**
Gel properties	Increase gel hardness (Hoover & Manuel, 1996a)
Granule morphology	No change in shape and size of starch granules (Hoover & Manuel, 1996a)
Relative crystallinity	Increase XRD intensities (Hoover & Manuel, 1996b)
Gelatinisation characteristics	Increase in gelatinization temperature, increase gelatinization temperature range, and increase enthalpy of gelatinization (Chung et al., 2009)
Enzymatic hydrolysis	Decrease (Gunaratne & Hoover, 2002)
Swelling power and solubility	Reduced swelling power, reduced hydration (Chung et al., 2009)
Pasting properties	Increased pasting temperature, reduced initial peak viscosity, reduced amylose leaching, reduced breakdown viscosity and retrogradation (Chung et al., 2009)

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2.3.2 Amylose – lipid complexes

Fatty acids can interact with amylose to form amylose-lipid complexes (Nierle & El Baya, 1990). Formation of amylose-lipid complexes change the properties and usage of the starch and has nutritional and functional importance compared to native starch. Several common saturated fatty acids found in edible oil like palmitic acid, oleic acid, myristic acid and stearic acid can be used to produce amylose-lipid complexes. Saturated fatty acids usually have straight chain hydrocarbons with several carbon atoms ranging between 12–22 (Rustan & Drevon, 2005) (Table 2.3).

Table 2.3: The chemical structures of the saturated fatty acids commonly used in starch modification (Rustan, Drevon and Christian, 2005)

Chemical formula	Fatty acid Name
$CH_3(CH_2)_{10}CO_2H$	lauric acid
$CH_3(CH_2)_{12}CO_2H$	myristic acid
$CH_3(CH_2)_{14}CO_2H$	palmitic acid
$CH_3(CH_2)_{16}CO_2H$	stearic acid

Amylose forms a helical complex of fats and other compounds (complexing agents) possessing a hydrophobic moiety. Inclusion complex in other words single-helical amylose complex, has complexing agent's hydrophobic mobility within the helix's hydrophobic cavity. (Putseys et al., 2010). A carboxyl group is the hydrophilic head, whereas a hydrocarbon chain is the hydrophobic tail in fatty acids as shown in Figure 2.5.

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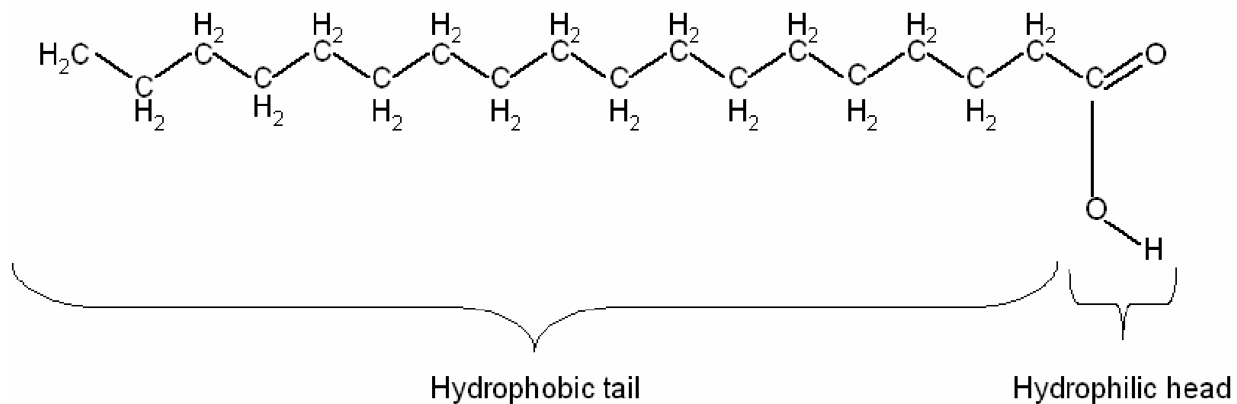

Figure 2.5. The structure of stearic acid showing the hydrophilic carboxyl group (head) and the hydrophobic carbon chain (tail).

The amylose chain shows a usual spiral supplying a helical configuration with six anhydro-glucose units per turn (Zobel, 1988 and Morrison, 1995). The outer surface of the chain is hydrophilic due to the hydroxyl groups of the glycosyl residues that are situated on the exterior of the helix. The inner cavity of the helix is a hydrophobic core with many CH- groups (Zobel, 1988) (Figure 2.6). The aliphatic fatty acid chain resides inside the helix of amylose (Godet et al., 1993; Obiro et al., 2012) and the helix turn is stabilized by intra and inter-molecular Van der Waals forces and hydrogen bonds (Rappenecker and Zugenmaier, 1981). The carboxylic head of the fatty acid lies beyond the amylose helix (Figure 2.6) due to steric hindrance and electrostatic repulsion (Godet et al., 1993).

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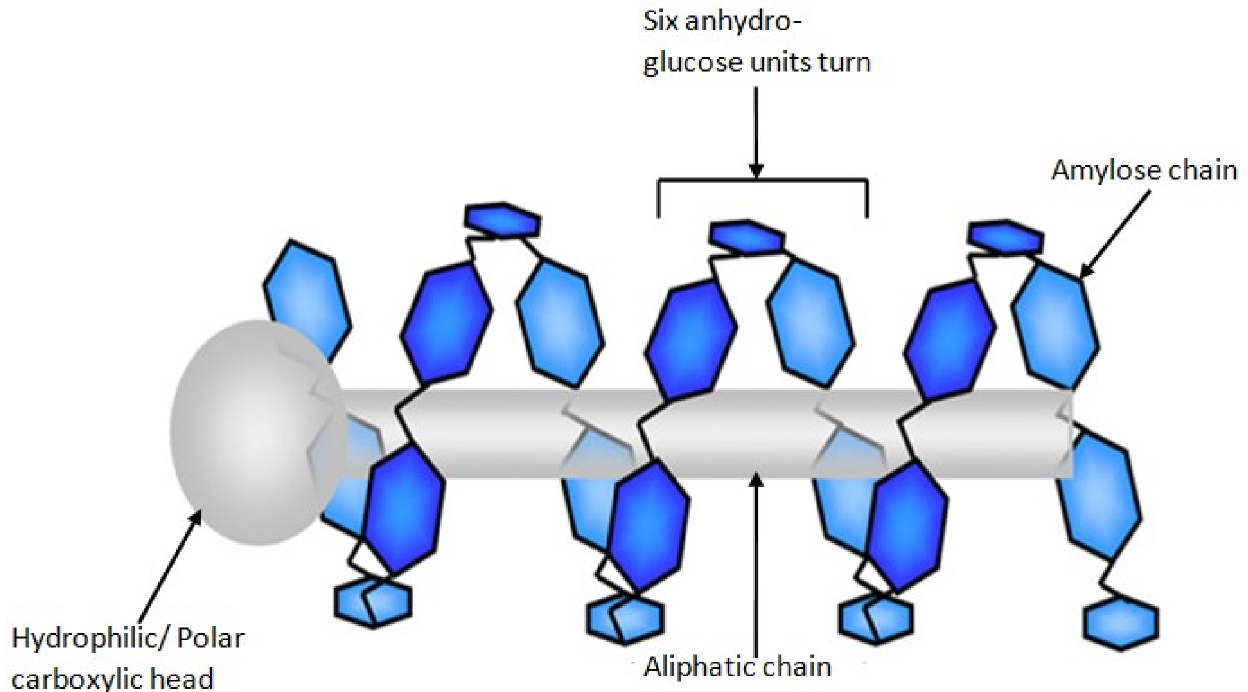

Figure 2.6: Schematic illustration of amylose helical complex with the polar carboxylic head located outside the helix and the whole aliphatic chain situated in the helix cavity (Putseys et al., 2010)

The formation of amylose–lipid complexes usually influence pasting, gelling, digestive properties, and fermentation rate of the starch. These amylose-lipid complexes form a firm network structure of intra-granular structures of the amylose-lipid complex. The rigid network structure caused an increase in hydrophobicity of the starch granules that prohibited water uptake, reduce swelling of the starch granule, eventually reduce initial paste viscosity, and completely collapse of the starch granules (Obiro et al, 2012) (Figure 2.7). Eliasson et al. (1988) recommended that the decrease in peak viscosity was due to amylose-lipid complex formation accompanied by the development of a layer made from amylose-lipid complex on the surface of the granule and due to a hydrophobic coating of fatty acids around the starch granule. This layer prevents leaching of amylose which has not formed complexes to exit the granule and limit the entry of water for reduced granular swelling.

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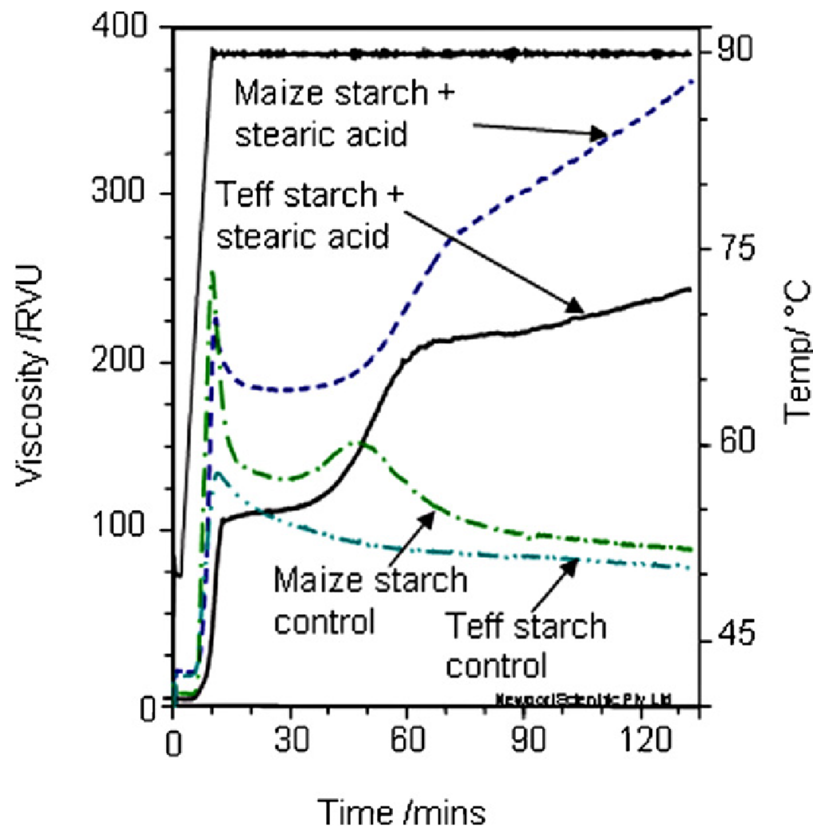
A



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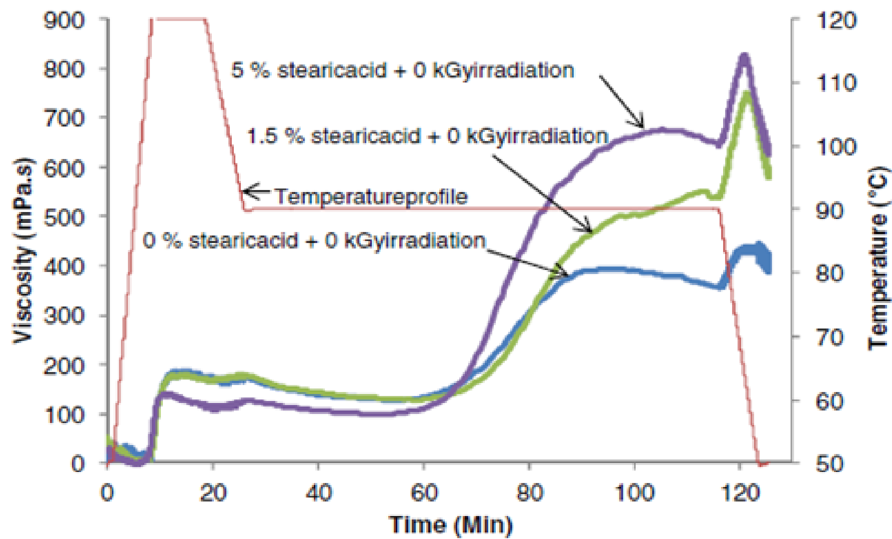

Figure 2.7: Effect of stearic acid (SA) addition on the paste viscosity of teff and maize starch pasted for a prolong time (130 mins) at 90°C [A] (Wokadala et al., 2011) and on prolonged pasted (120 min) of high amylose maize starch under pressure at high temperature [B] (Ocloo et al., 2016)

Investigation by Ocloo et al (2016) on pasting properties of high amylose maize starch showed a decrease in initial peak viscosity of high amylose maize starch with added stearic acid (Figure 2.7B). Prolonged pasting time results in more granule disintegration and amylose leaching to the slurry (Kaur and Singh, 2000) and formed more amylose lipid complexes as well as an increase in viscosity after the first pasting peak viscosity for both normal maize and teff (Wokadala et al., 2011) and high amylose maize starch (Ocloo et al., 2016).

The temperature of melting of the amylose-lipid complexes differentiate them as type I, type IIa and IIb (Biliaderis & Galloway, 1989) (Figure 2.8). According to Biliaderis & Galloway (1989), type I complexes are molded at about 60 °C and corresponded with endotherms having low range of dissociation temperature, around 98 °C. Type IIa and type IIb complexes were formed at about 90 °C and corresponded with two endotherms with advanced range of dissociation temperatures ~ 106 °C and ~ 120 °C respectively (Biliaderis & Galloway, 1989). Complexes of type I consisted of a partly organized structure without separate crystalline regions, but complexes of type II consisted of distinct crystalline and amorphous regions (Figure 2.8).

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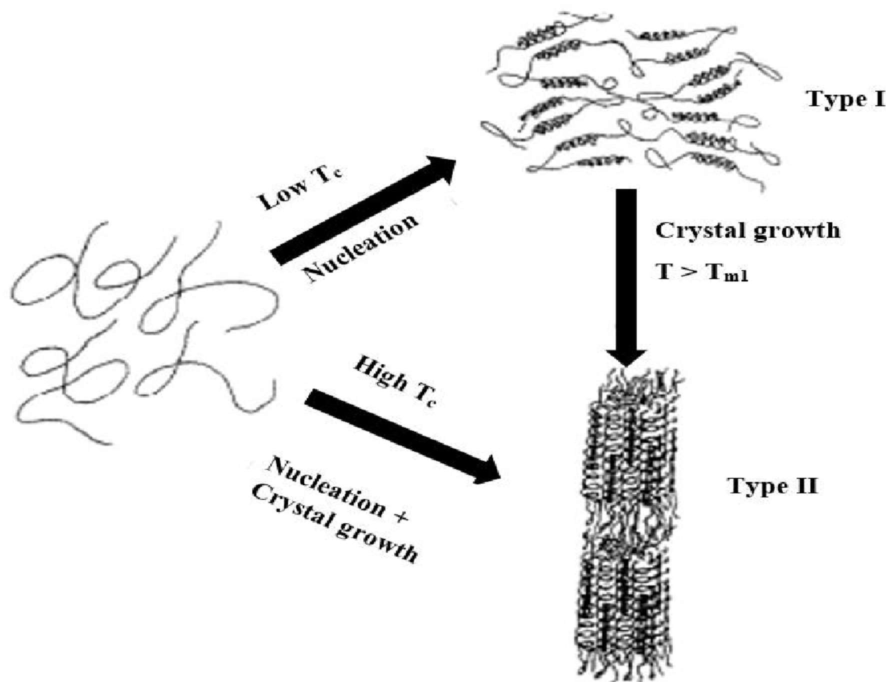

Figure 2.8: Diagrammatic representation of the step-by-step analysis of amylose-lipid complexation, developing Type I and Type II complexes; where T is temperature, Tc is the temperature of crystallization, and Tm1 is the temperature of melting of Type I complexes (Biliaderis & Galloway, 1989)

Amylose-lipid complex played an essential activity in maize and teff starches which has biphasic pasting phenomenon which included peak viscosities at short and long pasting times in the presence of S.A addition (Wokadala et al., 2012) (Figure 2.7). According to research done by D'Silva et al. (2011), stearic acid level of about 1.5 percent (w/w of starch) doubled the final viscosity during long pasting of maize and teff starches. Using X-ray diffraction, Wokadala, Ray, and Emmambux (2012) observed type II-V-amylose diffraction arrangements for starches pasted for a long time with addition of stearic acid.

Amylose-lipid complexes restrict starch granules to swell and reduce the accessibility of enzymes to hydrolyse the starch (Singh *et al*., 2002). The amylose-lipid complexes prevent the starch molecules from fitting to the enzyme binding site to be hydrolysed. The alpha 1-4 glycosidic bonds seem to be within the amylose helix, and this limits access to the enzymes for hydrolysis (figure

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2.9). Table 2.4 summarizes the influence of fatty acids on starch and its functional properties. Fatty acids addition promotes the creation of amylose-lipid complex, and these are responsible for the variations in the useful properties of starch, such as, increase in the viscosity during pasting, reduced retrogradation and reduced *in vitro* starch digestibility (Livesey et al., 2008; Barclay et al., 2008).

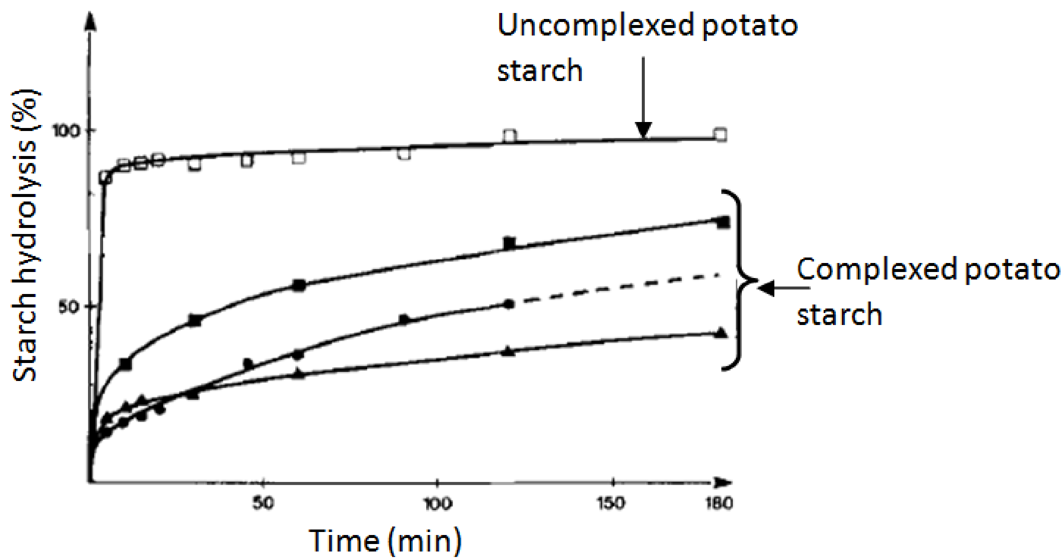

Figure 2.9: Effect of glycerol-monostearate on the starch digestibility of potato starch (Holm et al., 1983)

Creation of amylose-lipid complex causes a decrease in starch retrogradation (Godet et al., 1996). Retrogradation is the re-aligning of amylose and amylopectin chain through H-bonding after gelatinization during the cooling of the starch paste to gel (Karim et al., 2000). Amylose re-associates more rapidly to form firmer gel consistency than the amylopectin that forms weak gels (Damodaran et al., 2007). Amylose-lipid complexes hinder the re-association of amylose-amylose interactions, amylose-amylopectin interaction to form double helices, thus less likelihood to form junction zones for gelling (Zhou et al., 2007).

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Table 2.4: A summary of the effects of addition of fatty acid to the functional properties of starch

Functional properties	Impact of fatty acids
Swelling power and initial peak viscosity during wet heat processing	Formed hydrophobic layer around the starch and inhibits water penetration through the granule pores hence reduced swelling and initial peak viscosity (Nierle & El Baya, 1990)
Setback viscosity during cooling and final viscosity	Reduced breakdown, reduced setback viscosity and increased final viscosity (Maphalla & Emmambux, 2016)
Gel stability	No gel formation (D'Silva et al., 2011)
Relative crystallinity	Increased due to V-complex formation (Maphalla & Emmambux, 2016)
In vitro starch digestibility	Decreased in vulnerability to enzyme hydrolysis (Hasjim and others, 2010)

Combination of physical modifications such as fatty acid addition with irradiation was considered to produce modified starch with specific functional properties (Ocloo, Minnaar, & Emmambux, 2016). The dual modification of stearic acid and gamma irradiation of starch exhibited variations in the molecular structure of Hylon VII starch and thus recommended the potential use of this dual modification in enhancing the functional properties of starch (Ocloo et al., 2016). Gamma irradiation improved solubility, absorption capacities of oil and water and decreased the ability to swell (at 90 °C and 95 °C) of high amylose maize starch. Also, the added stearic acid to high amylose maize reduced the influence of gamma irradiation on the solubility and transition endotherms of Hylon VII. Gamma irradiation alone, stearic acid addition alone and their combination transformed the X-ray diffraction pattern and microstructure of Hylon VII (Ocloo et al., 2016) (Figure 2.10).

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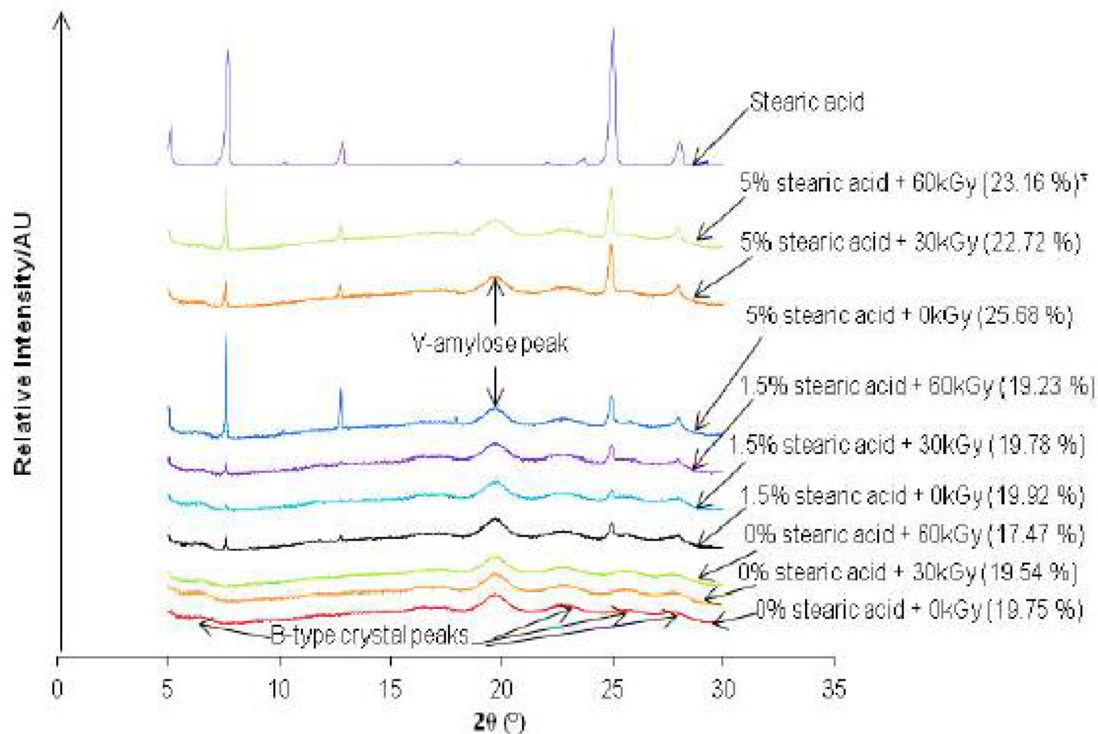

Figure 2.10. Effects of gamma irradiation, stearic acid alone and in combination on the X-ray crystallinity of Hylon VII starch (Ocloo et al., 2016).

*Treatment conditions were stearic acid (0, 1.5 & 5 %) added to Hylon VII starch, and then irradiated at 0, 30 and 60 kGy).

2.4 Starch Digestibility

Digestibility in foodstuffs has been a burning issue over the years. It was previously believed that all foods with starch was hydrolyzed by salivary α-amylase initially and then later by pancreatic α-amylase in the small intestine which led to the production of maltose, maltotriose and extra branched oligosaccharides known as alpha - limit dextrins, and later changed into glucose using maltase-glucoamylase (MGAM) brush border enzymes and sucrase-isomaltase (SI) (Lee et al., 2013).

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The kinetic constituent of digestion of starch is primarily linked with the glycaemic index, which strongly influence the postprandial metabolism. Starchy foods are assumed to have high postprandial blood glucose response. Glycaemic index is the tool to measure that and it's also for characterization and classification depending on the physiological response they produce (Jenkins et al., 2002; Miao et al., 2015).

An endo-acting enzyme alpha-amylase hydrolyzes only alpha 1, 4 starch bonds to yield maltodextrins of different molecular dimensions. The binding site size of alpha amylases differ from various sources (Robyt 2009). The starch molecules possess non-reducing end which generates glucose by using amyloglucosidase which is an exo – splitting enzyme. It also hydrolyses alpha 1, 6 bond at a slower rate (Hiromi et al. 1966). Starch with alpha 1, 6 glycosidic bonds can only be hydrolyzed by a debranching enzyme. Beta amylase is the only type of enzyme that is exo–acting that hydrolysis alpha 1, 4 starch bond and alpha-maltose (Robyt 2009).

The bacteria *Aspergillus niger* produces pancreatic alpha-amylase and amyloglucosidase which forms part of amylolytic enzymes that hydrolyse starch faster than other amylolytic enzymes such as alpha-amylase. Native starch granules could be hydrolyzed on time by amylolytic enzymes because amylolytic enzymes have starch-binding domain as the popular function, this was shown by structural analysis (Christiansen et al. 2009). Christiansen and other (2009) and Guillen and others (2010) suggested that the following functions could be available to starch-binding domain to enable the enzyme to hydrolyze granular starch swiftly: (a) improve interactions that exist between starch granules and enzyme; (b) interrupt the starch granule's crystalline structure.

The digestibility rate of starch granules is controlled by several causes: polymorphic composition, amylose content and size of the starch granule. Generally, A-type starches with crystalline structure (e.g., maize and rice starches) are rapidly digested by the enzyme than B-type starches (e.g., potato starch), and C-type starch (e.g., green banana starch) (Figure 2.11) (Jane 2003). The A-type starch being more susceptible may be because its amylopectin chain is shorter with branch chains (DP 6-12) and the branch points found in both crystalline and amorphous regions. Such characteristics of amylopectin structurally results in less complete crystallinity of type A starches (Jane 2003; 2006).

In addition, there are channels or pinholes in A-type starch granules which means internal structures are more porous (Fannon et al. 1992; Huber and BeMiller 2000; Jane 2006), this increase

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the weakness of such starches to hydrolysis as enzymes can diffuse inside the granules. Planchot et al. (1995) and Fuwa et al., (1979), showed that α-amylases hydrolyze A-type starch granules using "inside-out" route, i.e., attack at fragile points (e.g., pinholes) on the granule surface by the α-amylase, they form tunnels in the granules internally, and then from the inside out they hydrolyze the granule. Nevertheless, the process of attacking the granules with tunnels before attacking the granule surface (inside-out) route can't hydrolyse B-type and C-type starches when using α-amylase. However, alpha-amylase hydrolysis of starch primarily occurs at the periphery of B- and C- type starches. The discrepancies may be explained by the fact that the starch granules of type B- and C- does not have porous structures like starches of type A (Fannon et al. 1992; Huber and BeMiller 2000; Jane 2006).

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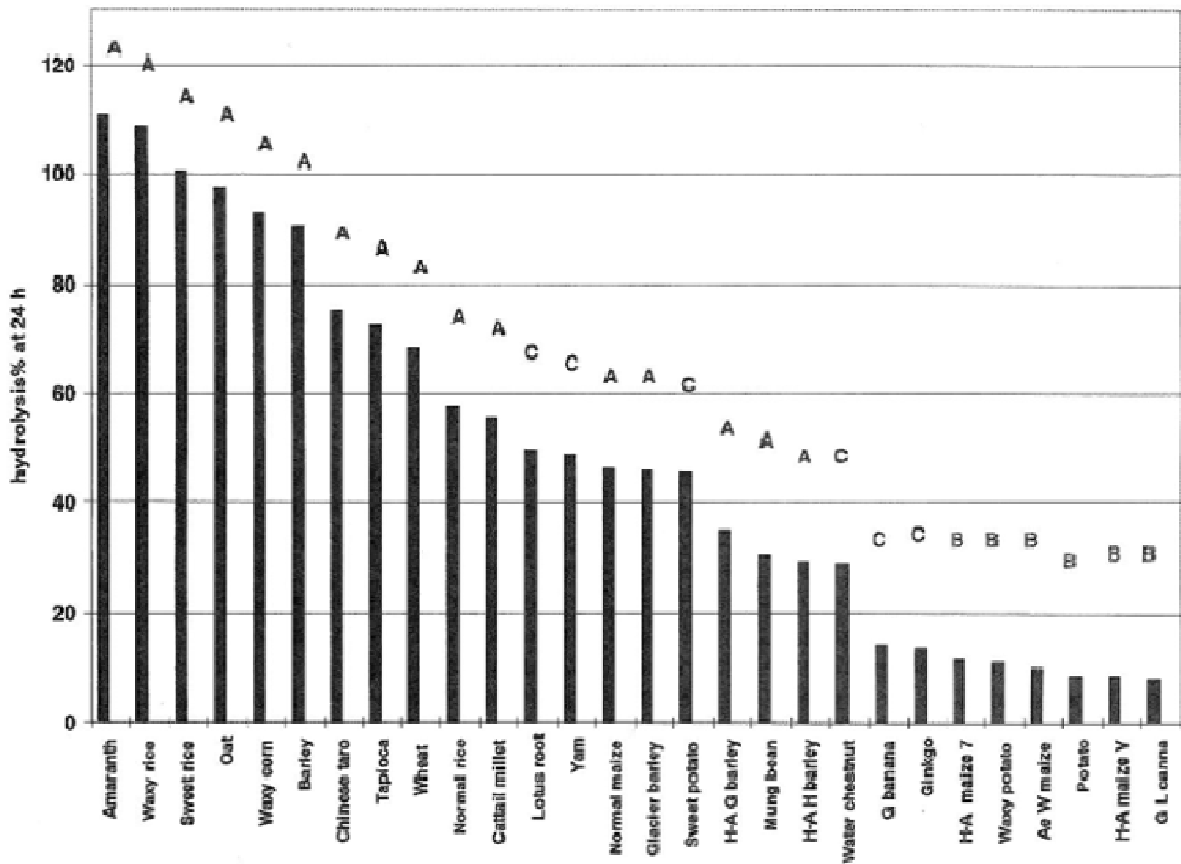

Figure 2.11. Relative enzyme digestibility of selected starch of diverse crystalline structures (Jane 2003). The letter overhead each bar shows the type of starch crystallinity.

[Porcine pancreatic α-amylase (120 U/20 mg starch in 9 mL phosphate buffer, pH 6.9) was used in the hydrolysis of starch at 37 °C for 24 h.]

Starch is usually separated into three portions as regards digestibility: quickly digesting starch also known as rapidly digestible starch (RDS), gradually digesting starch also known as slowly digestible starch (SDS), and resistant starch (RS). Rapid digesting of starch results in rise of blood glucose levels. High glycaemic index (GI) in foods is associated with elevated fast digestion, which is an estimation of how rapidly glucose in the food is released from the optimum amount of carbohydrate accessible for consumption by the body. High GI foods have reduced satiety and can cause excess food consumption leading to the high risk of obesity (Ludwig and others 1999). High GI foods were also associated with higher risk of type 2 diabetes (Shimotoyodome and others

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2010) and heart related diseases (Liu et al., 2000). Therefore, high GI foods have negative influence on human health.

Gradual digestion of starch is done slowly in the intestine, and resistant starch is undigested in upper GIT, resulting in greater satiety and less immediate effect on glucose levels in the blood. Thus, its outcome is lower GI. Since resistant starch goes through the small intestine intact, it is referred to as dietary fibre. As a result, rise in satiety levels leads to hunger and obesity reduction. (Willis and others 2009). Table 2.5 summarizes the starch fractions below.

Table 2.5: Starch classification (adapted from Englyst et al., 1992)

Starch fraction	Fast digestible starch (RDS)	Slowly digestible starch (SDS)	Resistant starch (RS)
Place and timeline for digestion (*in vitro*)	Mouth and small Intestine. First 20 min;	Small intestine only: Between 20 - 120 min;	Main action in colon. After small intestine digestion
Examples	Food products prepared afresh	waxy maize starch, millet and legumes	Cooked and cooled rice, bread that is staled
Amount (g/100 g dry matter)	Cooked hot potato: 65g	Cooked millet: 28g	Raw potato starch: 75g
Main functional Property	Provides fast energy source to the body	Provides gradual and sustained energy source lower blood glucose level	Influence gut health (e.g., fermentation of prebiotics to produce SCFA)
Structure	Specifically Amorphous	Amorphous and crystallinity	Depending on the RS type, specifically crystalline

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2.5 Resistant Starch (RS)

RS is that part of starch that is impervious to hydrolysis by the enzyme in the small intestine and moved to the colon for intestinal microflora fermentation (Englyst et al. 1982; Englyst & Macfarlane, 1986). A summary of the types of resistant starch is represented in the table 2.6 below. Resistant starch can be divided into five categories according to their structure and properties:

RS1: Physically inaccessible starches

RS1 denotes starch ensnared in cell-wall materials, protein matrix, and extra physical blockades that decrease the availability of amylolysis of starch (Zhang and Hamaker 1998; Rooney and Pflugfelder 1986; Ezeogu and others, 2005). Resistant starch in pasta is a typical example, the outcome is the decreasing of the postprandial reaction of plasma-glucose in humans than white bread made from the similar ingredient (Granfeldt and Bjorck 1991). The greater resistant starch content observed in the pasta is because of the compressed protein matrix than products of wheat (Fardet and others, 1998; Kim and others, 2008).

RS2: Native starch granule with polymorphs (B- or C-type)

Native starch with a polymorphic B- or C- type has extra resilience to digestion by enzymes than the starch of type A (Kimura and Robyt 1995; Jane 2003). Resistant starch type 2 has a disadvantage in terms of food application, which is the resistant starch content reduces considerably after processing it thermally (Kishida and others, 2001; Bornet and other, 1989). The existence of long chain double helical crystallite makes high-amylose starch to have a gelatinisation conclusion temperature (Tc) of 130 °C. After cooking, the starch retains its semi-crystalline structure with even up to 40 % resistant starch content (Jiang and others, 2010a). Subsequently, high-amylose starch is a better basis of resistant starch type 2 for several products of food (Li and others, 2008; Hasjim and others, 2010). Heat moisture treatment which is inclusive of hydrothermal treatment could be used to promote the proliferation of high amylose resistant starch content (Liu et al. 2007).

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RS3: Retrograded starch

During retrogradation, two adjoining starch polymers recrystallize to create double helices, of which the structure is more resilient to hydrolysis by enzymes (Jane and Robyt 1984; Sievert and Pomeranz 1989; 1990). Amylose is favoured for resistant starch type 3 preparation, as they retrograde at a quicker rate and become crystallites and more resilient than amylopectin molecule (Eerlingen and Delcour 1995; Sievert and Pomeranz 1989). Freezing/thaw cycles, debranching and partial acid hydrolysis improves starch retrogradation and can therefore be used to improve the resistant starch type 3 content (Sievert and Pomeranz 1989; Chiu and others, 1994; Lehmann and others, 2002; Thompson and Brumovsky 2002; Chung and others, 2003; Lehmann and others, 2003; Hasjim and Jane 2009).

RS4: Starch with Chemical modification

Chemical reformation of starch can be used to develop resistant starch. Modification of starch using octenyl succinic anhydride makes starch clusters become hydrophobic group interfering with the binding site of the amylolytic enzymes (Zhang and Hamaker 1998; Han and BeMiller 2007; He and others, 2008). Another active method of processing resistant starch type 4 is starch cross-linking (Woo and Seib 2002; Xie and others, 2006; Al-Tamimi and others 2010). Starch cross-linking excessively raises the temperature of gelatinization and reduces the granular swelling, thus improving the starches resilience to hydrolysis by enzymes (Woo and Seib 2002; Xie and others, 2006).

RS5: Amylose-lipid complex (ALC)

Another process used in developing resistant starch was a reaction between amylose and lipids to form a single-helical complex structure (Gelders et al. 2005; Hasjim et al. 2010). Naturally, ALCs exist in small quantities in starch (Morrison et al., 1993), but development can be increased by the introduction of external fatty acids (stearic acid), which facilitate the creation of ALCs during heat processing (Obiro, Ray & Emmambux, 2012). Meanwhile a lipid portion is now present that also has its requirement for enthalpy heating. The complex can dissociate when heated, but it will return to its complex nature when cooled (Hasjim et al., 2013).

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Table 2.6: Summary of the types of resistant starch

RS type	Attributes	Examples
1	Starch physically unavailable	Grains or seeds left without milling or partially milled
2	Granular starch with structures that then make digestion sluggish	High amylose starch or raw leguminous, green banana and potato starches
3	Starch retrogradation	Stalled bread, rice that has been cooked and allowed to cool
4	Starches modified with chemicals	Partly indigestible starches like free oxidation, esterification and hydroxyl groupings identified through beta bond formation by gamma irradiation
5	Amylose–lipid complexes	Starches which have complexed amylose chains with a lipid portion

RS is also part of dietary fiber (FAO / WHO 2010) and is therefore a choice to proliferate dietary fiber whiles the sensory properties is not compromised on the cereal-based food (Aigster et al., 2011). It is estimated that ten to twenty percent of the daily intake of carbohydrates must be resistant starch for the human body to gain the benefits of resistant starch physiologically (Hasjim et al., 2013). Because of the related health benefits of consumption of resistant starch, methods to increase resistant starch content must be developed in both processed food and food that has starch as an ingredient

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2.5.1. Health Benefits of RS

Health benefits of resistant starch in both human and animal subjects have been thoroughly studied. Features:

a. Lowering insulin responses and postprandial plasma-glucose levels

Impact on variability of RS styles on human subjects about insulin responses and postprandial plasma-glucose levels has been examined. Studies have revealed that consumption of foods containing resistant starches results in appreciable lower response of insulin and/or postprandial plasma-glucose in relation to their control starch respectively (Behall and others, 2006; Al-Tamimi and others, 2010). Subsequently, diet intake with higher resistant starch levels could help regulate insulin levels and human blood glucose levels for people especially with diabetes (Behall and others, 2006; Al-Tamimi and others, 2010; He and others, 2008; Hasjim and others, 2010).

b. potential to reduce obesity and improving serum lipid profile

Evidence from *in vivo* studies shows that resistant starch intake lowers cholesterol intensities and overall serum lipids (Cheng and Lai 2000; Dedeckere and others, 1993). Consumption of resistant starch has also been proven to facilitate lipid oxidation (Shimotoyodome and others, 2010; Higgins and others, 2004). Therefore, intake of resistant starch can decrease accumulation of lipids and inhibit obesity eventually.

c. Improves health of the colon

Investigations of resistant starch in the colon showed that the intestinal microflora ferments resistant starch that enters the colon to bring about short-chain fatty acids, which are metabolites (Zhao and others, 2011; Ferguson and others, 2000; Martin and others, 1998). Furthermore, the consumption of resistant starch increases the volume of faeces that dilutes possible carcinogens and decrease their colonic exposure (Ferguson and others, 2000; Phillips and others, 1995; Hylla and others, 1998). Such biological influence of resistant starch has been linked to its enhancement in colon health and colorectal cancer avoidance (Zhao and others, 2011; Topping and Clifton 2001; Le Leu and others, 2007).

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2.6. Colonic Fermentation

Non-digestible dietary fibre pass undigested in the upper gastrointestinal tract and are fermented by anaerobic gut microbiota in the cecum and large intestine. The human colon houses varied bacterial population based on their nutrition and physiological effect whereby species that belong to the genera *Bacteriodes, Bifidobacterium* and *Eubacterium* dominate in numbers. (Fint et al., 2012). Colonic bacterial growth is based on the substrate available to it, thus can be dietary or endogenous in nature from either carbohydrate or from protein (Macfarlane et al., 1992). The use of the substrate in large intestine includes the number of bacterial interactions that are related and leads to development of numerous finished products. The kind of fermentation product produced is correlated with the quantity of the substrate accessible, the bacteria involved metabolic pathways and degree of degradation (Cummings & Macfarlane 1991). In the colon, bacteria are mainly divided into two broad based groupings, harmful bacteria, and beneficial bacteria. Beneficial bacterial such as Bifidobacterium and Lactobacillus spp. generate unfavourable situations for the development of pathogenic microorganism. Though enterobacteria and clostridia are beneficial, they can be unwelcomed sometimes. This can be caused by intrusive activity or development of certain metabolites that could be toxic (Balows and others, 1991). Stimulating the growth of beneficial microbe and the inhibition of harmful microbe is a noticeable advantage to the host and there is currently a concern to introduce health-promoting substrates to the diet.

Babies are born without gut flora, but microbes from the mother and the nearby environment conventionalize the gut immediately after birth. The bacterial community conformation remains unbalanced until the infant gets to the age of 3–4 years when it matures.

Outcome of fermentation involves several metabolic groups (Wilkinson et al., 2012), the main groups are short chain fatty acids (SCFAs) (Warren et al., 2018). SCFAs are an essential waste product for the microbial population, it’s mandatory to equilibrate the production of redox equivalent in the anaerobic gut environment (van Hoek and Merks, 2012). About 95 percent of the SCFAs formed in the colon are quickly absorbed by colon epithelial cells whereas the five percent remaining are excreted in the faeces (Topping and Clifton 2001). Everyone has a specific microbiome that influences the composition of its physiology and genotype (Zoetendal et al., 2001).

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2.6.1. Short chain fatty acid production (SCFA)

Development of SCFA is difficult to quantify *in vivo*, so most investigations with fecal microbiota as inoculum were performed *in vitro*. Nevertheless, *in vitro* fermentation varies from *in vivo*, as: i) diversity varies drastically through microbiota isolation, and ii) products accrue throughout fermentation (den Besten et al., 2013). The predominant microbes in the colon are the three dominant phyla: Firmicutes (gram-positive), Bacteroidetes (gram-negative), and Actinobacteria (gram-positive). The Bacteroidetes phylum primarily produces acetic acid and propionic acid, while the phylum Firmicutes produces butyric acid as its principal metabolic product (Macfarlane and Macfarlane, 2003). Proximal colon is the place for some bacterial actions where the supply of substrates are maximal. Table 2.7 shows an *In vitro* batch fermentation presentation of several dietary fibers using human fecal inocula. The supply of substrates decreases with respect to the distal colon and free water extraction increases microbial products by causing diffusion of substrates. This makes the proximal portion of the colon a primary fermentation place. Saccharolytic bacteria belonging to the phyla Bacteroidetes and Firmicutes help ferment non digestible carbohydrate to produce SCFA, and gases under low pH at the proximal colon (Gibson et al., 2017).

Proteins and amino acids fermentation by bacteria like Bacteroidetes which are main fermenters happens at distal portion inside the colon via subordinate degraders like bacteria which are proteolytic in nature. Amino acids and proteins breakdown and results in branched-chain fatty acids (BCFA), together with possibly poisonous waste products like volatile sulfur compounds, amines, and phenolic compounds (Millet et al., 2010).

Earlier research suggested the ability of resistant starch to moderate *in vitro* development of SCFA in animal and human feeding studies (Birt and others, 2013; Nugent 2005). Several human investigations have found a rise in SCFA, specifically butyrate, faecal excretion when resistant starch was used for supplementation (Nugent 2005; Young and Le Leu 2004; Birt and others, 2013). Most studies did not convincingly indicate all types of resistant starch increases SCFA development in equal manner. For four weeks, forty-six healthy adults were given twenty-five grams of non-starch polysaccharide alone plus twenty-two grams of resistant starch from a mixture of sources [*Himalaya* 292, leguminous can and HAMS (resistant starch type 2; HI-MAIZE 260

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RS)]. In all, acetic acid, butyric acid, and total short chain fatty acid concentrations were meaningfully advanced in the resistant starch group, associated with baseline levels and control (non-starch polysaccharide alone group). There were, however, a significant mark of inter-individual response disparities, with variations in concentration and excretion (McOrist et al. 2011).

In animal models and human studies, it is expected that RS do not easily reproduce the influence on SCFA development recorded *in vitro*. Types of resistant starch, esterification and food types should be considered since resistant starch is not a homogeneous category. Recent *in vitro* studies investigating the degradation of resistant starch type 3 by various bacteria from different plant sources have recorded variations of concentrations of short chain fatty acids (Purwani et al. 2012). Many factors affect the development of short chain fatty acids, such as the assimilation site, digesta's moisture content and the rate of transit (Salonen et al., 2014).

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Table 2.7: *In vitro* batch fermentation performance of various dietary fibers using human fecal inocula.

Dietary Fibre/substrate	Fermentation Rate	Dominant SCFA	Microbiota composition	Reference
Resistant starch type 2	Slow	Acetate↑ and Butyrate↑	*Bifidobacterium* ↑, *Blautia* ↓, *Dorea* ↓, *Bacteroides* ↓	Plongbunjong and others, 2017; Yang and others, 2013
Resistant starch type 3	Slow	Acetate↑ and Butyrate↑	*Bifidobacterium* ↑	Arcila and others, 2015; Jonathan and others, 2012; Plongbunjong And others, 2017
Resistant starch type 4	Slow	Acetate↑ and Butyrate↑	*Bifidobacterium*↑	Bae et al., 2013; Thompson et al., 2011
Arabinoxylan	Partial Fermentation	Acetate↑ and Butyrate↑	*Bacteroides*↑, *Coprococcus*↑, *Faecalibacterium*↑	Yang and others, 2013; Chen and others, 2017; Rumpagaporn and others, 2015,2016
Xyloglucan	Partial Fermentation	Propionate↑ and Butyrate↑	*Lachnospiraceae*↑, *Bacteroides*↑	Tuncil and others (2017)

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Inulin	Partial Fermentation	Acetate↑ and Butyrate↑	*Bacteroides*↓, *Bifidobacterium*↑, *Catenibacterium*↑, *Collinsella*↑, *Dorea*↓	Yang and others (2013)
Pectin	Fast	Acetate↑ and Propionate↓	*Bacteroides*↓, *Bifidobacterium*↑, *Dorea*↓, *Parabacteroides*↓	Bang et al., 2018; Ferreira-Lazarte et al., 2018; Jonathan et al., 2012; Yang et al., 2013
Guar gum	Partial Fermentation	Acetate↑, Propionate↓ and Butyrate↑	*Roseburia*↑	Jonathan et al., 2012; Yang et al., 2013
Glucomannan	Fast	Acetate↑, Propionate↑ and Butyrate↓	N/A	Jonathan et al. (2012)
β-glucan	Partial Fermentation	Acetate↑ and Butyrate↑	*Coprobacillus*↑, *Dorea*↓, *Lactobacillus*↑, *Enterococcus*↑	Hughes et al., 2008; Jonathan et al., 2012; Yang et al., 2013
Cellulose	Very limited	Acetate↑ and Butyrate↑	*Bacteroides* sp.↑, *Ruminococcus* sp.↑,	Chassard et al., 2010; Jonathan et al., 2012

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			Enterococcus sp.↑	
Fructo-oligosaccharides	Fast	Acetate↑ and Butyrate↑	*Lactobacilli*↑ and *Prevotella*↑	Chen et al., 2017; Jonathan et al., 2012; Li et al., 2015
Galacto-oligosaccharides	Fast	Propionate↑ and Butyrate↑	*Bifidobacteria*↑	Li et al., 2015

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2.6.2. SCFA production and bacterial pathways

The bacteria in the gut can degrade resistant starch into monomers/glucose to be fermented throughout the anaerobic intestinal setting. Embden-Meyerhof-Parnas is the main pathway for the bacteria metabolite (glycolysis, i.e. sugars that contain 6-carbon) and the pentose-phosphate route (i.e. sugars that contain 5-carbons), this transforms the monosaccharides to phosphoenolpyruvate (PEP) (Fischbach and Sonnenburg, 2011). Consequently, PEP is transformed to products to be fermented like organic acids or alcohols.

Figure 2.12 explains the diagrammatic overview of the three routes on how to expel excess decreasing equivalents:

A: Pyruvic acid is reduced into lactic acid thus reducing NADH (1), pyruvic acid: ferredoxin oxidoreductase and hydrogenase (2a) or NADH: ferredoxin oxidoreductase and hydrogenase to reduce its equivalent into hydrogen molecule (2b), and basic anaerobic electron transport chain for the reduction of NADH (3). B, C: diagrammatic summary of the fabrication of acetic acid, propionic acid, and butyric acid from carbohydrates. B: Acetic acid is formed either straight from acetyl CoA or through the Wood-Ljungdahl route with formate. Propionic acid is created from phosphoenolpyruvate over the succinate decarboxylation path or over the acrylate route in which lactic acid is abridged to propionic acid. C: Acetyl CoA is reduced by 2 molecules to generate butyric acid using butyrate-kinase enzyme or using acetic acid utilizing enzyme butyryl- CoA: acetate-CoA-transferase.

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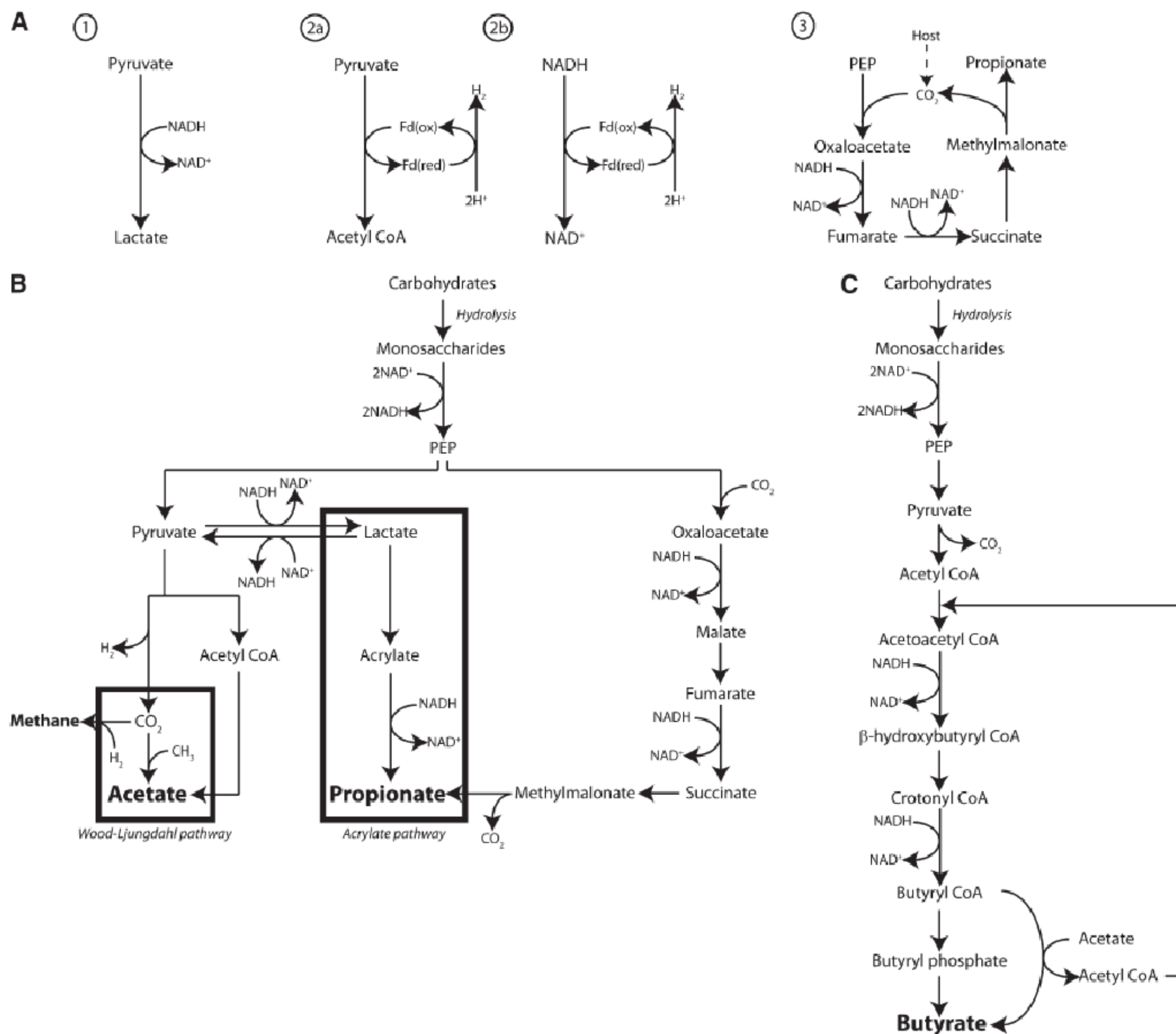

Figure 2.12: Schematic overview of the 3 paths that bacteria in the gut uses to expel excess reducing equivalents

2.6.3. Energy source from SCFAs

Short-chain fatty acids are basically energy sources. Short chain fatty acids deliver to humans about ten percent of calories daily (Bergman, 1990). Quantifications of CO_2 development in colon epithelial cells that has been isolated have revealed that colon epithelial cells get sixty seven percent of their energy source from short chain fatty acid oxidation (Roediger, 1982). The predominant idea is that colon epithelial cells desire butyric acid to acetic acid and propionic acid

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and oxidization of it to CO_2 and ketone bodies (Roediger, 1982). This is because of the colonocytes comparative high affinity to butyrate.

Ruminants rely on 80 percent of their energy on SCFAs (Bergman, 1990). After transforming propionic acid into propionyl-CoA by propanoate: CoA ligase (AMP-forming), propionyl-CoA is transformed to succinyl-CoA in three successive steps catalyzed by propionyl-CoA carboxylase, methyl malonyl-CoA epimerase, and methyl malonyl-CoA mutase. Tricarboxylic acid (TCA) cycle receives Succinyl-CoA which is transformed to oxaloacetate, gluconeogenesis forerunner (Bloemen and others, 2010).

The luminal butyrate is transferred by the monocarboxylate transporter 1 (MCT1) along with a proton from the colonic lumen to the colonocytes cytosol (Hadjiagapiou et al., 2000; Ritzhaupt et al., 1998). Butyrate is then transmitted to undergo b-oxidation via MCT1 or simple diffusion into the mitochondria. The 2 new molecules of acetyl CoA will release 2 ATP molecules or be metabolized along the HMG-CoA synthase path into ketone bodies. MCT4 (and MCT5) are believed to be elaborate in the efflux of butyrate and other SCFAs (acetate, propionate), ketone bodies, or monocarboxylic acids (pyruvate, lactate) by basolateral membrane

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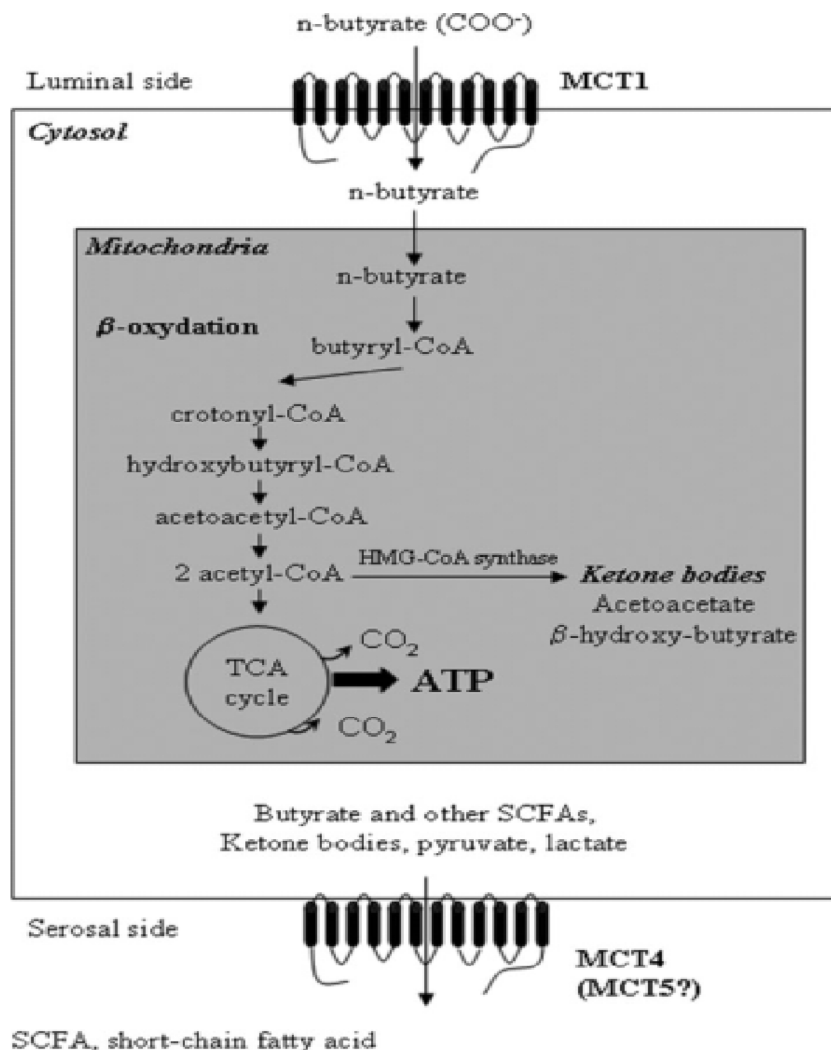

Figure 2.13. Butyrate b-oxidation in the mitochondria of colonocytes path.

2.6.4 SCFA and Immune function

Current investigation into SCFA's immune function and its function in controlling response to inflammation and immune system has arisen. This seems to be an interesting area because earlier work had established that probable function of butyrate in immune instruction, Butyrate can impede nuclear factor kappa β (NF-kB) activation in macrophages and prevents acute myeloid leukemia by preventing histone deacetylation (HDAc) (Luhrs et al., 2002; Maeda, Towatari, Kosugi and Saito, 2000). Eukaryotic transcription factor NF-kB is included in regulating the plethora of natural cellular functions, involving responses of inflammation and immunity. HDAc prevention also serves a function and epigenetic pathways in a specific inflammatory signaling

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route (Flint et al., 2012). Lately, two investigations have emphasized a likely role for propionic acid and butyric acid in regulatory T cell development and role, through inhibition of HDAc at the whole-animal level (Arpaia et al., 2013; Furusawa and others, 2013).

Short-chain fatty acids role also spreads to peripheral immune function. A current experiment showed that acetate in a murine gut model mediates joint inflammation via inflammatory development and assembly of IL-1b, which is partially dependent on FFAR2.90

2.7. Gut microbiota and resistant starch

Understanding of beneficial bacteria in the gut in relation to human health is well recognized. Good bacteria can affect nutritional benefits and immune function (Sekirov and others, 2010), appetite control pathways, as seen in many experiments on mice (Lyte and others, 2016), Illnesses like mental health issues (Flowers & Ellingrod 2015) and obesity and the underlying physiological discrepancies, (Arora & Backhed 2016; Kobyliak and others, 2016). Food intake is believed to affect the microbial populations in the gut (Birt and others, 2013), thus, the possibility of resistant starch impacting gut microbiota is of concern. DNA investigations on RS indicates that effects of resistant starch on intestinal microbiota on colonic pH, composition of SCFA, reduction of detrimental metabolites (e.g., bile acids, phenols, and ammonia) and enzymatic behavior correlated with bacterial breakdown pathway has been postulated. Improvements in sequencing stages and molecular techniques on culture-independent focused on 16S rRNA analysis provided for more comprehensive studies of how bacterial communities relate with the diverse types of starch (Martinez and others, 2010). *Ruminococcus bromii* is associated with abundance and substantial in the fermentation of complex carbohydrates like RS in humans (Abell et al. 2008).

The effect of approximately twenty-five gram of resistant starch type 3 or non-starch polysaccharide (wheat bran) or a weight-loss diet on colonic microbiota in obese men was investigated (Walker and others, 2011). Making use of 16S rRNA gene sequencing, bacterial profiles were established as being stable over time for a given diet within an organism, but significantly changing between diets. Notwithstanding the absence of a veritable control group, *R. Bromii* improved most in resistant starch diet (seventeen percent of overall bacteria verses 3.2 percent during the non-starch polysaccharide diet), whereas changes in *Oscillibacter guillermondii* bacteria were also found during the resistant starch diet and *Eubacterium rectale* was improved

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with the resistant starch diet to around ten percent of overall bacteria during the weight loss portion).

2.8. Concluding Remarks

- Heat-moisture treatment of starch induces molecular rearrangement and chain interaction, as well as the intra and inter-molecular chain interaction with water before and after treatment. Heat-moisture treatment produces gelling starches, and these are not desirable in some foods.

- Stearic acid addition to starch encourages the establishment of amylose-lipid complexes and these improve the non-gelling tendencies of starch and hence are desirable where gelling starches are not applicable, for example as fat replacers.

- Amylose-lipid complexes have a significant impact on starch's physico-chemical and rheological properties, promoting high starch viscosities during pasting, reduced breakdown and hence reduced retrogradation, higher relative crystallinity, and augmented inhibition to enzyme digestibility.

- Unmodified maize meal, on the other hand, is a health problem because of its rapidly digested starch and high glycaemic index, making it inappropriate for persons with diet-related non-communicable illnesses like type 2 diabetes and obesity.

- Amylose-lipid complexes which is known as resistant starch type 5 is also considered as dietary fibre. The dietary fibre has been endorsed as possibly useful in terms of faecal fermentation to produce short chain fatty acids.

- A number of investigations have concentrated on relationship between dietary fibre and the creation of SCFA as well as the bacteria responsible for the fermentation, but there are very limited information on the utilization of amylose-lipid complex by the gut bacteria.

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3.0 HYPOTHESIS AND OBJECTIVES

3.1 HYPOTHESIS

3.1.1 Hypothesis 1:

Maize starch and maize meal treated with stearic acid and heat moisture treatment alone and their combination will promote the formation of amylose-lipid complex (ALC). This complex formed will reduce peak, breakdown, and final viscosity, produce non-gelling product. The complex formation will also reduce starch digestibility, decrease the EGI (estimated glycaemic index). Amylose-lipid complexes have been shown to lower glycaemic and insulinemic responses (Hasjim et al., 2010). Amorphous amylose and most A-type polymorphism starches are further impervious to amylolytic enzyme hydrolysis than amylose-lipid complexes. The molecular structures of the complexing lipid and the amylose determine enzyme resistance to amylose-lipid complexes. (Gelders et al., 2005; Kitahara et al., 1996) and on the crystalline structure of the amylose-lipid complex (Seneviratne & Biliaderis, 1991).

3.1.2 Hypothesis 2

The indigestible residues of modified and unmodified maize starch and maize meal will be fermented in the colon by the gut microbiome to produce gases and short chain fatty acids under low luminal pH. The indigestible residues which can be called resistant starch or dietary fibre have been known to generate short chain fatty acids such as acetate, propionate, and butyrate (Bird *et al* 2000). Low luminal pH is known to impede the development of pathogenic bacteria and the reduction in the production of secondary bile acids (O'Hara & Shanahan, 2006; Videla et al., 2001; Galvez et al., 2005) as well as increase in the absorption of mineral. Production of SCFAs is due to reduction of the pH. SCFAs are beneficial to the gut by providing the energy source as well as regulating glucose homeostasis, immunomodulation, appetite regulation and obesity (Scholz-Ahrens et al., 2007; Rose et al., 2007).

3.1.3 Hypothesis 3

Indigestible residues of modified and unmodified maize starch and maize meal in the gut would act as a prebiotic for the gut microbiome. This prebiotic will promote the growth of some of the bacteria in the gut.

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It is known from research that prebiotics are responsible for the development and the growth of most microbes in the gut since most of the microbes utilized the prebiotics (Leitch et al, 2007). In the gut, most bacteria that predominantly utilize prebiotics belong to the phyla Firmicutes, Bacteroidetes and Actinobacteria (Kovatcheva-Datchary et al, 2009). Most bacteria that falls under these phyla promotes the production of acetate, propionate, and butyrate which promotes gut health (Martínez et al. 2010).

3.2 OBJECTIVES

3.2.1: Objective 1:

To investigates the effects of heat-moisture treatment with added stearic acid on the *in vitro* starch digestibility and functional properties *(reduced peak viscosity, increased final viscosity, reduced breakdown viscosity and non-gelling tendencies)* of maize meal and maize starch with the aim of producing a lower Glyceamic Index maize meal and maize starch

3.2.2: Objective 2:

To determine the effects of indigestible residues from unmodified and modified (with and without stearic acid and HMT) maize meal and maize starch produce SCFA during *in vitro* faecal fermentation with the aim of promoting health benefits.

3.2. 3: Objective 3:

To determine the effect of indigestible residues from modified and unmodified (with and without stearic acid and heat-moisture treatment) maize meal and maize starch on the human gut microbiota during *in vitro* faecal fermentation.

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4.1. In Vitro Starch Digestion and Physicochemical Properties of Maize Starch and Maize Meal Modified by Heat-Moisture Treatment and Stearic Acid

Abstract

The physicochemical and nutritional properties of heat-moisture treated (HMT) maize starch and maize meal with stearic acid (SA) were studied. The addition of SA followed by HMT produced nongelling starch and maize meal porridge with reduced pasting viscosity. Heat-moisture treatment significantly ($P \leq 0.05$) decreased the starch hydrolysis, increased resistant starch, and lowered estimated glycaemic index of both maize meal and maize starch with SA. These changes are due to a more organized crystalline structure between starch polymers and well as the formation of amylose–lipid complexes as shown by differential scanning calorimetry and X-ray diffraction. There seemed to be a synergistic effect between HMT and stearic addition as HMT promoted more starch polymer interaction compared to amylose–lipid formation for stearic acid addition. These results suggested that HMT combined with SA can be used to manufacture starch-based functional ingredients and foods with reduced glycaemic index

This part of the study has been published:

Asare, I. K., Mapengo, C. R., & Emmambux, M. N. (2021). In Vitro Starch Digestion and Physicochemical Properties of Maize Starch and Maize Meal Modified by Heat-Moisture Treatment and Stearic Acid. Starch-Stärke, 73(3-4), 2000128.

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4.1.1. INTRODUCTION

Maize meal is considered a staple and traditional diet in Southern Africa and other sub-Saharan countries (Van der Merwe 2001). It is prepared from maize flour through milling and contains about 70 to 75% starch (Arendt & Zannini 2013). Maize meal can be cooked as soft porridge and as a stiff pap. Soft porridge is a thin gruel which flows but stiff pap is a soft gel, that does not flow. Most staple foods like maize and cassava are considered to be high glycemic index (GI) foods (Kouamé et al, 2014) and have GI of about 74. The high glycaemic index is because of high percentage of starch assimilation that results in rapid absorption of glucose to increase blood glucose level. There are some concerns over the consumption of high GI foods, as the latter triggers hyper-postprandial glucose responses and has been linked with type 2 diabetes (Benmoussa et al 2004).

Starch can be divided into 3 portions; starch digested quickly (RDS), starch digested gradually (SDS), and starch resistant to digestion (RS). Englyst et al. (2003) stated that RDS and SDS are starch portions which are hydrolysed to dextrins by α-amylase within 20 to 120 mins after assimilation, respectively. SDS is gradually assimilated all through the small intestine, resulting in a slow and lengthy release of glucose into the bloodstream, associated with low glycemic response. RS is the fraction that is not hydrolysed after 180 min and continuous to the large intestine where it is fermented by gut microbiota (Englyst et al. 2003). RS is also sub-divided into 5 categories (Jiang & Jane 2013). RS 1 is physically unavailable starch to be assimilated. RS 2 is composed of native granules with structures making the starch slow to digest (Jiang & Jane 2013). RS 3 is retrograded starch. RS 4 are chemically improved starches (Rombo et al., 2004). RS 5 can be considered a starch with amylose-lipid complexes (ALC) (Hasjim et al., 2013). ALCs are said to be dietary fibre and thus have health benefits (Codex Alimentarius, 2010). ALCs contribute towards controlling postprandial glycemic and insulinemic responses and preventing colon cancer (Nugent, 2005).

Amylose-lipid complex is formed through an interaction between a fatty acid and the hydrophobic core of the amylose helix (Gelders et al., 2004). Fatty acids fit into a category of lipids which are considered amphiphilic compounds (Campbell & Farrell, 2003). The ALC is generally

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strengthened by intermolecular bonding between the amylose glucose residues, water molecules and ligand (Gelders et al., 2006).

Heat moisture treatment (HMT) is a procedure that includes treatment of starch at low moisture levels (<35% moisture w/w) for a certain period (15 min–16 h) and at temperatures (84–120 °C) below the temperature of gelatinisation and above glass transition temperature. HMT induces molecular rearrangement of amylose–amylose interaction (AM-AM) within the amorphous domain, amylose-amylopectin side-chain interaction (AM-AMP) and the complex formed between the amylose helix and the endogenous lipid to form amylose-lipid complexes (Adebowale and Lawal, 2003).

Sievert and Pomeranz (1989) reported HMT reduced enzyme susceptibility when they produced RS from normal and waxy starches at 18% moisture and a temperature of 110°C.
Chung et al. (2009) worked on RS levels of corn, pea, and lentil starches and reported that RS levels increased from 4.6%, 10.0%, 9.1.% to 12.3%, 14.5%, 14.7% respectively after HMT (120 °C, 1h and 30% moisture content). Sang and Seib (2006) stated that subjecting Hylon V starch (about 50% amylose) to concurrent HMT (45% moisture, 110°C, 4 h) and phosphorylation (sodium trimetaphosphate/sodium tripolyphosphate) improved RS by 19% and reduced SDS and RDS by 12% and 6%, respectively. HMT of starches has been examined as a method of increasing the percentage of SDS and RS due to the altered crystalline nature making the glycosidic bonds inaccessible for enzyme hydrolysis (Chung et al., 2009).

Several researchers have studied the effects of stearic acid addition alone and HMT alone on starch (Mapengo et al. 2019). There is little information about the effects of addition of stearic acid (SA) together with heat-moisture treated (HMT) of maize meal and starch on their digestibility. It was therefore hypothesized that SA and HMT together for both starch and maize meal will reduce the digestibility of both starch and maize meal and the pasting as well as gelling properties. The current study investigates the effects of SA plus HMT on the *in vitro* starch digestibility of maize meal and starch and their health benefit to consumers.

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4.1.2. MATERIALS AND METHODS

4.1.2.1. Materials

Superfine maize meal was obtained from Rainbow Chicken Limited (RCL) (Pretoria, South Africa). It contained 15.3 % moisture, 8.2% protein, 1.1% fat content, 0.65 % Ash and a total starch of 81.3%. Commercial normal starch, Amyral® with 12.9 % moisture, 0.64% protein, 0.31% fat content, 0.08 % Ash and total starch content of 95 % was obtained from Tongaat Hulett® Starch (Edenvale, South Africa). Stearic acid (SA) with CAS number of 57-11-4 was acquired from Sigma–Aldrich Company (St. Louis, MO, USA). The reagents used for this work were analytical standard.

4.1.2.2 Methods

4.1.2.2.1 Adding stearic acid (SA) into starch and maize meal

Stearic acid (SA) (1.5% w/w) was incorporated into samples using the method of Chang et al. (2013). Pure ethanol was used as a solvent to dissolve SA, and then the powdered samples were added. The blend was placed in the water bath and shaken at 50 °C for 30 min at 120 rpm. The samples were placed in an oven for solvent evaporation (at 40 °C).

4.1.2.2.2. Heat-moisture treated (HMT)

The starch and maize meal samples with and without SA were put together with approximately 20% moisture which is the desired moisture for HMT. The starch mixture of insoluble particles was heated at 110 °C for 16 hr (Maache-Rezzoug et al., 2008) in an oven.

4.1.2.3 Analyses

4.1.2.3.1. Starch and maize meal pasting properties

Samples were pasted according to Wokadala et al. (2012) with modification using a cup and a stirrer (ST24-2D/2V/2V-30) in a Rheometer (Physica MCR 101, Anton Paar, Germany). Starch slurry (10 % w/w) were pasted by stirring primarily at 960 rpm at 50 °C for 10 s, heating to 91°C at 7.5 min at 160 rpm and holding at 91°C for 15 min at 160 rpm. The paste was allowed to cool from 91 °C to 50 °C at 7.5 min, then allowed to stand for 1min at 50 °C. After which the pastes

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were freeze-dried. The freeze-dried samples were then crushed using mortar and pestle. Samples were kept in an airtight container at 5 °C before analysis.

4.1.2.3.2. Pasted starch texture

The texture of the pasted starch was analysed with a modified procedure from Bultosa and Taylor (2004b). The produced paste was emptied into small sphere-shaped vessel of diameter 39.5 mm and a depth of 11.5 mm. The depth of each dish was increased by approximately 5 mm by paper tape around its rim. The gels were kept at 25 °C overnight in covered containers. After the paper tape was removed, excess gel above the rim was removed by cutting the surface to be smooth using a wire cheese cutter. A 20 mm cylindrical probe was used to analyse the gel firmness using texture analyzer EZ-L, Shimadzu (Tokyo, Japan). Infiltration effect of the gels were 5 mm deep and 2 mm/s speed at a temperature of 25 °C using a plunger (diameter) and then withdrawn from the sample. The maximum force and least force were taken as the firmness and adhesiveness respectively.

4.1.2.3.3. In vitro starch digestibility and estimated glycemic index

A modification of the Goňi et al. (1997) procedure was used. The cooked and freeze-dried starch and maize meal which contains 50 mg of the starch was used according to the user's manual. HCl-KCl buffer (pH 1.5) and a solution of 0.2 ml comprising 1mg pepsin (Sigma-Aldrich P7000- 100 G) was added to the samples. Samples were then allowed to stay for 60 min at 40 °C with constant shaking. The rest of the reagents and enzyme solutions were added as per the steps/ sequence outlined by Dolapo & Emmambux (2017). Concentrated glucose was estimated using glucose oxidase-peroxidase kit, and the degree of in-vitro starch digestion was calculated as the percentage of the total starch assimilated at specific time intervals between 0 and 180 min.

The starch hydrolysis kinetics was estimated based on the equation by Goňi et al., (1997)

$$C = C_{\infty} (1 - e^{-kt})$$

where C is the concentration at time t, C_{∞} is the starch hydrolyses percentage after 180 min, k is kinetic constant (min^{-1}) and t is time (min). The parameters k and $C\infty$ were calculated for each treatment based on the data obtained from the in-vitro hydrolysis technique. Jaisut et al. (2008) proposed an equation to compute the area under the curve (AUC) as follows:

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$AUC = C_\infty (t_f - t_0) - (C_\infty / k)(1 - \exp(-k(t_f - t_0))$

t_f is the final time (180 min), t0 is the initial time (time 0).

The hydrolysis index (HI) = $\frac{\text{AUC of the sample}}{\text{AUC of the reference sample}} \times 100$

The estimated glycemic index (EGI) = 39.71+ 0.549 HI (Goňi et al., 1997)

Starch digested quickly, starch digested gradually, and resistant starch fractions were estimated using the enzymatic hydrolysis technique (Englyst et al., 1992). Starch digested quickly is calculated as a fraction of starch assimilated at 30 min, and starch digested slowly was calculated as the fraction of starch digested at 120 min. RS was the entirety of starch undigested and deducted from total starch.

Equations are as follows: RDS = G_{30} x 0.9; SDS = ($G_{120} - G_{30}$) x 0.9; RS = Glucose x 0.9

4.1.2.3.4. Thermal properties

Pasted and unpasted starch and maize meal were analysed using a differential scanning calorimeter (DSC) system (HP DSC827e, Mettler Toledo, Greifensee, Switzerland) (Wokadala et al., 2012). Aluminium pan was used in weighing the starch powder (10 mg, db) and distilled water was added in a proportion of 1:3 (w/w) starch-to-water to make a homogeneous suspension. Pans were then sealed and were allowed to stay at room temperature for at most 24 h. Scanning was done at a high pressure (using N_2 at 4 MPa) and a temperature of 25 °C to 140 °C at a rate of 10 °C/min. The standard used was Indium with Tp=156.6 °C and ΔH of 28.45 Jg^{-1} and an empty pan was used as reference.

4.1.2.3.5. X-ray diffractions for starch crystallinity

X'Pert PANalytical diffractometer (Eindhoven, Netherlands) was used for the wide-angle X-ray diffraction scattering on cooked and uncooked starch and maize meal. The cooked samples were freeze-dried and allowed to stay for five days, with an estimated relative humidity of 95% at 25 °C using glycerol (Wokadala et al., 2012). The conditions for the XRD to operate well were 45 kV, 40 mA, CuKα (0.154 nm). The overall level of crystallinity was resolved as the percentage incorporated territory peaks of crystallinity to the absolute coordinated area on a straight baseline (Wokadala et al., 2012).

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4.1.2.4. Data Analysis

A factorial design (2*2) was used which entailed SA at two levels, i.e., 0 and 1.5 % (w/w) and heat-moisture treatment at two levels (0% and 20% w/w moisture). The work was done in three independent experiments. MANOVA was used to determine the significant differences between SA addition and HMT sample. The data for starch and maize meal were analysed separately. Fischer's LSD Test at $P \leq 0.05$ was used to compare the means.

4.1.3. Results and discussion

Starch and maize meal with stearic acid (SA) addition and heat moisture treated alone and in combination had pasting properties which are shown in Figure 4.1.1 and Table 4.1.1. Compared to SA addition, HMT and the combination treatment, starch without treatment had significantly ($P<0.05$) higher peak and breakdown viscosities (Table 4.1.1, Figure 4.1.1a). The paste of raw starch produced a strong gel with a compression force of 6.43 N (Table 4.1.1). Addition of SA also produced a significant ($P \leq 0.05$) reduction in breakdown viscosity during cooling (Table 4.1.1, Figure 4.1.1a), and a non-gelling starch paste (Table 4.1.1) compared to the native starch. Similar findings have been reported (Mapengo et al. 2019) for raw maize starch and starch with added SA. Richardson et al., (2003) highlighted that monoglycerides coated the starch granules to increase its hydrophobicity. This layer reduces the capacity of the granules to imbibe water, thus reducing swelling and producing a reduced peak viscosity. The non-gelling starch also suggests that amylose-stearic acid complex was formed since amylose intermingles with SA which hinders the development of junction zones, and the latter is an integral part for gel formation (Gelders et al., 2004).

Heat moisture treatment (HMT) without SA further reduced the peak viscosity and produced a stronger gel than that of raw starch and showed no difference in the breakdown viscosity as compared with SA addition (Table 4.1.1, Figure 4.1.1a). Adding SA followed by HMT further reduced the peak viscosity, breakdown viscosity and viscosity during cooling with a non-gelling paste compared to the raw starch (Table 4.1.1, Figure 4.1.1a). The reduction of peak viscosity suggests that HMT reduced the amylose leaching and starch granule swelling (Liu et al., 2000). HMT alone increased gel hardness, and this is ascribed to the increased cross-linking between the starch polymer chains to form more junction zones (Liu et al., 2000). There was an interactive

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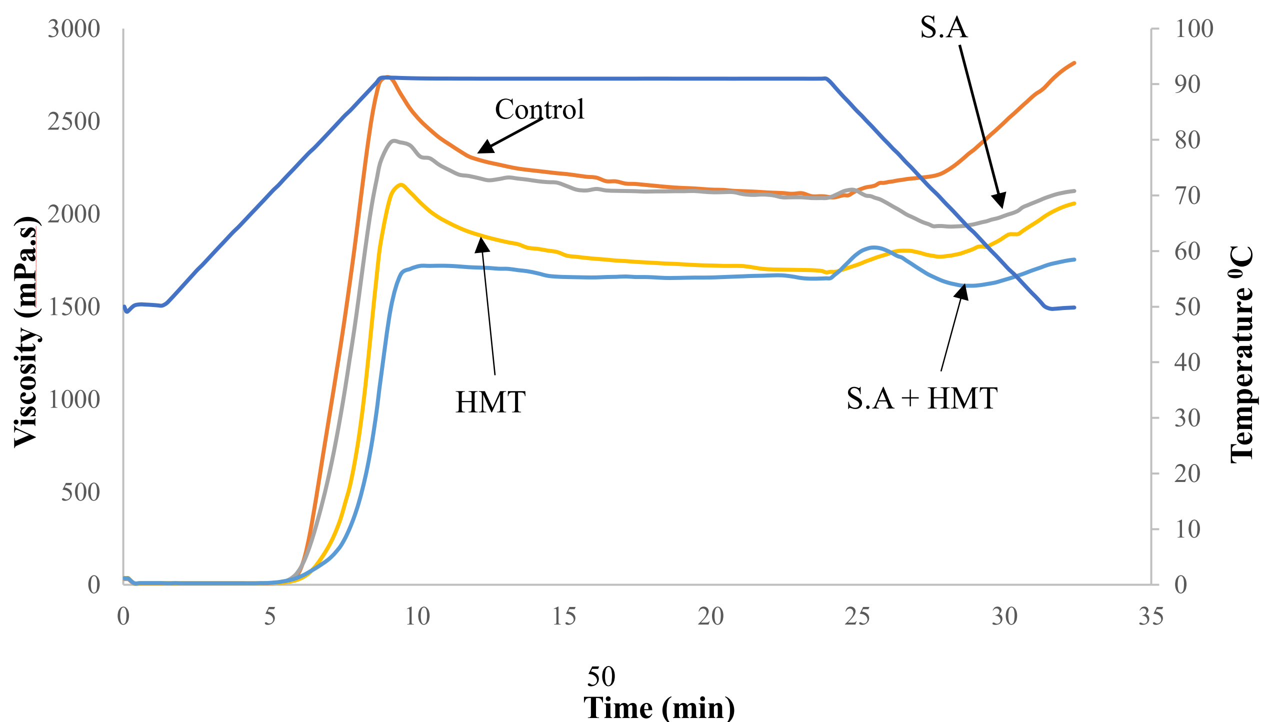
effect between SA and HMT at the peak viscosity and increased in viscosity during cooling. The other parameters showed no interactive effect.

Untreated maize meal showed no peak and breakdown viscosities, but there was an increased viscosity during holding, final viscosity and increase in viscosity during cooling when pasted (Table 4.1.1, Figure 4.1.1b). Addition of SA to maize meal led to significant changes in pasting properties compared to the raw maize meal (Table 4.1.1, Figure 4.1.1b). Addition of SA produced a non-gelling material (0.39N compressive force) compared to gel for control (1.47 N compressive force). HMT of maize meal significantly ($P \leq 0.05$) decreased the viscosity during holding, final viscosity and viscosity during cooling (Table 4.1.1, Figure 4.1.1b) as related to the raw maize meal. HMT treated maize meal formed a gel after cooling the paste for 24 h at 25°C with a compression force of 1.57 N (Table 4.1.1). There was also an interactive effect between the SA and HMT at highest viscosity during holding and the final viscosity. The combination treatment (HMT with added SA) exhibited a lower viscosity during holding, final viscosity and viscosity during cooling compared to the raw maize meal (Table 4.1.1, Figure 4.1.1b) but were like HMT treatment alone (Table 4.1.1, Figure 4.1.1b). However, the combination treatment did not gel (Table 4.1.1). The great difference in the pasting properties between starch and maize meal can be ascribed to their amylose, lipid, and protein content.

A: Maize starch



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B: Maize meal

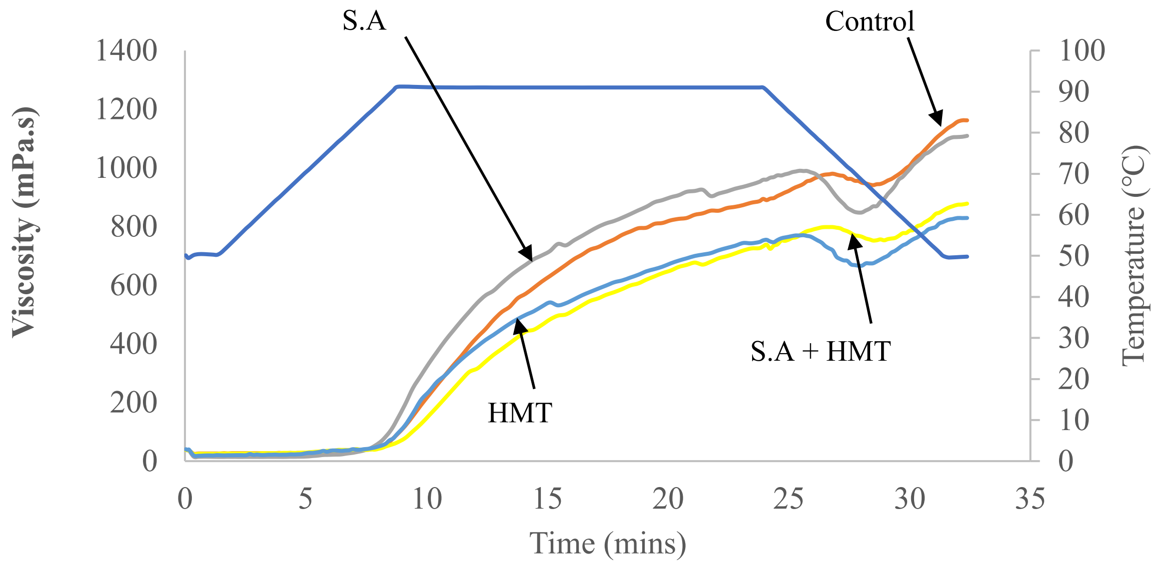

Figure 4.1.1: Effects the stearic acid and heat moisture treatment alone and in combination on the pasting properties of maize starch (A) and maize meal (B)

S.A (stearic acid) curve for maize starch and maize meal was added with 1.5% (w/w); HMT (Heat moisture treatment) curve for both maize starch and maize meal, treated at 110°C, 16 hrs and a moisture content of 20%; S.A + HMT curve for both maize starch and maize meal stands for combination treatment

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Table 4.1.1: Effects of HMT and SA on the pasting and gel properties of starch/maize meal.

Sample	Treatments: Heat Moisture Treatment (20%)	Treatments: Stearic Acid (1.5%)	Pasting temperature (°C)	Peak viscosity (mPa.s)	Trough (mPa.s)	Breakdown viscosity (mPa.s)	Highest viscosity during holding (mPa.s)	Final viscosity (mPa.s)	Increase in viscosity during cooling (mPa.s)	Firmness (Force /N)	Visual Perception
Maize Starch	None	0	68 ± 0.03^{c}	2756 ± 33^{d}	2129 ± 47^{b}	627 ± 14^{c}	2212 ± 19^{b}	2904 ± 125^{c}	690 ± 112^{c}	6.43 ± 0.33^{d}	Gel
	None	1.5 %	74 ± 0.50^{b}	$2354 \pm 45^{c*}$	1956 ± 185^{ab}	399 ± 139^{b}	2093 ± 54^{b}	2103 ± 30^{b}	$198 \pm 8^{a*}$	0.84 ± 0.13^{b}	Paste
	HMT	0	74 ± 0.82^{b}	$2155 \pm 3^{b*}$	1718 ± 42^{a}	437 ± 45^{b}	1843 ± 59^{a}	2075 ± 26^{b}	$278 \pm 13^{b*}$	4.86 ± 0.36^{c}	Gel
	HMT	1.5 %	71 ± 2.20^{a}	1725 ± 5^{a}	1626 ± 1^{a}	99 ± 6^{a}	1797 ± 17^{a}	1723 ± 43^{a}	111 ± 11^{a}	0.48 ± 0.04^{a}	Paste
Maize Meal	None	0	89 ± 0.51^{y}	ND	ND	ND	1006 ± 12^{y}	1199 ± 20^{y}	270 ± 2^{z}	1.47 ± 0.09^{y}	Gel
	None	1.5 %	84 ± 1.46^{x}	ND	ND	ND	$1013 \pm 124^{y*}$	$1178 \pm 97^{y*}$	193 ± 15^{y}	0.39 ± 0.02^{x}	Paste
	HMT	0	87 ± 0.45^{xy}	ND	ND	ND	$770 \pm 40^{x*}$	$843 \pm 50^{x*}$	144 ± 4^{x}	1.57 ± 0.33^{y}	Gel
	HMT	1.5 %	85 ± 0.81^{x}	ND	ND	ND	786 ± 22^{x}	863 ± 48^{x}	77 ± 4^{w}	0.51 ± 0.12^{x}	Paste

Mean values ± standard deviation of three independent experiments (n=3)
Means with different superscript alphabets are significantly different at $P \leq 0.05$; HMT = heat moisture treatment; Stearic acid (SA); *Interaction effect between SA and HMT; Breakdown=Initial peak – Trough; Gel is when the paste cannot be poured freely after 24 hr but a paste can be poured out freely; ND = not detected.

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The effects of SA addition and HMT alone and in combination on the enzymatic hydrolysis of pasted and freeze-dried starch and maize meal are represented in Figure 4.1.2 and Table 4.1.2. There were noticeable differences in the digestibility curves of both starch and maize meal treatments. White bread was a reference sample. After 30 mins, untreated starch and maize meal had about 60-70% of starch hydrolysed. Maximum hydrolysis of about 80% was observed at 60 mins for both untreated starch and maize meal, and then it reached a plateau. C_∞ (C infinity) indicates starch digested (until 180 min) expressed as a percentage. The raw starch and maize meal after 180 mins of digestion had 89% and 94 % respectively and a corresponding estimated glycaemic index (EGI) of 91 and 90 respectively (Table 4.1.2). This showed that not all the starches were digested. The estimated rapidly digestible starch (RDS), slowly digestible starch (SDS) and the resistant starch (RS) for both starch and maize meal were 77, 16, 7 and 76, 16, 8% respectively. The kinetic constant (K) gives an indication of the amylolytic digestion and the intrinsic susceptibility of the starch. Low k values suggest a slow rate of digestion, while high k values represent a rapid rate of digestion. The values from Table 4.1.2 and Figure4.1.2 showed that starch in raw starch and maize meal were rapidly digested and can thus be considered as high GI.

For starch with added SA alone, there was a gradual increase in the rate of starch digestion followed by a gradual decrease until it plateaued at about 60-90 mins, but in maize meal, there was a plateau after 60 mins of digestion (Figure 4.1.2 A&B). The first 5 mins saw gradual digestion until it reached 60 mins (Figure 4.1.2). After 180 mins, starch and maize meal with added SA had about 71 and 70 % starch digested (Table 4.1.2) and a corresponding EGI of 73 for both. The RDS, SDS and RS of added SA to starch and maize meal were 47, 22, 31 and 46, 25, 29 % (Table 2) respectively. There was notable ($P \leq 0.05$) improvement in the SDS and RS values with SA addition than control, but RDS displayed a significant ($P \leq 0.05$) reduction than the control. The starch K value (Table 4.1.2) was 0.1 $min^{-1,}$ and maize meal was also 0.08 min^{-1}. This means that maize meal and starch (with SA) were slowly digested related to the control. Lower starch digestibility suggests that there was a restriction by the enzymes to breakdown the starch polymer. This could be because of the addition of SA which complexes with the amylose to form amylose–SA complex, restricting alpha-amylase enzyme availability to the alpha 1,4 glycosidic bonds. Cui and Oates (1999) reported that the development of amylose-lipid complex decreases the digestibility of starch and modulates lower glycaemic response. There was a significant ($P<0.05$) interactive effect

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between SA and HMT in terms of RDS, SDS and RS for starch. In maize meal, the interactive effect occurred for RDS and RS only but not SDS.

Heat moisture treatment (HMT) alone of starch and maize meal also showed a plateau after 60 mins of hydrolysis in starch (Figure 4.1.2A) and after 30 mins of hydrolysis in maize meal (Figure 4.1.2B). Both HMT starch and maize meal after 180 mins were 54 and 64 % (Table 4.1.2) and a corresponding RDS, SDS, and RS values as 46, 22, 32 and 42, 25, 33 respectively (Table 4.1.2). The K value for both samples were 0.05 min^{-1} and 0.04 min^{-1}, suggesting a reduced susceptibility to enzymatic digestion and this corresponds to an EGI of 70 for both samples. There was a decrease in the RDS values in relation to the control (Table 4.1.2), but SDS and RS displayed a significant ($P \leq 0.05$) increase than the control. Chung et al., (2009) reported similar results and concluded that HMT induced the starch chains to intermingle and form double helices to maximize the stability of the granule to disruption. This is probably the reason for the rise in the SDS and RS values.

SA addition in combination with HMT of starch showed a gradual increase of hydrolysis from 5 mins to 60 mins after which it became a plateau. Maize meal was also digested for the first 5 mins to 30 mins and then reached a plateau (Figure 4.1.2 A&B). After 180 mins of hydrolysis, the starch and maize meal were 57 % and 70 % (Figure 4.1.2) with corresponding EGI value of 67 for both and hydrolysis index (HI) of 50 % also for both (Table 4.1.2). This can correspond to a medium and low eGI, respectively. The RDS, SDS and RS for both starch and maize meal were 43, 20, 37 and 44, 18, 38, respectively (Table 4.1.2). The K value of both starch and maize meal was 0.04 min^{-1} and 0.05 min^{-1}, suggesting a slow rate of hydrolysis. There was significant ($P \leq 0.05$) reduction in the RDS value but increase in the SDS and RS than the control.

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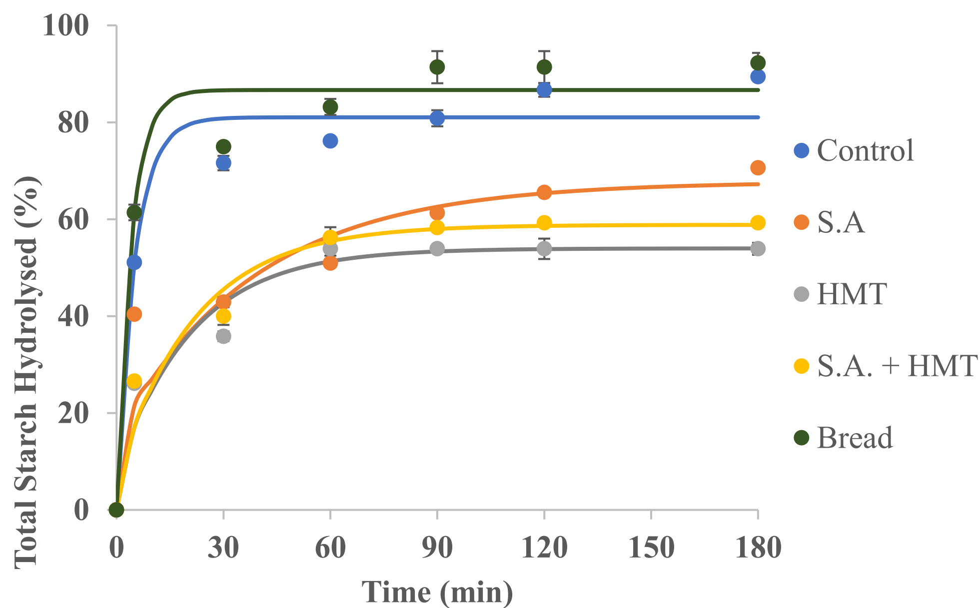
A: Maize starch



B: Maize meal

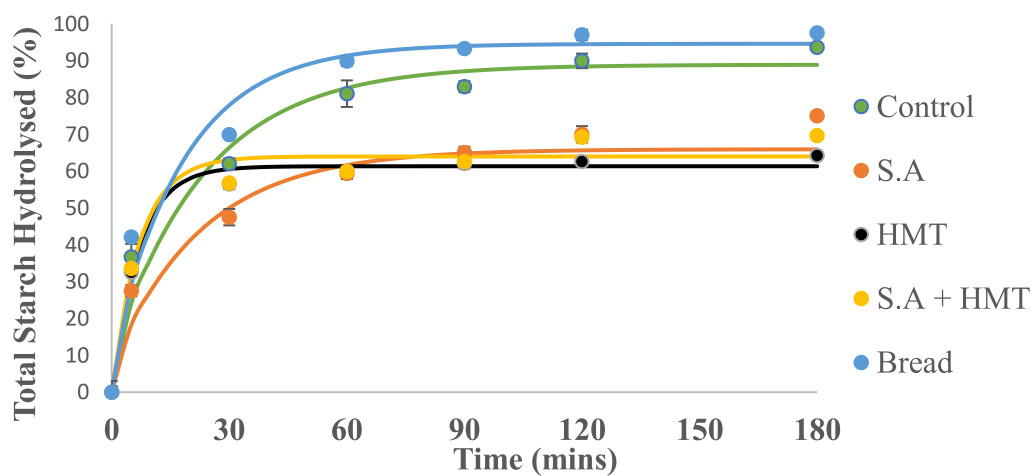


Figure 4.1.2: Effects of stearic acid addition and heat moisture treatment alone and in combination on the starch hydrolysis of maize starch (A) and maize meal (B) after 180 mins

S.A (stearic acid) curve for maize starch and maize meal was added with 1.5% (w/w); HMT (Heat moisture treatment) curve for both maize starch and maize meal, treated at 110°C, 16 h and a moisture content of 20%; S.A + HMT curve for both maize starch and maize meal stands for combination treatment

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Table 4.1.2: Effect of HMT and SA addition on the fractions of starch hydrolyzed and the digestibility kinetics of starch and maize meal

Sample	Treatment									
	HMT (%)	Stearic Acid (%)	C∞ (%)	K (min^{-1})	AUC	HI (%)	EGI	RDS (%)	SDS (%)	RS (%)
Starch	None	None	89±0.50^{b}	0.25±0.02^{c}	14025±0.02^{c}	93±1.53^{d}	91±0.84^{d}	77±1.2^{b}	16±1.43^{a}	7±0.23^{a}
	None	1.5%	71±0.21^{c}	0.10±0.01^{b}	10218±16.1^{b}	62±0.20^{c}	74±0.11^{c}	47±2.83^{a*}	22±1.30^{c*}	31±1.81^{b*}
	20 %	None	54±0.01^{a}	0.05±0.00^{a}	8652±34.8^{a}	55±0.01^{b}	70±0.35^{b}	46±1.68^{a*}	25±0.81^{bc*}	29±2.77^{b*}
	20 %	1.5%	57±0.17^{a}	0.04±0.00^{a}	9063±75^{a}	50±0.24^{a}	67±0.13^{a}	43±1.11^{a}	20±0.21^{b}	37±1.11^{c}
Maize meal	None	None	94±1.98^{x}	0.20±0.01^{y}	14032±230.7^{y}	90±1.39^{z}	89±0.76^{z}	76±1.59^{x}	16±0.87^{w}	8±2.45^{w}
	None	1.5%	70±1.53^{w}	0.08±0.01^{w}	10448±148.7^{w}	60±0.35^{y}	72±0.43^{y}	45±1.86^{w*}	22±2.50^{xy}	33±0.59^{x*}
	20 %	None	64±0.76^{y}	0.04±0.00^{w}	10452±18.2^{w}	55±0.01^{x}	70±0.01^{x}	44±3.1^{w*}	26±1.17^{y}	30±1.87*
	20 %	1.5%	70±0.77^{w}	0.05±0.01^{w}	10949±15.1^{x}	50±0.14^{w}	67±0.08^{w}	44±1.52^{w}	18±0.19^{wx}	38±1.39^{y}
Bread			92±2.09^{x}	0.25±0.02	15096±248.6	100±0.00	95±0.01	82±1.51	15±2.16	3±0.40

Mean values ± standard deviation of three independent experiments (n=3)

Different alphabets with the same superscript in the same column are significantly different ($p \leq 0.05$)

HMT = Heat Moisture Treatment. Stearic acid= SA; EGI = estimated glyceamic index (was calculated using the equation (39.71+ 0.549HI) according to Goni et al., [22]. HI = Hydrolysis index expressed as a percentage; K (min^{-1}) = kinetic constant deals with rate of digestion per minute; White wheat bread was the reference sample to calculate EGI; *means interactive effect.

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The melting endotherms for starch and maize meal are shown in Figure 4.1.3, with the significant parameters namely, To (onset temperature), Tp (peak temperature), Tc (conclusion temperature) and ΔH (enthalpy of gelatinization) recorded in Table 4.1.3. The raw starch and maize meal before pasting had To, Tp, Tc and ΔH as 64.5 °C, 69.6 °C, 75.7 °C and 8.2 J/g for starch, 63 °C, 67 °C, 73 °C and 0.9 J/g for maize meal respectively (Table 4.1.3). Figure 4.1.3 (C & D) shows the XRD results of unpasted starch and maize meal. XRD results for raw starch had 2θ degree peaks of about 6°, 10°, 12°, 15°, 17° and 23° (Figure 4.1.3C) and that of maize meal had 2θ degree peaks of about 5.8°, 11.2°, 15°, 17.2° and 23° (Figure 4.1.3D) respectively. The core diffraction peaks for both raw starch and maize meal before pasting were 15° and 23° and a double peak at 17° (Figure 4.1.3a & b). Both starch and maize meal had an A-type proposed crystalline organisation (Mapengo et al., 2019).

Addition of SA alone to starch before pasting produced a significant increase of ΔH value as 19.3 J/g and maize meal as 3.2 J/g (Table 4.1.3) than the control ΔH of 8.2 J/g. One endothermic peak (Figure 4.1.3A&B) was observed with Tp of 68.9 °C (Table 4.1.3), this means that the gelatinisation endotherm merged with pure SA since the melting point of SA is 69 °C and starch is between 64 °C and 80 °C. The XRD peak at 21° for both starch and maize meal corresponds to uncomplexed pure SA (Gelders et al., 2005). There was another peak at 7° and 12.3° for both starch and maize meal (Figure 4.1.3C&D) suggesting that addition of SA to both starch and maize meal produced V-type complex formation (Figure 4.1.3C&D). V-type amylose does not promote retrogradation for gel formation, as shown in Table 4.1.1. Starch and maize meal with added SA had crystallinity values significantly ($P \leq 0.05$) increased from 18.3 and 19.8 to 25.4 % and 25.8 % (Table 4.1.5) respectively. (Relative crystallinity estimated excludes pure SA).

Starch and maize meal with heat moisture treatment (HMT) before pasting showed one endotherm (Figure 4.1.3A &B). That endotherm signifies the gelatinisation temperature of unpasted starch and maize meal. The gelatinisation enthalpy significantly ($P \leq 0.05$) increased for both starch and maize meal to about 16.2 J/g and 1.5 J/g (Table 4.1.3) than that of control.

XRD pattern for control was similar to HMT unpasted starch and maize meal (Figure 4.1.3C & D). The HMT starch and maize meal had relative crystallinity of 22.7 % and 22.3 % (Table 4.1.5) respectively. This is significantly ($P<0.05$) higher than the control of both starch and maize meal. Increased in relative crystallinity can be ascribed to the development of crystalline helices which

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are in double form for amylopectin side chain as well as partial number of complexes formed from the amylose of starch and maize meal reacting with endogenous lipids (Hoover, 2000).

Adding SA followed by HMT starch and maize meal before pasting showed a significant ($P \leq 0.05$) increase than the raw sample with ΔH being 22.8 J/g (Table 4.1.3) for starch for the melting endotherm with Tp of 68.9℃ (Table 4.1.3). Maize meal before pasting had two endotherms with ΔH of about 1.25 J/g and 8.46 J/g at Tp of 70℃ and 93℃, respectively (Table 4.1.3). The second endothermic peak was ascribed to amylose-lipid complex type I (Sun et al., 2014). Amylose in the maize meal interacted with the lipids inside to form a complex known as amylose-lipid complex. The second endotherm noticed by the DSC was in pact with the XRD small peak at 7º (Figure 4.1.3D). This small peak corresponds with amylose-lipid complex (Godet et al., 1993).

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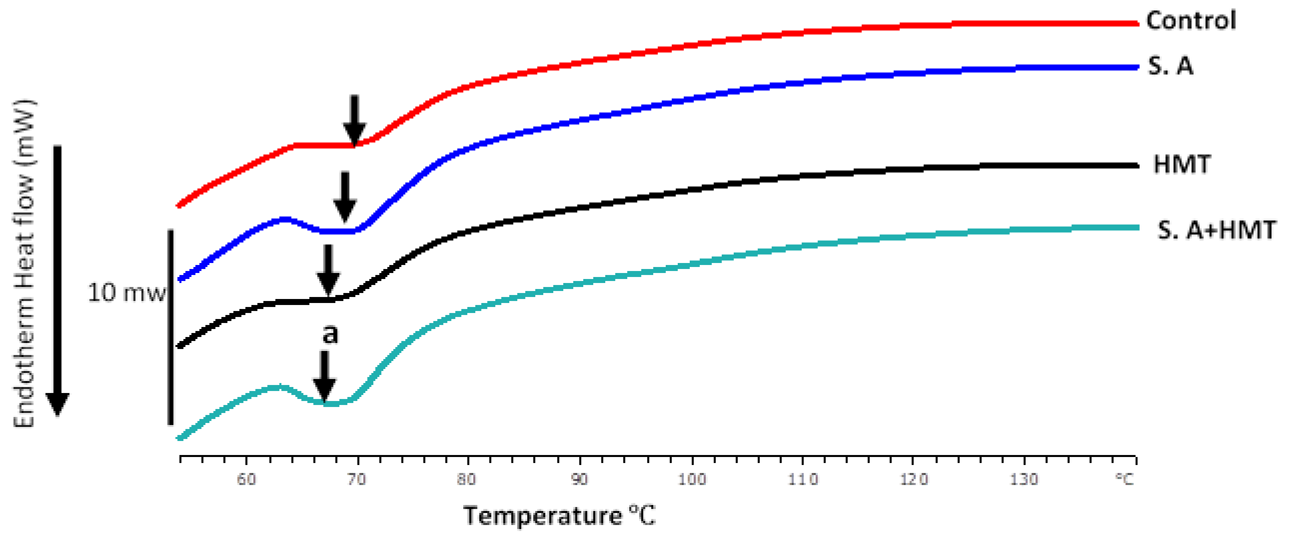
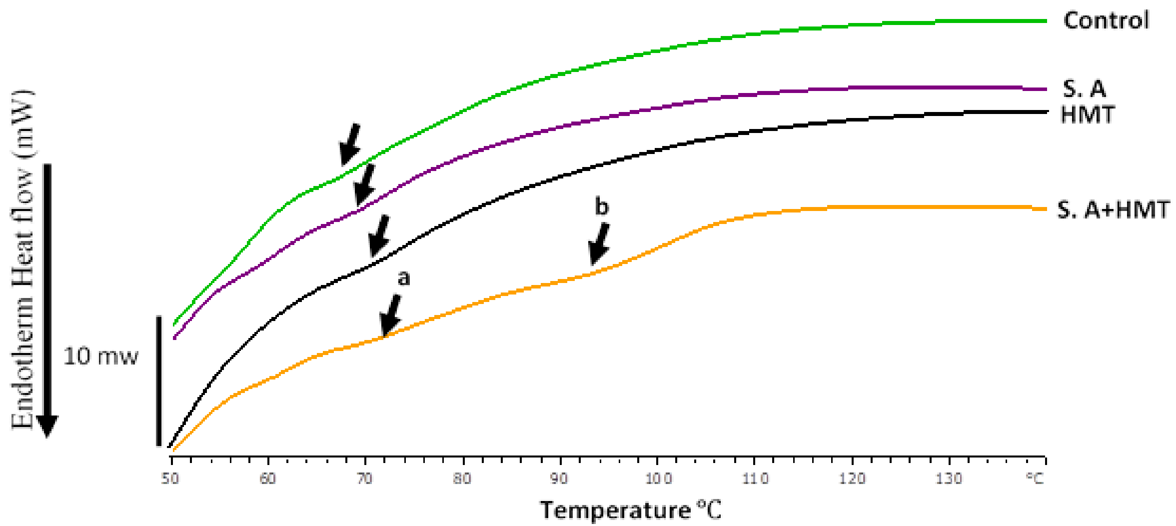
A: Unpasted maize starch



B: Unpasted maize meal



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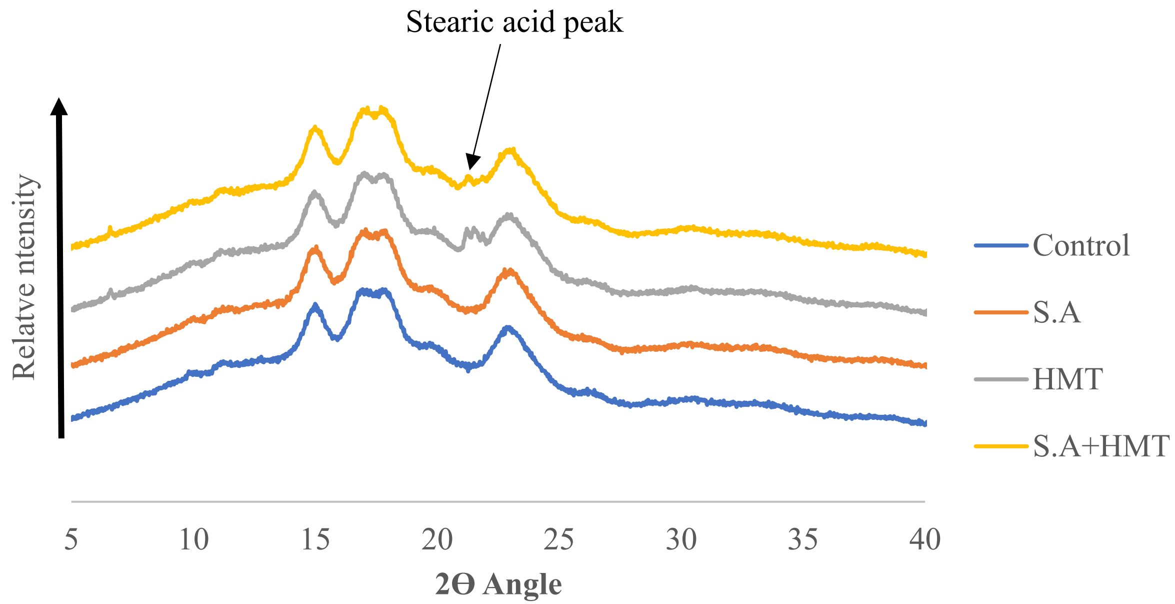
C: Unpasted maize starch



D Unpasted maize meal

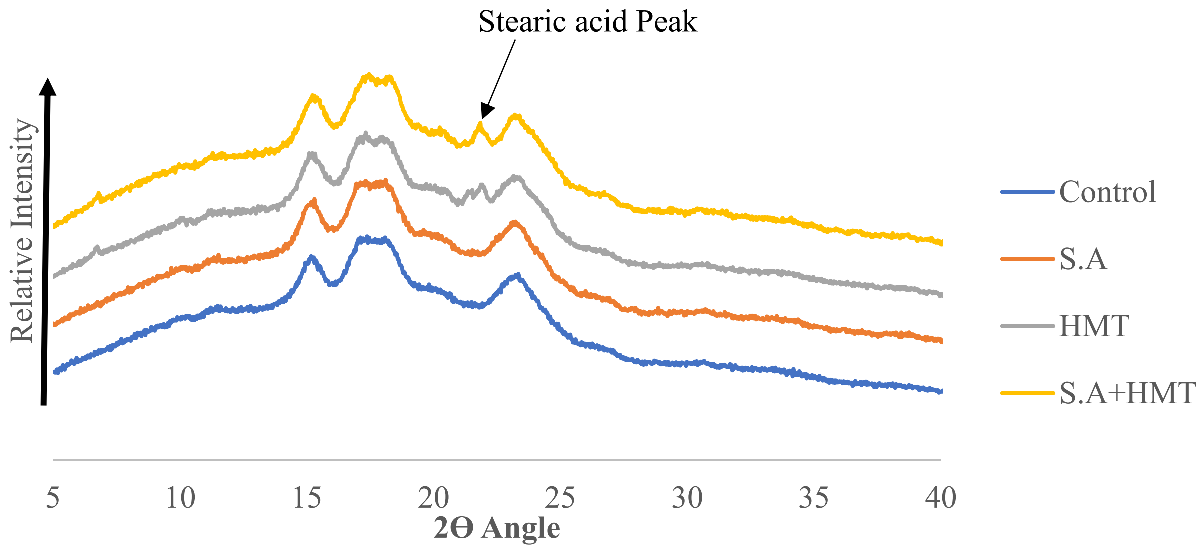


Figure 4.1.3**.** Effects of Stearic acid and heat moisture treatment alone and in combination on the thermal properties (A and B) and X Ray diffractogram (C and D) of unpasted maize starch and unpasted maize meal

S.A (stearic acid) curve for maize starch and maize meal was added with 1.5% (w/w); HMT (Heat moisture treatment) curve for both maize starch and maize meal, treated at 110°C, 16 hrs and a moisture content of 20%; S.A + HMT curve for both maize starch and maize meal stands for combination treatment

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Evaluation of starch and maize meal after pasting was also represented in Figure 4.1.4(A&B) and Table 4.1.4. Maize starch and maize meal in Figure 4.1.4(A&B) after pasting showed one endothermic transition peak with To, Tp, Tc and ΔH for starch as 119 °C, 125 °C, 132 °C and 3.86 J/g (Table 4.1.4) and maize meal also had 97 °C, 102 °C, 110 °C and 1.3 J/g (Table 4.1.4) respectively. Both starch and maize meal did not show any endotherm between 65°C and 85°C; this means the starch and starch in maize meal had gelatinized during pasting for 32 minutes (Figure 4.1.1 A&B). Figure 4.1.4(C&D) represents X-ray diffraction of starch and maize meal after pasting. Raw starch and maize meal had 2θ angle peaks at 6.9°, 11.5°, 15.6°, 17.4° and 21°; their relative crystallinity was 5.2 % and 5.75 % (Table 4.1.5) respectively.

Addition of SA to both starch and maize meal showed two endothermic peaks in starch (Figure 4.1.4A) and one peak in maize meal (Figure 4.1.4B) above 90 °C. The ΔH value significantly ($P \leq 0.05$) augmented than the control. Starch had ΔH value of 23.8 Jg^{-1} and 0.19 Jg^{-1} and maize meal had 1.9 Jg^{-1} (Table 4.1.4) at an endothermic peak of about 106 °C and 122°C and 103°C. Endotherms with dissociation temperatures of about (98-105) °C, (106-109) °C and (110-120) °C correspond to type I, IIa and IIb amylose-lipid complexes (Wokadala et al., 2012). The two endothermic peaks observed in the SA addition to starch and pasted agrees to type I and IIb amylose-lipid complex, and pasted maize meal also agrees to type I amylose-lipid complex (Table 4.1.4) (Wokadala et al., 2012). Pasted starch and maize meal had diffraction peaks of 2θ degrees at 7.5°, 12.7° and 19°, which is a major peak in determining amylose-lipid complexes (Figure 4.1.4C&D). Grouping of A-type and V-type X-Ray crystallography patterns was observed when pasted starch and maize meal with added SA were displayed (Figure 4.1.4C&D). This result confirms the amylose-lipid complexes detected through DSC in Figure 4.1.4(A&B). Amylose-lipid complex structures form a rigid crystalline structure which appreciates hydrogen bonds (Arijaje & Wang 2016). These amylose-lipid complexes contribute to the decrease in peak and breakdown viscosities (Figure 4.1.1A). This also suggests that the conformational changes were due to development of amylose-lipid complexes which restricted the action of alpha-amylase enzyme. This conformational change also showed that the enzyme could not access the alpha 1,4 glycosidic bonds for starch hydrolysis (Figure 4.1.2A&B). Pasted starch and maize meal with added SA significantly ($P \leq 0.05$) increased the relative crystallinity from 5.20 % and 5.75% to 8.35 % and 8.70 % (Table 4.1.5) than the control.

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HMT treated starch after pasting showed two transition endotherms (Figure 4.1.4A) and one endotherm for HMT maize meal (Figure 4.1.4B). The gelatinisation enthalpy for starch paste was 0.9 J/g and 6.17 J/g and maize meal was 1.6 J/g (Table 4.1.4) for the endothermic peak with Tp of 106°C and 127°C, (Table 4.1.4). This corresponds to amylose-lipid complex type I and type IIb respectively (Wokadala et al., 2012). Pasted maize meal had one endothermic peak of temperature 105 °C (Table 4.1.4) which corresponds to type I amylose-lipid complex. The XRD results followed the same pattern as control but showed a rise in the relative crystallinity value from 5.20 % and 5.75% to 7.50 % and 6.40 % (Table 4.1.5) for starch and maize meal. The high relative crystallinity is linked with the changes of the amorphous region to a more crystalline nature of starch (Hoover 1993). This can also be related to the low peak viscosity, breakdown, and final viscosity (Figure 4.1.1A and Table 4.1.1) and highest viscosity during cooling and final viscosity (Table 4.1.1 and Figure 4.1.1B). The more crystalline nature also suggests that the alpha-amylase enzyme could not access the alpha 1,4 glycosidic bond in both starch and maize meal (Figure 4.1.2A & B) for hydrolysis resulting in a reduction of RDS and a rise in RS and SDS values (Tables 4.1.2).

Adding SA to HMT starch and maize meal after pasting is represented in Figure 4.1.4(A&B). Pasted Starch had two transition endotherms (b & c) temperatures as 106 °C and 121 °C (Table 4.1.4), this corresponds to type I and IIb amylose-lipid complexes. Pasted maize meal had one endothermic peak of temperature 115 °C (Table 4.1.4), this agrees with amylose-lipid complex type IIa (Biliaderis & Galloway (1989). The gelatinisation enthalpy for pasted starch was 1.1 J/g and 16.85 J/g, maize meal had 37 J/g (Table 4.1.4) for this endothermic peak.

The XRD peaks of the combination treatment followed the same pattern as SA addition alone (Figure 4.1.4C&D). Both starch and maize meal had significantly ($P \leq 0.05$) increased relative crystallinity from 5.20 % and 5.75 % to about 12.8 % and 11.42 % (Table 4.1.5) respectively. The V-type crystallite (amylose-lipid complexes) can be associated with low pasting peak and breakdown viscosities observed in Figure 4.1.1A. This was because of the rigid network of structures in granules due to amylose-lipid complexes. This network of structure prevented granule swelling and leaching of amylose (Seo et al., 2015). These changes due to HMT with stearic acid addition also showed there are molecular changes in starch for restriction of the enzyme hydrolysis of the alpha 1,4 glycosidic bond to increase in RS, SDS and reduce RDS (table 2).

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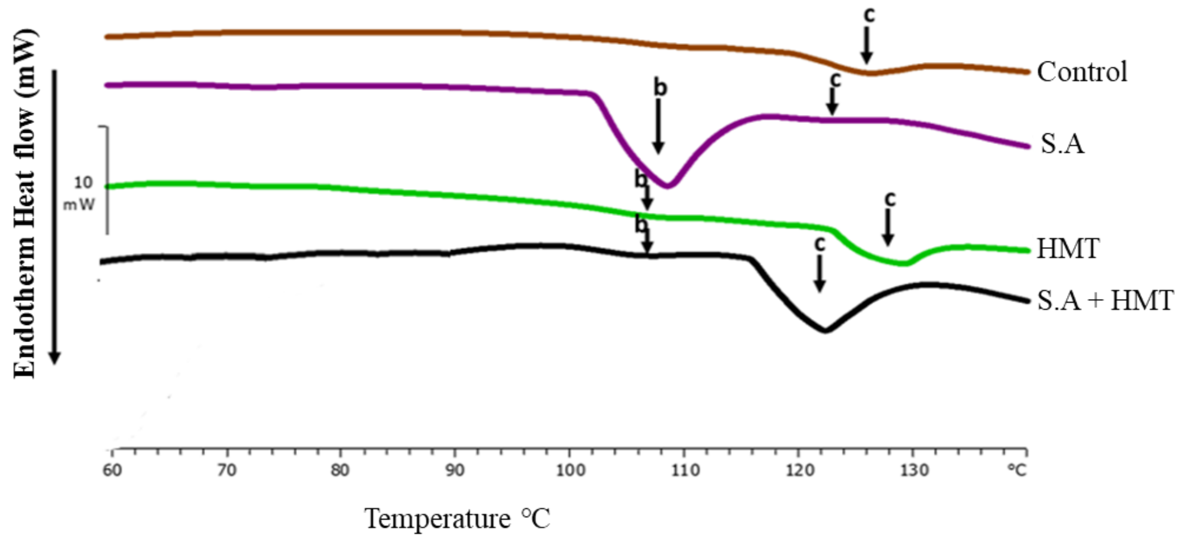
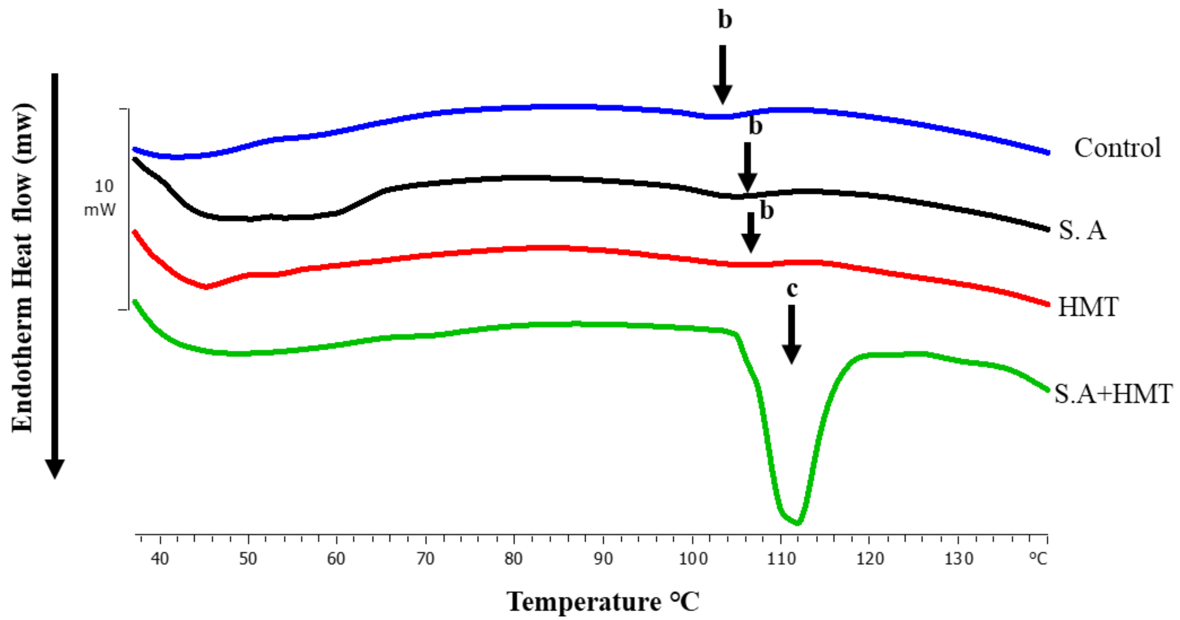
A: Pasted maize starch



B: Pasted maize meal



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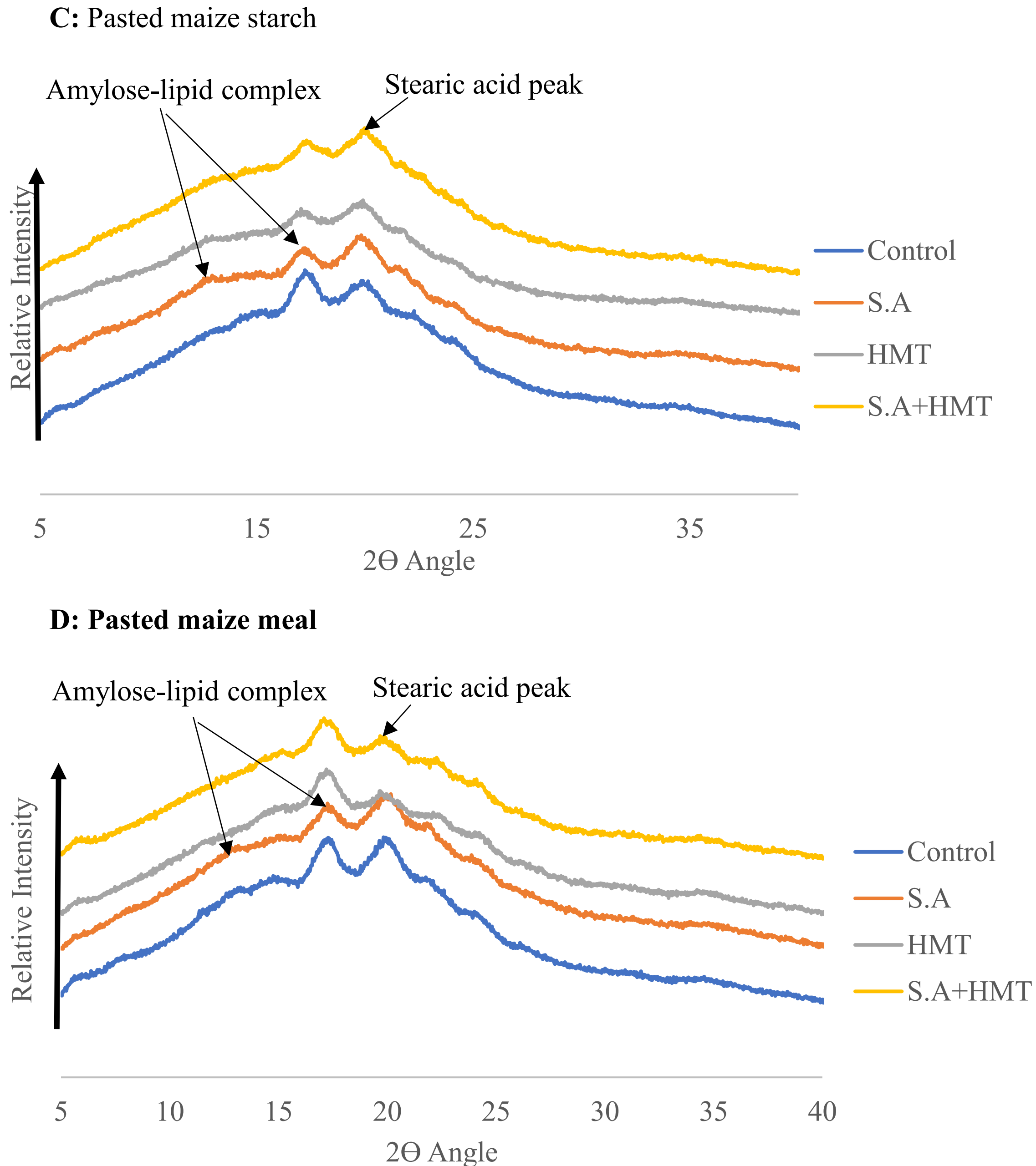

Figure 4.1.4: Effects of Stearic acid and heat moisture treatment alone and in combination on the thermal properties (A and B) and X Ray diffractogram (C and D) of pasted maize starch and unpasted maize meal

S.A (stearic acid) curve for maize starch and maize meal was added with 1.5% (w/w); HMT (Heat moisture treatment) curve for both maize starch and maize meal, treated at 110°C, 16 hrs and a moisture content of 20%; S.A + HMT curve for both maize starch and maize meal stands for combination treatment

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Table 4.1.3: Effects of HMT and SA addition on the thermal properties of starch and maize meal before pasting

Sample	Treatments		1st Endothermic Peak				2nd Endothermic Peak			
	HMT (%)	SA (%)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
Starch	None	None	64.50±0.2[b]	69.60±0.1[c]	75.70±0.38[b]	8.2±1.78[a]	-	-	-	-
	None	1.5%	63.30±0.1[a]	68.60±0.1[b]	75.20±0.80[ab]	19.3±1.39[c]	-	-	-	-
	HMT	None	63.00±0.4[a]	68.30±0.3[ab]	74.60±0.16[ab]	16.2±0.83[b]	-	-	-	-
	HMT	1.5%	63.10±0.1[a]	68.00±0.1[a]	73.90±0.79[a]	22.8±0.90[d]	-	-	-	-
Maize meal	None	None	63±0.6[w]	67±1.5[w]	73±1.2[w]	0.9±0.1[w]		-	-	-
	None	1.5%	63±0.6[w]	69±1.0[wx]	76±0.2[x]	3.2±0.7[y]		-	-	-
	HMT	None	62±0.2[w]	70±1.1[x]	75±0.5[x]	1.5±0.4[x]		-	-	-
	HMT	1.5%	64±0.6[w]	70±1.0[x]	76±0.6[x]	1.25±0.1[x]	83±0.1	93±0.5	102±0.7	8.46±0.2

Mean values± standard deviation of three independent experiments (n=3)
Different alphabets with the same superscript in the same column are significantly different ($p \leq 0.05$)
* = Interaction effect.
- = Not Detected
HMT= Heat Moisture Treatment S.A = Stearic acid

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Table 4.1.4: Effects of HMT and SA addition on the thermal properties of starch and maize meal after pasting.

Sample	Treatments		1st Endothermic Peak				2nd Endothermic Peak			
	HMT (%)	SA (%)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
Starch	None	None	-	-	-	-	119±2[b]	125±0.4[b]	132±1.2[b]	3.86±0.1[b]
	None	1.5 %	101±1[a]	106±2[a]	114±1[a]	23.8±0.4[b]	118±0.2[b]	122±0.4[a]	125±1.0[a]	0.19±0.1[a]
	HMT	None	101±1[a]	106±1[a]	113±1[a]	0.9±0.1[a]	123±0.6[c]	127±1.3[b]	131±0.6[b]	6.17±0.2[c]
	HMT	1.5 %	101±1[a]	106±1[a]	111±2[a]	1.1±0.1[a]	114±1.6[a]	121±0.2[a]	126±0.6[a]	16.85±0.2[d]
Maize meal	None	None	97±1[w]	102±1[w]	110±1[w]	1.3±0.5[w]	-	-	-	-
	None	1.5 %	97±1[w]	103±1[w]	108±1[w]	1.9±0.4[x]	-	-	-	-
	HMT	None	97±0.3[w]	105±1[w]	110±1[w]	1.6±0.3[x]	-	-	-	-
	HMT	1.5 %	-	-	-	-	106±0.6	111±5.3	115±0.3	37±0.4

Mean values± standard deviation of three independent experiments (n=3)
Different alphabets with the same superscript in the same column are significantly different (p≤0.05)
* = Interaction effect.
- = Not Detected
HMT= Heat Moisture Treatment
SA = Stearic acid

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Table 4.1.5: Effects of HMT and SA addition on wide-angle x-ray diffraction scattering on starch and maize meal before pasting and after pasting.

Treatments		Before pasting		After pasting	
		Starch	Maize meal	Starch	Maize meal
Heat Moisture treatment (%)	Stearic Acid (%)	Relative Crystallinity (%)	Relative Crystallinity (%)	Relative Crystallinity (%)	Relative Crystallinity (%)
None	None	18.3 ± 0.78^{b}	19.8 ± 1.63^{w}	5.20 ± 0.28^{a}	5.75 ± 0.35^{w}
None	1.5 %	25.4 ± 1.41^{a}	25.8 ± 1.27^{xy}	8.35 ± 0.21^{b}	8.70 ± 0.42^{x}
HMT	None	22.7 ± 1.63^{ab}	22.3 ± 0.78^{wx}	7.50 ± 0.28^{b}	6.40 ± 0.28^{w}
HMT	1.5 %	27.1 ± 1.27^{a}	27 ± 1.41^{y}	12.80 ± 0.42^{c}	11.42 ± 0.35^{y}

Mean values± standard deviation of three independent experiments (n=3)
Different alphabets in the same column are significantly different ($p\leq0.05$)

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The effects (in terms of the trends) of SA alone and heat moisture treatment alone and their combination on both starch and maize meal were similar, but starch showed a bit higher effect than maize meal. The main difference between starch and maize meal during pasting can be ascribed to their lipid and protein contents. Amylose acts as a diluent and inhibitor to swelling in the company of lipids. This means differences in the chemical composition of starch and maize meal could influence their pasting behaviours and gel properties. Protein was also primarily responsible for the differences in the pasting behaviour of starch and maize meal. Protein forms hydrophobic interaction relation with the starch in the maize meal to cover the granules preventing it from swelling, hence no peak and breakdown viscosities were noted in maize meal.

To further understand the functional properties associated with different treatments between maize starch and maize meal, principal component analysis (PCA) was performed. It is first noted that the maize meal and maize starch are from commercial sources and the starch was not extracted from the specific maize meal used in this research. Nonetheless, some researchers (Aparicio-Saguilán et al., 2006; Milašinovi´c-Šeremeši´c et al.,2012) reported that the pasting properties, molecular weight and gelatinization properties of normal starches were not significantly different among native maize starches isolated from different varieties. The first two principal components contributed about 92% of the total variation in maize starch (Figure 4.1.5A). The plot in Figure 4.1.5A was used to identify possible clusters visually. The different starch samples can be divided into four groups. The starch with stearic acid alone (cluster A) was strongly associated with high peak temperature for type I ALCs. High peak temperature for type II ALCs, summation enthalpy for type I and type II ALCs and resistant starch content was strongly associated with maize starch-containing stearic acid followed by infrared HMT (cluster B). The high k-value for starch digestibility, rapidly digestible starch content, high EGI, and gel strength were associated with untreated maize starch (cluster C). A high amount of slowly digestible starch and relative crystallinity was associated with heat-moisture treated starch without stearic acid (cluster D). The PCA of maize starch below showed many similarities with some slight variations for maize meal. The first two principal components contributed about 87% of the total variation in maize meal. The PC plot in Figure 4.1.5B was used to identify possible clusters visually. The different maize meal samples can be divided into four groups. The relative crystallinity, high peak temperature for type II ALCs, and thermal stability of ALCs (increased enthalpy of melting for all ALCs) were associated with maize meal with stearic acid (cluster A). The maize meal with stearic acid and

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HMT (cluster B) was associated with high content of slowly digestible starch. The high content of rapidly digestible starch and EGI were associated with untreated maize meal (cluster C). Increased final viscosity, gel strength, and k-value (starch digestion kinetic) were associated with heat-moisture treated maize meal (cluster D). The similarity suggests that the properties shown by maize meal in terms of the pasting, starch digestibility, and thermal properties are mainly due to the starch polymers in the maize meal. The effects (in terms of the trends) of SA alone and HMT alone and their combination on both starch and maize meal were similar, except in the starch digestibility fractions. Heat-moisture treated maize starch with stearic acid was more associated with RS and type II ALCs, while heat-moisture treated maize meal with stearic acid was mostly associated with SDS. Untreated maize meal was also associated with RS, which could be explained by the high content of protein and fat. The protein and lipids have been reported to favor the formation of ternary complexes (formation of starch–lipid complexes, the formation of protein–lipid complexes, and the disulfide bond-linked protein aggregates) (Zhang, & Hamaker, 2003). These protein interactions have been reported to restrict starch hydration during pasting and the denatured or hydrolyzed proteins that interact with starch chains during cooking/pasting result in complexes can be inhibiting enzyme-substrate formation hence slowing down starch digestion (Zhang, & Hamaker, 2003). The interactions between maize meal components explain the different pasting profiles observed in Figure 4.1.1 as well as the different hydrolysis profiles in Figure 4.1.2 between maize starch and maize meal.

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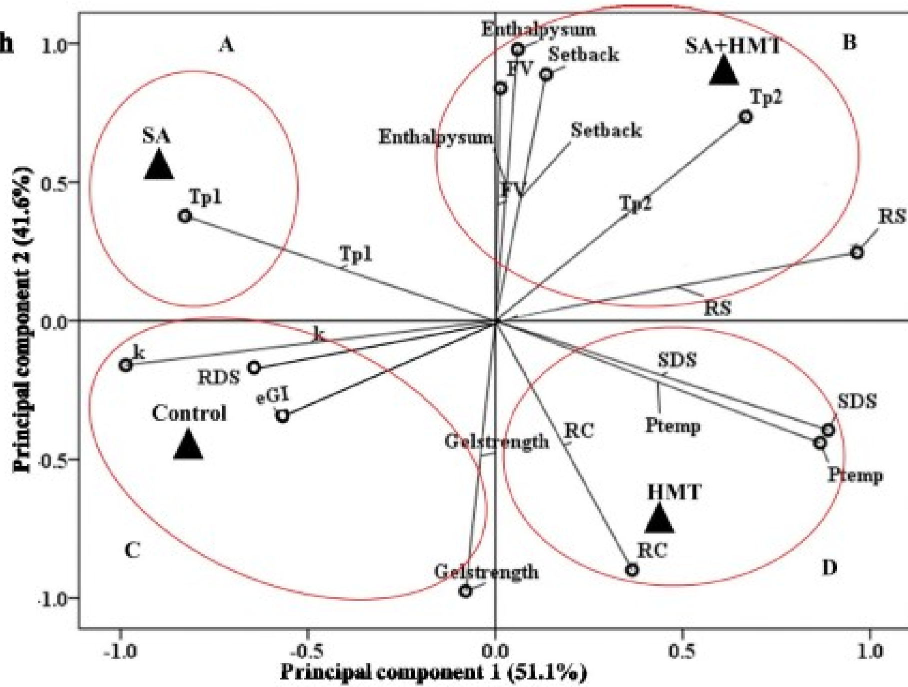

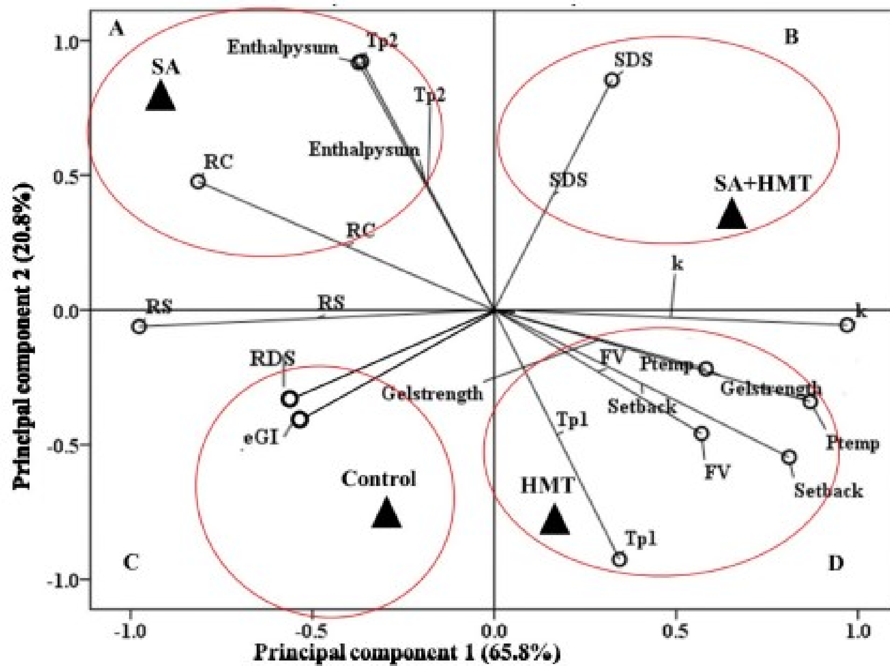

Figure 4.1.5: Principal component analysis: A, score plot describing overall variation in the first and second components in maize starch samples; and B score plot describing overall variation in the first and second components in maize meal pasted samples. (Ptemp = pasting temperature; Setback = setback viscosity; FV

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= final viscosity; RC = relative crystallinity for pasted samples; RDS = rapidly digestible starch; SDS = slowly

4.1.4. Conclusion

Amalgamation of SA and HMT has potential to yield a medium eGI starch and maize meal. The heat-moisture treatment most likely encourages the rearrangement of starch chains and the development of more ordered double-helical amylopectin side-chain clusters within the starch granules while the presence of SA facilitates amylose-lipid complex formation. These changes make the alpha 1-4 glycosidic bonds not as much accessible for enzymatic hydrolysis thereby making starch and maize meal less susceptible to enzyme digestion. Amalgamation of SA and HMT of starch and maize meal produces a soft non-gelling porridge for longer shelf life. Retrogradation can limit porridge shelf life as they become hard, and this is not preferred. The main difference between the maize meal and starch was the non-starch components present in the maize meal namely, protein and fat. This significantly affected the digestibility of both samples to a large extent.

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4.2 In vitro faecal fermentation of indigestible residues from heat -moisture treated maize meal and maize starch with stearic acid

Abstract

The effects of resistant starch type 5 (amylose-lipid complex, ALC) from both maize starch and maize meal on the production of short-chain fatty acids (SCFAs) during in vitro human faecal fermentation were determined. Maize starch and maize meal were modified with heat moisture treatment (HMT) alone, stearic acid (SA) alone, combination treatment (SAHMT), and their control. They were then digested to obtain indigestible residues. Fructooligosaccharides and a blank were used as positive and negative controls. The results showed the production of SCFAs (acetate, propionate, and butyrate) from the indigestible residues containing amylose lipid complexes during in vitro faecal fermentation. The concentrations of the three SCFAs was lower than FOS in most cases. Regarding the indigestible residues, Fermentation of the indigestible residue from the combination treatment produced a significantly higher concentration of SCFAs compared to the other treatments (SA alone and HMT alone) and the control. Reduction in pH values was observed as well as increased production of gas. Acetate and butyrate levels were higher compared to propionate levels after fermentation of RS 5 from both maize starch and maize meal. There was a positive correlation between the gas produced and the SCFAs (acetate, propionate, and butyrate) but a negative correlation with the pH. In conclusion, indigestible residues containing ALC (or RS 5) produced SCFAs during in vitro faecal fermentation suggesting that ALC are good substrates for fermentation in the lower gut.

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4.2.1 Introduction

Resistant starch (RS) is the starch that escapes digestion in the small intestine and gets into the large intestine with potential to be fermented by the gut microbiota (Murphy et al., 2008). RS can be categorized into five classes; namely, RS 1 is starch that is physically unavailable to digestive enzymes; RS 2 is composed of native granules with structures making the starch slow to digest (Jiang & Jane, 2013); RS 3 is retrograded starch (Luckett & Wang, 2012); RS 4 are chemically modified starches (Rombo et al., 2004); and RS 5 can be considered as starch with amylose-lipid complexes (ALC) (Hasjim et al., 2013). Some of the potential health benefits of resistant starch include controlling postprandial glycemic and insulinemic responses and preventing colon cancer (Higgins, 2004).

ALC is formed through an interaction between the fatty acid and hydrophobic core of the amylose helix (Arijaje & Wang, 2016). The aliphatic chain of the fatty acid lies within the amylose helix (Obiro et al., (2012) and the helix turn is stabilized by intra and inter-molecular van der Waals forces and hydrogen bonds. The carboxylic head of the fatty acid is located outside the amylose helix. This carboxylic head is prevented from entering the helix by steric hindrance and electrostatic repulsion (Godet et al., 1993). According to Singh *et al*. (2010), ALC reduced the accessibility of enzymes to hydrolyze the starch, thus preventing the starch molecules from fitting to the enzyme binding site to be hydrolyzed. The α-1-4 glycosidic bond appears to be found inside the amylose helix and is made inaccessible to enzymes. Thus, ALC reaches the large intestine, and their fate in lower GIT is not well understood. ALC is considered as dietary fibre and is, therefore, an option to increase dietary fibre without compromising the sensory properties of cereal-based foods (Aigster et al., 2011). The fermentation of dietary fibre by gut microbiota results in the generation of gas and production of short-chain fatty acids (SCFA). It can also provide selective substrates for the growth of specific groups of bacteria that may enhance the intestinal health of the host.

Heat moisture treatment (HMT) is a technique that involves treatment of starch at low moisture levels (<35% moisture w/w) for a specified period (15 min–16 h) and at temperatures (84–120 °C) above its glass transition temperature (57 °C) but below the gelatinization temperature (69 °C). HMT of starches has been examined as a method of increasing the percentage of RS due to the altered crystalline nature, making the glycosidic bonds inaccessible for enzyme hydrolysis (Chung

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et al., 2009). HMT causes amylose-amylose interaction (AM-AM) within the amorphous domain, interaction between the side - chain of amylose-amylopectin (AM-AMP) which is a molecular rearrangement, and complex formation between the amylose helix and the endogenous lipid to form amylose-lipid complexes (Gunaratne & Hoover, 2002; (Hoover et al., 1993). It also induces side-chain interactions of amylopectin-amylopectin (AP-AP) and has further been shown to have effects on the levels of rapidly digestible starch (RDS), slowly digestible starch (SDS) and RS (Chung et al., 2009). Sievert and Pomeranz, (1989) prepared RS from normal and waxy starches by HMT at 18 % moisture and temperature of 110 °C. They reported that HMT reduced enzyme susceptibility of the ordinary and waxy starches.

Chung et al. (2009) showed that the RS levels of ,maize, pea, and lentil starches increased from 4.6%, 10.0%, 9.1.% to 12.3%, 14.5%, 14.7% respectively after HMT (120°C, 1h and 30% moisture content). Sang and Seib, (2006) reported that subjecting Hylon V maize starch (about 50% amylose) to concurrent HMT (45% moisture, 110°C, four h) and phosphorylation (sodium trimetaphosphate/sodium tripolyphosphate) increased RS by 19% and decreased SDS and RDS levels by 12% and 6%, respectively.

Research has shown that RS reaching the large intestine can be fermented by the gut microflora to produce short-chain fatty acids (SCFA), which include acetate, propionate, and butyrate (Ferguson & Senie, 2000; Zhao et al., 2011; Holscher, 2017) that are of biological significance to human health. It was proposed that depending on specific structural features of resistant starch, a distinct fermentation profile could be observed (Phillips & Von Tungeln, 1995). Besides, the consumption of RS increased the faecal bulk, which can have a dilution effect on potential carcinogens and reduce their exposure to the colon (Topping & Clifton, 2001; Ferguson & Senie, 2000). These physiological effects of RS have been related to its improvement on colon health and prevention of colorectal cancer (Topping & Clifton, 2001; Zhao et al., 2011). Hence, it is necessary to maintain a balance in the composition of colonic microbiota for improved health.

There has been no systemic study to measure the potential of ALC as carbon sources for the utilization of human gut microbiota and production of SCFA. In this study using human faeces (from 3 different donors in the age group of 30-35) as a model for colonic composition, the

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objective was framed to quantify the fermentation patterns and products (SCFA) of unhydrolyzed residues of maize starch and maize meal modified with heat moisture treatment and stearic acid compared with the rapidly fermentable substrate, fructooligosaccharides (FOS).

4.2.2. Materials and Methods

4.2.2.1 Materials

Commercial superfine maize meal was purchased from a local supermarket (Pretoria, South Africa). Its proximate composition consisted of 15 % moisture, total starch of about 81 %, ash content of 0.65 %, a fat content of 1.1 % and a dietary fibre of 4.7 %. Commercial maize starch, Amyral® with 12.9 % moisture, total starch content of 95 %, ash content of 0.08 %, fat content of 0.31 % and dietary fibre content of 9.0 % was obtained from Tongaat Hulett® Starch (Edenvale, South Africa). Stearic acid with CAS number 57-11-4 was purchased from Sigma–Aldrich (St. Louis, MO, USA) and all other analytical grade reagents from Merck Chemicals (Germany).

4.2.2.2 Methods

4.2.2.2.1 Modification of maize starch and maize meal

Stearic acid (1.5% w/w in absolute ethanol) was added to 100 g of each sample followed by the procedure of D'Silva et al. (2011). The mixture was incubated in the shaking water bath at 50 °C for 30 min with a speed of 120 rpm. After the incubation period, the mixture was then dried in a hot air oven at 40°C to evaporate the excess solvent and residues were stored at 4°C for further analysis.

Maize starch and maize meal were mixed with deionized water to give the desired moisture content of 20 % for heat-moisture treatment. The starch slurry was heated above the glass transition temperature (57 °C), but below the gelatinization temperature (69 °C) (Jacobs & Delcour, 1998), thus at a temperature of 110 °C for 16 hr (Kweon et al., 2000) in a hot air oven and the treated residues were used for further studies. The samples (maize meal and maize starch) were also treated with stearic acid and followed by heat moisture treatment for testing their effect on GIT microbiota.

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4.2.2.2.2 Upper GIT enzyme hydrolysis

The Goňi et al. (1997) method was used with slight modification. A sample size of 50mg treated samples were used for the upper GIT analysis. Boiling water (1mL) was added to each sample for dispersion followed by addition of 10ml HCl–KCl buffer (pH 1.5) and 0.2 mL of a solution containing 1mg pepsin (Sigma–Aldrich P7000-100G). The samples were incubated at 40°C for 60 min with constant agitation. After the incubation period, 10 ml of Tris-maleate buffer (pH 6.9) was added to the solutions and adjusted to 25 mL. An aliquot of 0.1 ml was taken for 0 min before the addition of 5 mL tris-maleate buffer (pH 6.9) containing 2.6 IU of pancreatic α-amylase with activity of 19.6 units/mg (Sigma–Aldrich A-3176) followed by incubation at 37 ℃ with constant shaking in a water bath. Aliquots of 0.1 ml were taken during the incubation period at different time intervals of 5, 30, 60, 90, 120 and 180 min. The tubes containing the solutions were placed in boiling water for 15 min to inactivate the α-amylase. Then, 1 ml of 0.4 M sodium–acetate buffer (pH 4.75) and 90 µL of amyloglucosidase with an activity of 64.7 U/mg (Megazyme E-AMGDF) was added into the tubes and incubated at 60 ℃ for 45 min. After incubation, the tubes containing solutions were read in a UV Vis spectrophotometer at 510 nm using glucose to create the standard graph.

4.2.2.2.3. Differential Scanning Calorimeter (DSC) analyses

Thermal properties of unhydrolyzed/undigested maize starch/meal samples were measured using a differential scanning calorimeter (DSC) system (HP DSC827e, Mettler Toledo, Greifensee, Switzerland) described by Wokadala et al. (2012). Undigested residues and control samples (10 mg) were weighed in aluminum pans and thoroughly mixed with distilled water at a ratio of 1:3 (w/w) starch-to-water to make a homogeneous slurry. The pans were sealed and equilibrated for 24h at room temperature before scanning. Scanning was done from 25° C ℃ to 140° C ℃ under high pressure (4 MPa using N_2) at a rate of 10°C/min. Indium (Tp = 156.6°C, 28.45 Jg^{-1}) was used as an internal standard to calibrate the pan and an empty pan was the reference.

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4.2.2.2.4 In vitro faecal fermentation

Batch faecal fermentation was performed according to the methodology of Lebet et al. (1998) and Rose et al. (2010). Each substrate (50 mg undigestible residue from modified and unmodified maize starch and maize meal) was weighed in three test tubes for triplicate analysis. Faecal samples were obtained from three healthy volunteers (age 35-40) who fed on unspecified and varied diets and had not taken any antibiotics for past six months. Faecal samples were collected in plastic bags that were sealed after removing the air and immediately placed inside the anaerobic chamber (10% H_2, 5 % CO_2, and 85 % N_2; BactronEZ, SHEL LAB, Cornelius, OR) where all further procedures were performed within 2 h after collection. Faecal samples were used individually as per donor, and slurry prepared by homogenization with carbonate-phosphate buffer pH 6.8 ± 0.1 in a ratio of 1:3 (w/v) and further strained through four layers of cheesecloth. The samples (undigested residues of modified and unmodified maize starch and maize meal) with their controls (fructooligosaccharides and blank) were hydrated with 4 ml of carbonate-phosphate buffer pH 6.8 ± 0.1 and then inoculated with 1 mL of the faecal filtrate. The tubes were then sealed and incubated at 37 °C in an incubation chamber. The following was then analysed at specific times:

4.2.2.2.4.1 Gas production

At the incubation period intervals of 4, 8, 12, and 24 h of fermentation, assigned tubes were removed from the incubation chamber and gas measured using a plunger displacement of a syringe.

4.2.2.2.4.2 pH

The pH of the faecal inoculated samples were measured at the same time intervals during fermentation (0, 4, 8, 12 and 24 h) using a pH meter (Mettler Toledo, USA).

4.2.2.2.4.3 Short chain fatty acids (SCFAs)

Aliquots (1 mL) were taken from each substrate-inoculated faeces fermentation media for the SCFA analysis using a gas chromatograph with a flame ionization detector (GC-FID). The samples were thawed and centrifuged at 13000 x *g* for 10 min. Aliquots (400 µL) from fermented supernatant samples were combined with 100 µL of a mixture containing 50 mM 4-methyl-valeric

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acid No. 277827- 5 G, Sigma-Aldrich Inc., St. Louis, Mo., USA), meta-phosphoric acid (5%) and copper sulfate (1.56 mg/mL) used as an internal standard for SCFA analysis. The mixture was centrifuged at 1300 x *g* for 10 min. An aliquot of 0.2 µL was injected into a GC-FID (7890 A, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a fused silica capillary column (Nukol TM, Supelco No. 40369-03 A, Bellefonte, Pa., USA). The initial oven temperature was held at 50 °C for 2 min, ramped to 70 °C at a rate of 10 °C/min, to 85 °C at a rate of 3 °C/min, to 110 °C at a rate of 5 °C/min, to 290 °C at a rate of 30 °C/min, and finally held at 290 °C for 8 min. Helium was used as a carrier gas at a constant flow rate of 1 mL/min through the column. Quantification was performed based on the relative peak area of each SCFA in a fatty acid external standard mixture (Volatile Free Acid Mix, ten mM, 46975-U, Supelco), adjusting the quantity of each compound based on that of the internal standard.

4.2.3. Statistical Analysis

The experimental design was a 2*2 factorial design (stearic acid addition with two levels of 0% and 1.5% w/w and heat-moisture treatment with two levels of (0% and 20% w/w moisture). All the experiments were carried out in triplicates. Multivariate analysis of variance (MANOVA) was used to determine significant differences due to the addition of stearic acid, and heat-moisture treatment in maize starch and maize meal. The data for maize starch and maize meal were analyzed separately. Averages were compared using the Fischer's Least Significant Difference Test (LSD) at $P \leq 0.05$. Origin Pro version 2019b was used to fit the graphs for gases, pH and SCFAs.

The equation used was $Y = Yo + A1e^{-x/t1}$

Where Y is the dependent variable; x = independent variable; t = time in hr; K was derived from the graph as is the exponential decay rate constant (h^{-1}); tau = time at half-life of product production (h).

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4.2.4. Results and Discussion

Residues from upper gastrointestinal (G.I.) tract samples were used for thermal properties analysis (Table 4.2.1). The control maize starch and maize meal produced 7 and 8 % of unhydrolyzed residues, respectively after digestion. Addition of stearic acid alone, heat moisture treatment alone and their combination significantly increased the proportion of unhydrolyzed residue (Table 4.2.1) compared to the control. This is possible because stearic acid addition to starch during pasting formed amylose–stearic acid complexes that limit the hydrolysis of the alpha 1,4 glycosidic bond located in the amylose. Heat moisture treatment also causes fractions of amylose and amylopectin to form a double-helical structure during the process (Hoover, 2010). This increases the overall stability of the granule to disruption and thus lowers digestibility. The percentage of unhydrolyzed maize starch and maize meal from Table 4.2.1 agrees with previous work done on *in vitro* starch digestibility (Mapengo & Emmambux, 2020) of maize starch and maize meal where their resistant starches were calculated. The control maize starch and maize meal had RS of 7 and 8 %. Addition of stearic acid alone increased RS to 29 % for maize starch and 33 % for maize meal. Heat moisture treatment alone increased RS further to 32, and 33 % for both samples and their combination treatment had RS value of 37 and 38 % for both maize starch and maize meal, respectively. These values were the percentage RS that can potentially enter the large intestine for fermentation.

The melting endotherms of the unhydrolyzed control maize starch had Tp (peak temperature) of 106.5 °C and ΔH (enthalpy of gelatinization) as 1.98 J/g (Table 4.2.1). Addition of stearic acid alone, heat moisture treatment alone and their combination only showed one endothermic peak with Tp as 116.8 °C, 101.1 °C and 110.4 °C with their corresponding ΔH as 4.64 J/g, 9.12 J/g and 10.43 J/g respectively (Table 4.2.1) compared to the enthalpy of the control. Unhydrolyzed control maize meal showed Tp as 102.9 °C and its ΔH as 3.57 J/g (Table 4.2.1). Addition of stearic acid alone had Tp as 106.2 °C, and its ΔH as 8.05 J/g, heat moisture treatment alone showed two endothermic peaks with Tp as 100.1 °C 122.2 °C with their corresponding ΔH as 6.04 J/g and 3.21 J/g respectively. Combination treatment had one endotherm with Tp as 111.5 °C and enthalpy of 10.64 J/g (Table 4.2.1) respectively compared to the control. Endotherms with dissociation temperatures of about (98-105), (106-109) and (110-120) correspond to type I, type IIa and type IIb amylose-lipid complexes (Raphaelides & Karkalas, 1988). The results showed that both types I, and IIa and IIb ALC were present in the unhydrolyzed residues. More ALC was formed when stearic acid was added to the pasted maize starch. Pasted maize starch treated with HMT showed

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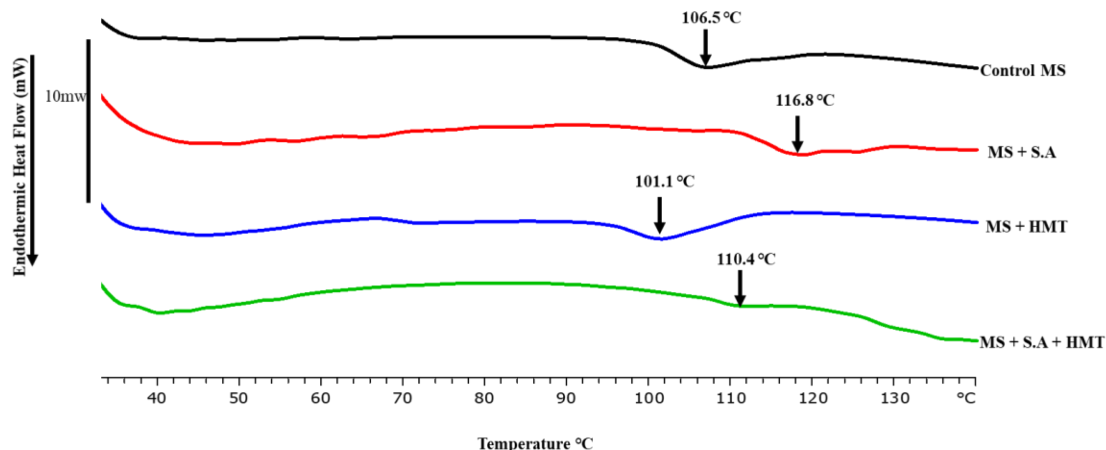
type I but maize meal showed type I and type IIb ALC (Figure 4.2.1). The pasted maize starch and meal with stearic acid followed by HMT form more type IIa and IIb ALC, respectively (Figure 4.2.1).

Table 4.2.1: Effects of stearic acid and heat moisture treatment alone and in a combination on the upper G.I. digestion and thermal properties of maize starch and maize meal

Sample	Treatment	Percentage Unhydrolyzed (%)	DSC Results			
			1s Endothermic peak		2nd Endothermic peak	
			Tp (°C)	Delta H (J/g)	Tp (°C)	Delta H (J/g)
Maize starch	Control	7	106.5	1.98	-	-
	S.A	29	116.8	4.64	-	-
	HMT	32	101.1	9.12	-	-
	S.A+HMT	37	110.4	10.43	-	-
Maize meal	Control	8	102.9	3.57	-	-
	S.A	33	106.2	8.05	-	-
	HMT	33	100.1	6.04	122.2	3.21
	S.A+HMT	38	111.5	10.63	-	-

Control = without treatment; S.A = stearic acid was at 1.5% (w/w); HMT = heat moisture treatment, 20% moisture at 110°C for 16 hr; S.A+HMT = combination treatment; - = Not detected.

A



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B

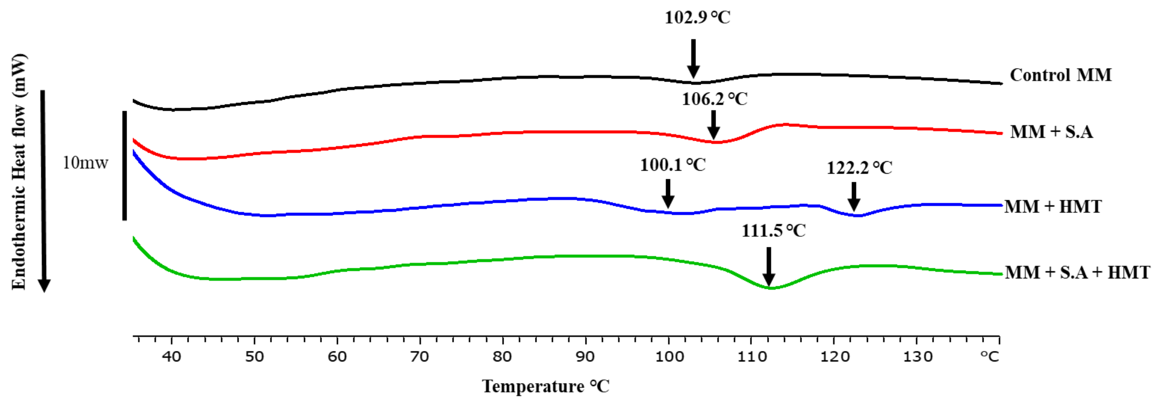

Figure 4.2.1: Effects of stearic acid and heat moisture treatment alone and in combination on the thermal properties of unhydrolyzed (A) maize starch (ms) (B) maize meal (mm).

S. A=stearic acid was 1.5% (w/w) as db of the starch content; HMT=heat moisture treatment,

20 % moisture at 110°C for 16hr; S. A+HMT=Combination treatment.

Production of gas from three donors during 24 hr *in vitro* faecal fermentation from unhydrolyzed residues of modified and unmodified maize starch and maize meal are shown in Figure 4.2.2. There was an increase in the gas production of both maize starch and maize meal (Figure 4.2.2) from all the three donors. The initial gas produced was 1 ml for all the donors (Figure 4.2.2) after 4 h, the production of gas increased significantly ($p \leq 0.05$) until 8 h, it then plateaued until the end of the faecal fermentation (Figure 4.2.2). The data was also fitted into $Y = Yo + A_1e^{-x/t1}$ equation to determine the reaction rate, half-life and total gas produced with R^2 greater than 0.99 except for blank. The negligible fermentation of the blank was expected as there was no substrate for fermentation by the faecal microbes.

Faecal fermentation of FOS produced significantly ($P<0.05$) higher total gas, but lower exponential decay constant, and higher half-life compared to indigestible residues from untreated and treated maize meal and maize starch (Table 4.2.2). This shows that FOS had a slower fermentation rate compared to the indigestible residues, although the final gas production was only 1 ml more than the indigestible residues. Comparing the indigestible residues from maize meal and maize starch, HMT with stearic acid seemed to show the highest gas production in some

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donors from both maize meal and maize starch. Indigestible residues from maize starch appeared to have a higher gas production compared to maize meal. Donor 2 and Donor 3 produced slightly higher gas than Donor 1.

A large amount of gas production *in vivo* can result in gastrointestinal discomfort, particularly in patients with visceral hypersensitivities, such as individuals with irritable bowel syndrome (Serra and Azpiroz, 1998; Dong et al., 2004). Though the treated RS samples were fully fermented, their fermentation were surprisingly rapid. This shows that bacterial amylases and glucoamylases are highly efficient at digesting starch from ALCs that enzymes could not. Some individuals may not be able to create equilibrium by flatulence when more gases are produced, and these could lead to gastrointestinal intolerance in them (Serra and Azpiroz, 1998).

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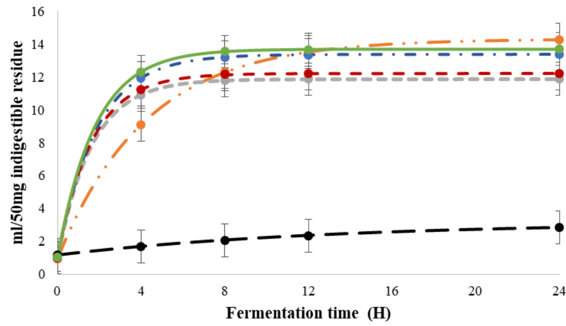
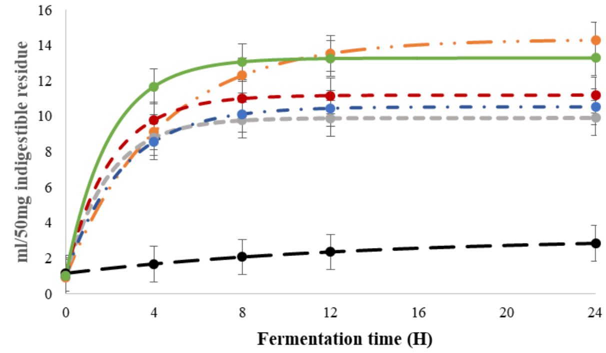
(Maize Starch) donor 1



(Maize meal) donor 1



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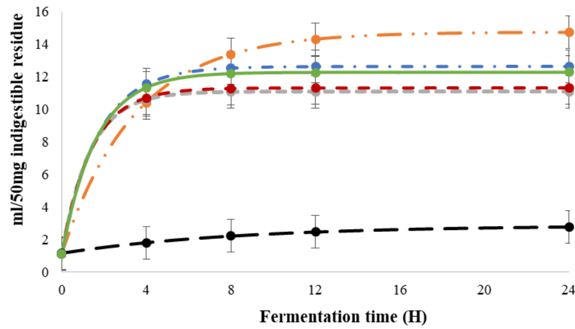

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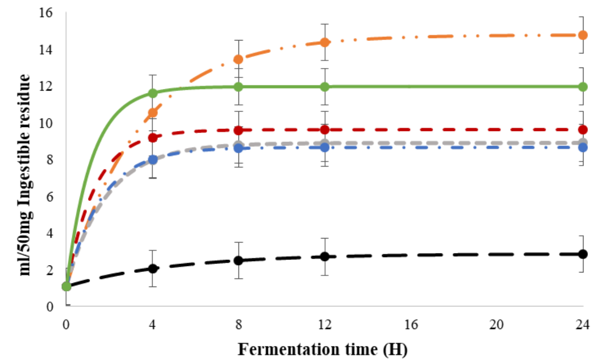
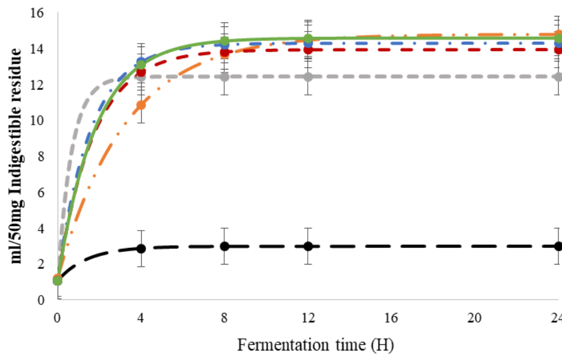


(Maize starch) donor 3



(Maize meal) donor 3

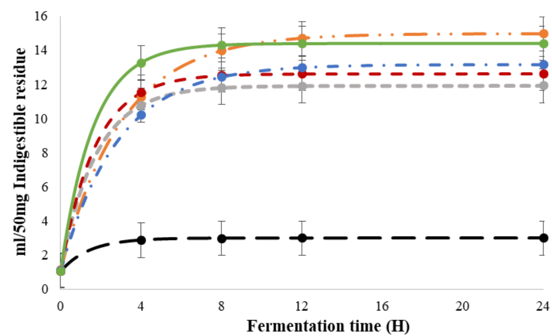


Figure 4.2.2: Effects of unhydrolyzed residue from unmodified and modified maize starch and maize meal on gas production from the three donors during their 24 hr in vitro faecal fermentation

Key: Blank black (— —), Control grey (-----), S. A. red (– – –) HMT blue (– · – ·)

S.A+HMT green (———), FOS orange (— · · —)

FOS = Fructooligosaccharides; S.A = Stearic acid addition at 1.5 % (w/w); HMT = heat moisture treatment at 20 % moisture at 110 °C for 16 hr; S.A+HMT = Combination treatment.

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Table 4.2.2: Effects of unhydrolyzed residue from unmodified and modified maize starch and maize meal on gas production from three donors during their 24 h in vitro faecal fermentation

Sample	Treatment	Donor 1				Donor 2				Donor 3			
		Yo (ml/50mg)	time (hr)	K (h^{-1})	Tau (hr)	Yo (ml/50mg)	time (hr)	K (h^{-1})	Tau (hr)	Yo (ml/50mg)	time (hr)	K (h^{-1})	Tau (hr)
Maize Starch	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	11.0±0.6^{a}	1.5±0.2^{a}	0.7±0.1^{b}	0.8±0.2^{a}	11.1±0.9^{a}	2.0±0.9^{a}	0.5±0.1^{c}	1.7±0.2^{a}	11.9±0.2^{a}	1.4±0.3^{a}	0.7±0.1^{a}	1.2±0.2^{a}
	S.A	11.8±0.3^{a}	1.8±0.4^{a}	0.6±0.1^{b}	1.0±0.1^{a}	12.1±0.3^{a}	2.5±1.0^{a}	0.4±0.2^{c}	1.7±0.2^{a}	13.5±0.2^{b}	2.2±0.4^{b}	0.45±0.2^{b}	2.1±0.1^{b}
	HMT	12.9±0.6^{b}	2.1±0.1^{b}	0.4±0.1^{b}	1.7±0.1^{b}	13.5±0.1^{b}	3.2±1.2^{b}	0.3±0.3^{b}	2.3±0.1^{b}	13.6±0.1^{b}	2.2±0.1^{b}	0.45±0.1^{b}	2.2±0.1^{b}
	SAHMT	13.4±0.9^{b}	2.8±0.4^{b}	0.3±0.2^{b}	2.2±0.1^{b}	13.8±0.2^{b}	4.9±0.2^{b}	0.2±0.1^{b}	3.0±0.1^{c}	14.2±0.4^{c}	3.4±0.4^{c}	0.29±0.2^{c}	3.0±0.1^{c}
	FOS	14.6±0.1^{c}	3.5±0.2^{c}	0.2±0.1^{a}	2.8±0.2^{c}	14.7±0.4^{c}	5.5±0.2^{c}	0.18±0.1^{a}	3.2±0.2^{c}	14.7±0.2^{c}	3.5±0.2^{c}	0.28±0.1^{c}	3.1±0.2^{c}
Maize Meal	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	8.5±0.3^{a}	1.2±0.2^{a}	0.6±0.1^{d}	1.4±0.1^{a}	8.9±0.1^{a}	1.1±0.4^{a}	0.9±0.1^{d}	1.2±0.1^{a}	10.9±0.1^{a}	1.8±0.1^{a}	0.6±0.1^{c}	1.2±0.1^{a}
	S.A	11±0.2^{b}	2.5±0.2^{b}	0.35±0.1^{c}	2.4±0.1^{b}	9.6±0.2^{b}	2.3±0.1^{b}	0.43±0.1^{c}	2.2±0.1^{b}	12.6±0.3^{b}	3.2±0.4^{b}	0.3±0.1^{b}	3.1±0.1^{b}
	HMT	10.9±0.8^{b}	2.4±0.2^{b}	0.4±0.1^{c}	2.2±0.2^{b}	8.7±0.1^{a}	1.0±0.2^{a}	1.0±0.1^{d}	1.1±0.1^{a}	13.2±0.5^{b}	3.0±0.5^{b}	0.3±0.2^{b}	3.1±0.2^{b}
	SAHMT	13.4±0.1^{c}	4.0±0.1^{c}	0.25±0.2^{b}	3.4±0.1^{c}	12.2±0.4^{c}	3.4±0.2^{c}	0.29±0.2^{b}	3.0±0.1^{c}	14.4±0.1^{c}	4.6±0.4^{c}	0.2±0.2^{a}	4.0±0.1^{c}
	FOS	14.7±0.4^{d}	5.5±0.2^{d}	0.18±0.1^{a}	4.1±0.1^{c}	14.7±0.2^{d}	4.5±0.2^{d}	0.2±0.1^{a}	3.4±0.2^{d}	14.9±0.6^{c}	5.0±0.5^{c}	0.2±0.1^{a}	4.1±0.2^{c}

Mean ± standard deviation of three independent replicates for three donors.
Different alphabetical letters in the same column are significantly different ($p \leq 0.05$)
This table was derived using this equation $Y = Yo + A_1e^{-x/t1}$ Yo = Maximum gas production; t_1=time (hr); tau=half-life; k = exponential decay constant (derived from the equation), ND=not determined as fitting curve
FOS = Fructooligosaccharides; S.A = Stearic acid was added at 1.5% (w/w); HMT = heat moisture treatment was at 20 % moisture at 110 °C for 16 hr; S.A+HMT = Combination treatment.

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The blank faecal sample without added substrate showed a slight increase in the mean pH value after the initial inoculum value for some samples and donors, though afterwards did not change up to the end of fermentation, (Figure 4.2.3). Fassler et al., (2006) also reported an increase in the pH of the blank by 0.1 units at the end of the in vitro faecal fermentation. There was a general reduction in the mean pH value from all the three donors for both maize starch and maize meal treatments as well FOS (Figure 4.2.3).

Colonic pH value may be associated with reducing the risk of colonic cancer (Duncan et al., 2002). In African ethnic populations, a low colon-cancer risk has been associated with a low faecal pH (Duncan et al., 2002). A lower pH may reduce the number of pathogenic bacteria in the intestine without influencing the quantity of Bifidobacteria (Walker et al., 2005). It can also increase the rate of absorption of minerals like calcium, magnesium, and sodium (Trinidad et al., 1996; Younes et al., 1996). Reduction in the mean pH value also decreases the activity of co-carcinogenic enzymes such as glucuronidases, glycosidases and 7a-hydroxylases (Thornton, 1981).

From Table 4.2.3, when the values obtained from pH was analysed using the $Y = Yo + A_1e^{-x/t1}$, it was noticed that the blank was not detected since there were no sample to ferment. Both maize starch and maize meal experience a sharp decline from the pH values. This was expected because it gave the idea for the production of SCFA. Kaur et al. (2011) also observed a similar trend when working on fermentable carbohydrates that produce SCFAs to reduce pH.

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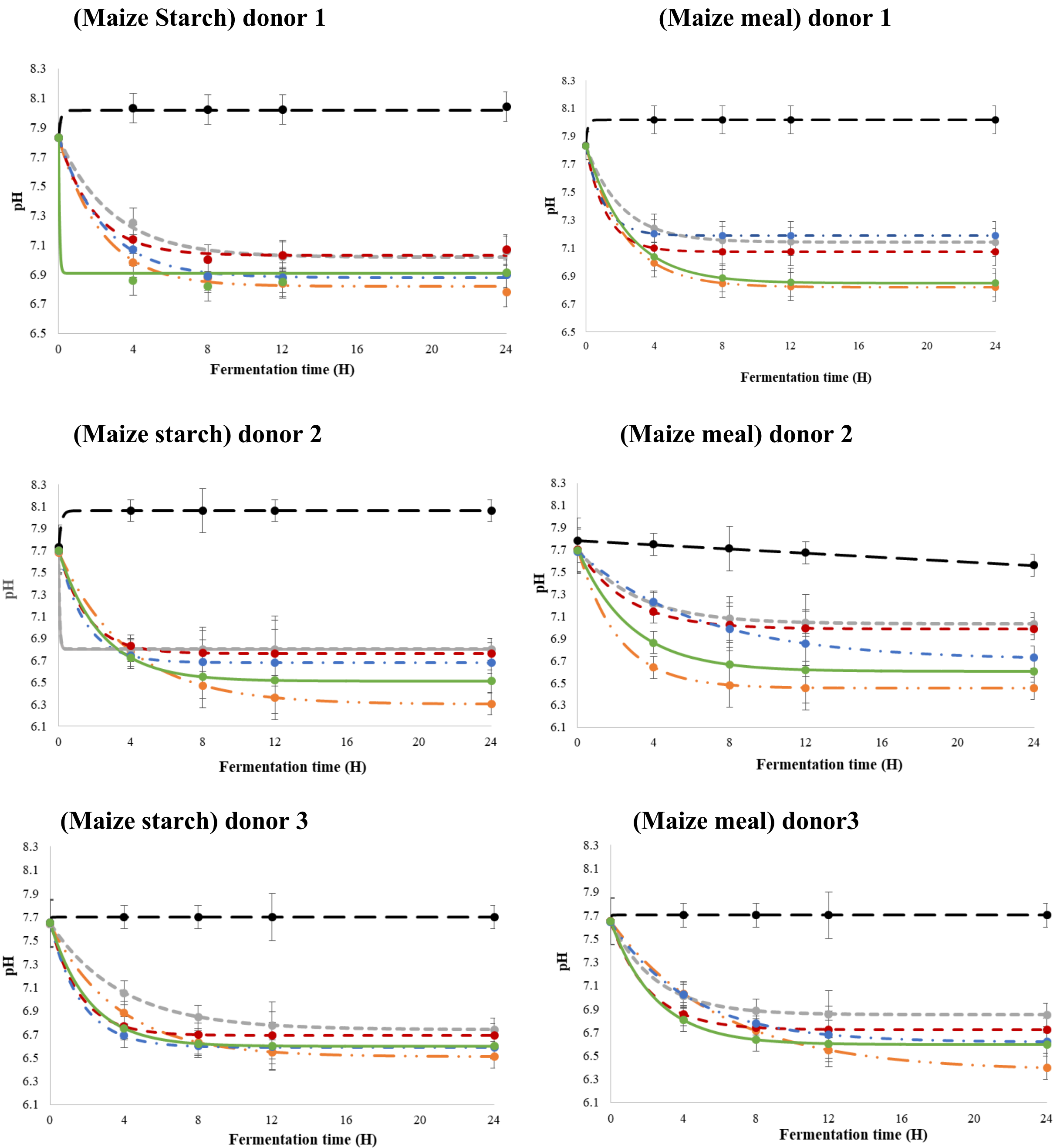

Figure 4.2.3: Effects of unhydrolyzed residue from unmodified and modified maize meal and maize starch on pH from the three donors during their 24 hr in vitro faecal fermentation.

Key: Blank black (— —), Control grey (-----), S. A red (– – –) HMT blue (– · – ·)

S.A+HMT green (———), FOS orange (— · · —)

FOS = Fructooligosaccharides; S.A = Stearic acid was added at 1.5% (w/w); HMT = heat moisture treatment at 20 % moisture at 110 °C for 16 hr; S.A+HMT = Combination treatment.

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Table 4.2.3: Effects of unhydrolyzed residue from unmodified and modified maize starch and maize meal on pH from three donors during their 24 h in vitro faecal fermentation.

		Donor 1				Donor 2				Donor 3			
Sample	Treatment	Yo	time (hr)	K (h^{-1})	Tau (hr)	Yo	time (hr)	K (h^{-1})	Tau (hr)	Yo	time (hr)	K (h^{-1})	Tau (hr)
Maize Starch	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	7.07±0.1^{c}	1.1±0.3^{c}	0.91±0.1^{d}	0.8±0.2^{c}	6.76±0.1^{d}	1.41±0.9^{d}	0.71±0.7^{d}	0.70±0.7^{c}	6.80±0.2^{c}	3.86±0.5^{b}	0.26±0.5^{c}	2.67±0.5^{c}
	SA	6.99±0.1^{c}	2.2±1.2^{ab}	0.45±0.2^{c}	1.0±0.5^{ab}	6.73±0.2^{d}	1.72±0.4^{d}	0.58±0.1^{c}	1.20±0.3^{c}	6.72±0.1^{c}	4.1±0.2^{a}	0.24±0.1^{c}	3.1±0.1^{c}
	HMT	6.86±0.2^{b}	3.0±0.3^{b}	0.33±0.1^{b}	1.7±0.4^{bc}	6.66±0.1^{c}	2.52±0.5^{c}	0.39±0.1^{b}	2.05±0.4^{b}	6.59±0.1^{b}	8.76±0.1^{a}	0.11±0.1^{b}	5.22±0.1^{b}
	SAHMT	6.82±0.1^{b}	3.5±0.4^{b}	0.29±0.2^{b}	1.81±0.3^{a}	6.50±0.1^{b}	3.45±0.9^{bc}	0.29±0.2^{a}	3.1±0.6^{a}	6.59±0.1^{b}	8.08±0.3^{a}	0.12±0.1^{b}	5.44±0.2^{b}
	FOS	6.79±0.1^{a}	3.9±0.2^{a}	0.26±0.1^{a}	2.8±0.1^{bc}	6.38±0.1^{a}	4.33±0.9^{c}	0.23±0.1^{a}	3.31±0.6^{a}	6.2±0.1^{a}	10.6±2.0^{c}	0.09±0.1^{a}	8.08±1.4^{a}
Maize Meal	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	7.14±0.1^{c}	1.40±0.4^{a}	0.71±0.1^{c}	0.82±0.3^{c}	7.08±0.1^{c}	1.28±0.5^{c}	0.78±0.1^{d}	1.58±0.3^{a}	6.9±0.1^{d}	2.61±0.3^{c}	0.38±0.1^{c}	2.51±0.2^{c}
	SA	7.03±0.1^{b}	2.77±0.2^{a}	0.36±0.1^{b}	1.82±0.1^{b}	6.81±0.2^{c}	1.80±0.4^{c}	0.56±0.1^{c}	2.25±0.3^{ab}	6.71±0.1^{c}	2.96±0.3^{c}	0.33±0.1^{c}	2.91±0.2^{c}
	HMT	7.14±0.1^{c}	1.41±0.3^{a}	0.71±0.1^{c}	0.67±0.2^{c}	6.69±0.1^{b}	2.45±0.7^{b}	0.41±0.1^{b}	3.16±0.5^{c}	6.60±0.1^{b}	4.92±0.5^{b}	0.20±0.1^{b}	3.72±0.4^{b}
	SAHMT	6.81±0.1^{a}	3.80±1.3^{a}	0.26±0.1^{a}	2.90±0.8^{a}	6.61±0.1^{b}	2.80±0.4^{b}	0.36±0.1^{b}	3.94±0.3^{ab}	6.60±0.1^{b}	4.91±0.4^{b}	0.20±0.1^{b}	3.88±0.3^{b}
	FOS	6.79±0.1^{a}	3.85±0.3^{a}	0.25±0.1^{a}	2.91±0.2^{a}	6.38±0.1^{a}	3.39±0.8^{a}	0.29±0.1^{a}	4.35±0.6^{b}	6.2±0.1^{a}	6.2±2.1^{a}	0.16±0.1^{a}	5.52±1.0^{a}

Mean ± standard deviation of three independent replicates for three donors.
Different alphabetical letters in the same column are significantly different ($p \leq 0.05$)
This table was derived from equation $Y = Yo + A_1e^{-x/t1}$ Yo = Minimum pH; t_1=time (hr); tau=time at Half-life; k = exponential decay constant (derived from the equation), ND=not determined as fitting curve; FOS = Fructooligosaccharides; S.A = Stearic acid added at 1.5% (w/w); HMT = heat moisture treatment at 20 % moisture at 110 °C for 16 hr; S.A+HMT = Combination treatment.

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Figures 4.2.4 and 4.2.5 show the effect of unhydrolyzed residue from unmodified and modified maize starch and maize meal on SCFA production from the three different donors during 24 h *in vitro* faecal fermentation. The data obtained were fitted into the equation with R^2 higher than 0.99 except for the blank since there was no substrate for human faecal microbiota to ferment. FOS was seen to have the highest production of acetate, propionate, and butyrate in all the three donors at the end of the overall fermentation (Figure 4.2.4 and Tables 4.2.4 - 4.2.6).

As expected, acetate production was dominant and higher than propionate and butyrate in both the maize starch and maize meal (Figures 4.2.4 & 4.2.5 and Tables 4.2.4 – 4.2.6). Among the donors, the acetate production was significantly ($p<0.05$) higher in Donor 1 compared to other donors. FOS resulted in significantly ($p<0.05$) higher production of acetate, about 50 % of the total SCFA compared to the maize starch and meal indigestible residues (Table 4.2.4). FOS had the highest fermentation rate suggesting that the fermentation was not as rapid asthought to reach the maximum acetate production, and lower K value with significantly higher tau value. The lower K value signifies the slow fermentation rate for all the three donors.

Regarding the maize starch and meal indigestible residues, the combination treatment had a significantly ($p<0.05$) higher acetate production when compared to the stearic acid (S.A.) and heat moisture treatment alone. The combination treatment also had low K value but not as low as that of FOS, and even higher time and high tau in all the three donors (Table 4.2.4). The chemical structure and physical form of dietary fibre are the critical factors that determine the fermentation rate (Gidley, 2013). No significant difference was observed between the maize starch and meal in terms of the production of acetate, in all the substrates for Donor 1. A synergistic effect was also found for combination treatment in terms of acetate production from fermentation of maize starch and meal indigestible residues.

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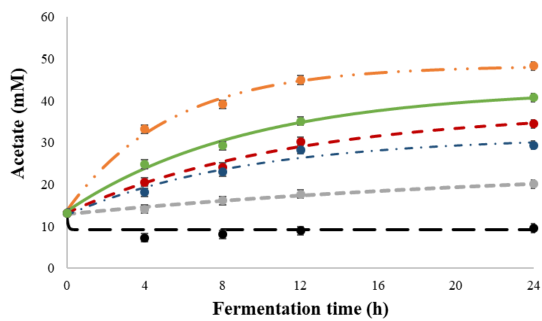
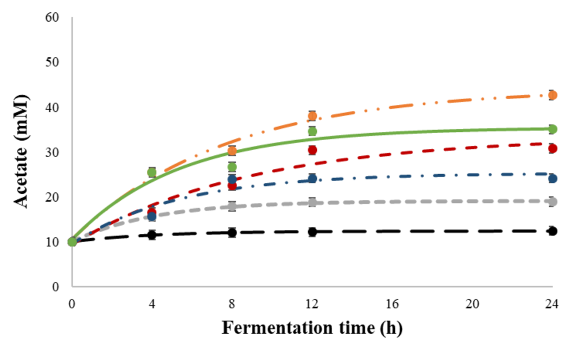
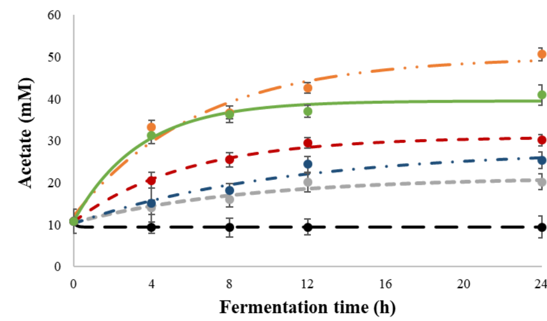
Maize starch (donor 1)



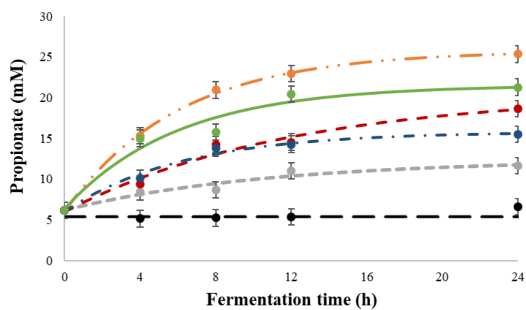
Maize starch (donor 2)



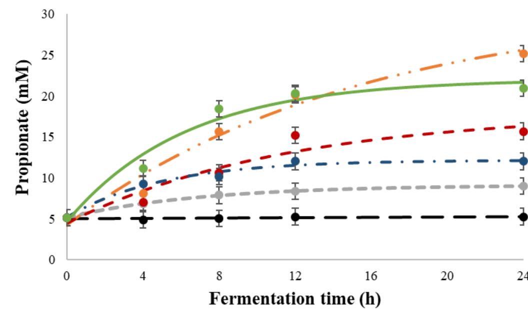
Maize starch (donor 3)



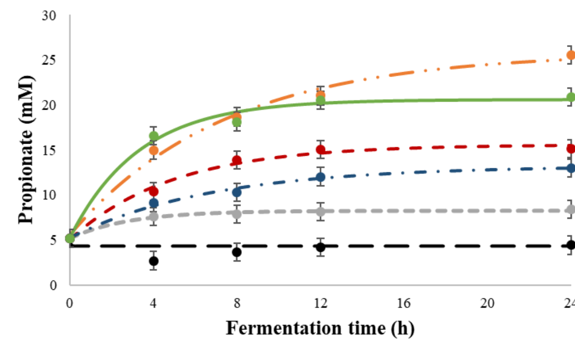
Maize starch (donor 1)



Maize starch (donor 2)



Maize starch (donor 3)



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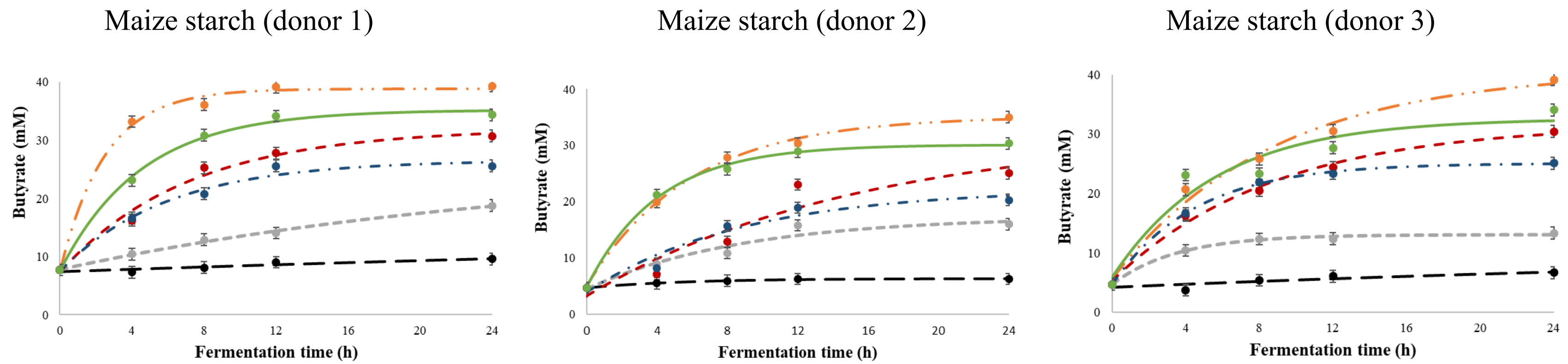
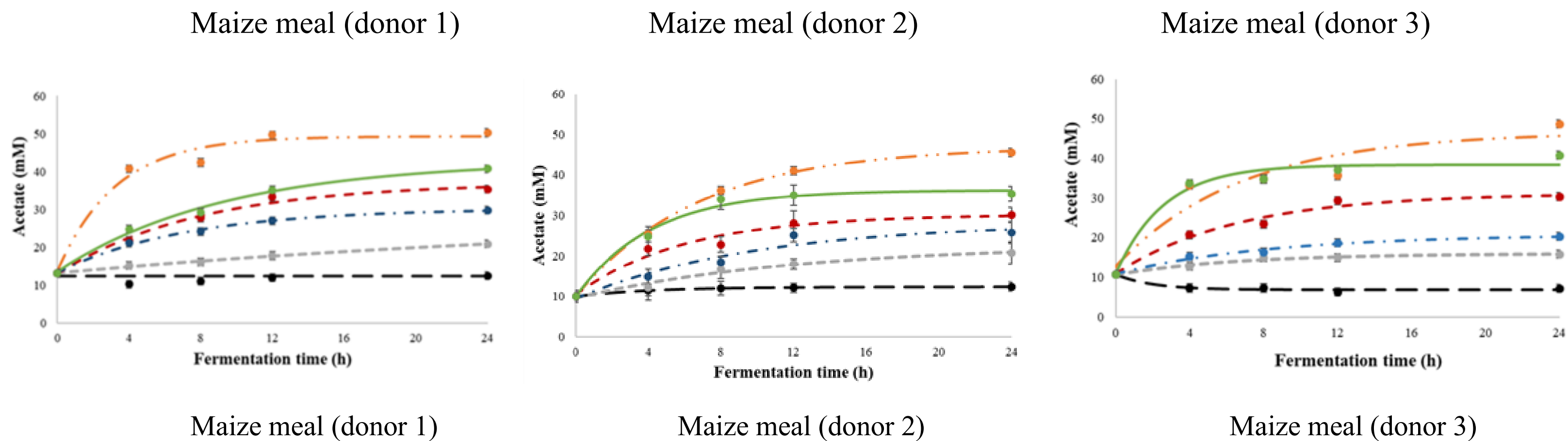


Figure 4.2.4: Effects of unhydrolyzed residues from unmodified and modified maize starch on SCFA production from the three donors during their 24 hr *in vitro* faecal fermentation.

Key: Blank black (— —), Control grey (-----), S. A red (– – –) HMT blue (– · – ·), S.A+HMT green (———), FOS orange (— · · —)

FOS = Fructooligosaccharides; S.A = Stearic acid was added at 1.5% (w/w); HMT = heat moisture treatment was at 20 % moisture at 110°C for 16 hr; S.A+HMT = Combination treatment.



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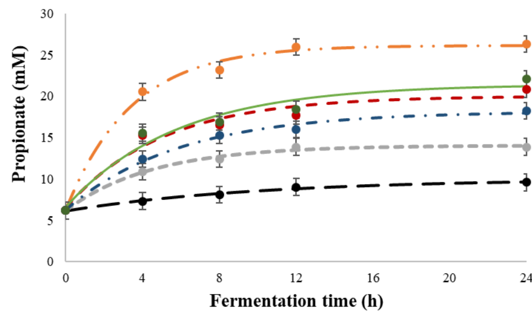
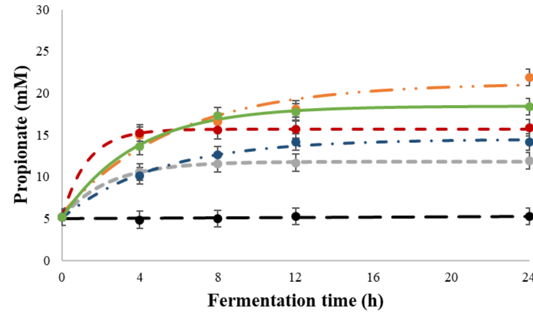
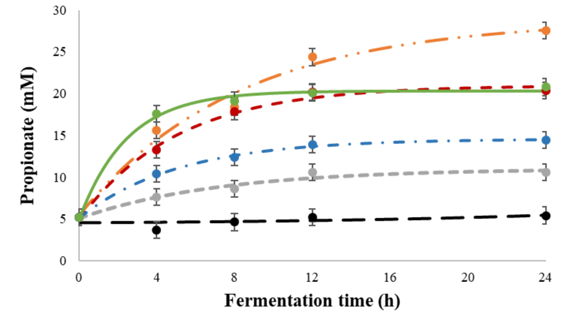
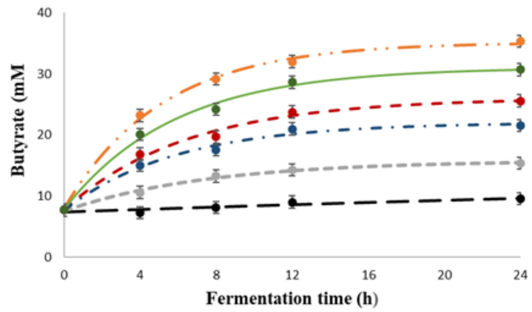
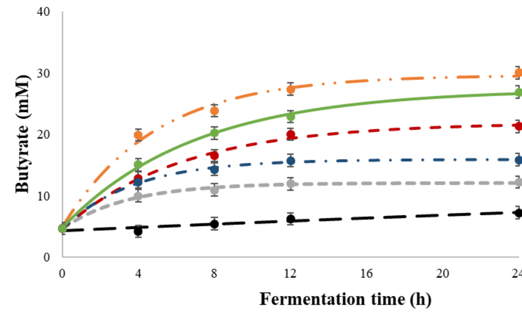






Maize meal (donor 1) Maize meal (donor 2) Maize meal (donor3





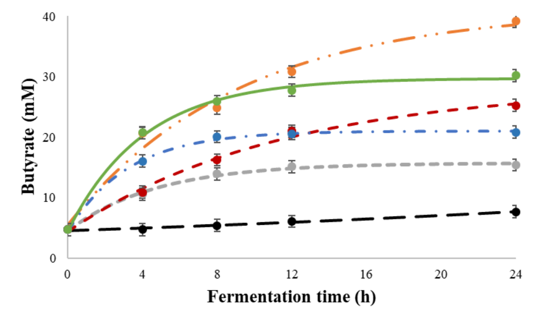


Figure 4.2.5: Effects of unhydrolyzed residues from unmodified and modified maize meal on SCFA production from the three donors during their 24 hr *in vitro* faecal fermentation.

Key: Blank black (— —), Control grey (-----), S. A red (– – –) HMT blue (- · – ·), S.A+HMT green (———) FOS orange (— · · —)
FOS = Fructooligosaccharides; S.A = Stearic acid added at 1.5% (w/w); HMT = heat moisture treatment was at 20 % moisture at 110°C for 16 hr; S.A+HMT = Combination treatment.

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Production of propionate for modified and unmodified maize starch and maize meal is presented in Figures 4.2.4 & 4.2.5 and Table 4.2.5. FOS had the overall highest significant ($p<0.05$) concentration in all the three donors compared to the indigestible residue. The indigestible residues from SAHMT showed significantly ($p<0.05$) higher production than the control in all donors in maize starch and maize meal treatments (Table 4.2.5). FOS had the highest time value to reach the maximum propionate with lower K value and the highest tau value, which was significantly different ($p<0.05$) from the maize starch and meal undigested residues. Regarding the indigestible residues, the combination of stearic acid and heat moisture treatment had the highest propionate production with corresponding higher time, low K value and high tau value which is significantly ($p<0.05$) different from the addition of S.A. alone and HMT alone and the control for both maize starch and maize meal in all the three donors (Table 4.2.5). The combination showed a synergistic effect in all the donors regarding the maize starch and meal indigestible residues.

Butyrate showed similar observations as that of acetate and propionate, graphically represented in Figures 4.2.4 & 4.2.5 and Table 4.2.6 for maize starch and maize meal indigestible residues, and FOS showed maximum production in all donors followed by indigestible residues from SAHMT. Indigestible residues from SAHMT maize starch and meal resulted in increased butyrate compared to the undigested residue of S.A. addition alone and HMT alone as well as residues from the control (Table 4.2.6). Butyrate produced by SAHMT indigestible residue were almost similar as FOS, especially in Donors 2 and 3. This could be attributed to the fermentation rate of the combination treatment (Pompei et al., 2008).

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Table 4.2.4: Effects of unhydrolyzed residue from unmodified and modified maize starch and maize meal on acetate production from three donors during their 24 h in vitro faecal fermentation.

Sample	Treatment	Donor 1				Donor 2				Donor 3			
		Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)
Maize Starch	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	20.1± 0.2^{a}	5.0± 1.7^{a}	0.2± 0.03^{a}	3.9± 1.2^{a}	18.9± 0.9^{a}	3.0± 0.8^{a}	0.33± 0.1^{a}	2.4± 0.6^{a}	20.1± 1.1^{a}	2.9± 1.1^{a}	0.34± 0.03^{a}	2.2± 0.8^{a}
	SA	34.5± 0.2^{c}	10.4± 0.9^{c}	0.1± 0.02^{b}	6.1± 0.4^{c}	30.8± 2.9^{c}	6.4± 0.3^{c}	0.16± 0.1^{c}	4.4± 0.2^{c}	30.3± 1.0^{c}	6.0± 0.6^{c}	0.17± 0.05^{c}	6.7± 0.4^{c}
	HMT	29.4± 0.3^{b}	8.5± 2.4^{b}	0.1± 0.02^{b}	5.4± 0.6^{b}	24.2± 1.3^{b}	4.3± 1.6^{b}	0.23± 0.1^{b}	3.4± 1.1^{b}	25.4± 0.4^{b}	4.0± 1.0^{b}	0.25± 0.04^{b}	4.3± 0.7^{b}
	SAHMT	40.8± 0.5^{d*}	12.2± 2.8^{d}	0.08± 0.02^{c}	7.9± 1.7^{d}	35.1± 1.0^{d*}	8.5± 0.4^{d}	0.1± 0.02^{d}	5.2± 0.2^{d}	40.9± 0.4^{d*}	8.2± 0.5^{d}	0.12± 0.1^{d}	8.4± 1.0^{d}
	FOS	50.3± 0.4^{e}	13.7± 0.5^{e}	0.07± 0.01^{c}	10.8± 1.2^{e}	42.5± 1.8^{e}	10.8± 0.5^{e}	0.09± 0.1^{e}	6.1± 0.3^{d}	50.6± 0.4^{e}	10.0± 0.7^{e}	0.1± 0.02^{d}	9.0± 0.5^{e}
Maize Meal	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	20.3± 1.7^{a}	5.5± 1.0^{a}	0.2± 0.2^{a}	1.7± 0.7^{a}	20.8± 0.6^{a}	2.5± 0.4^{a}	0.4± 0.1^{a}	1.7± 0.3^{a}	15.8± 0.2^{a}	2.8± 1.6^{a}	0.36± 0.1^{a}	2.3± 1.1^{a}
	SA	35.4± 0.5^{c}	8.6± 0.3^{c}	0.1± 0.1^{b}	5.1± 0.2^{c}	30.3± 0.6^{c}	5.1± 0.2^{c}	0.2± 0.2^{c}	4.1± 0.2^{c}	30.3± 0.2^{c}	6.3± 1.0^{c}	0.16± 0.2^{c}	5.9± 1.1^{c}
	HMT	29.8± 0.6^{b}	7.2± 0.3^{b}	0.1± 0.1^{b}	3.2± 0.2^{b}	25.8± 0.4^{b}	4.0± 0.8^{b}	0.25± 0.3^{b}	2.8± 0.5^{b}	20.3± 1.0^{b}	4.3.± 1.1^{b}	0.23± 0.1^{b}	3.5± 0.8^{b}
	SAHMT	40.8± 0.5^{d*}	10.9± 1.4^{d}	0.09± 0.1^{d}	7.1± 1.0^{d}	35.4± 1.1^{d*}	7.2± 0.7^{d}	0.13± 0.1^{d}	5.9± 0.9^{d}	40.8± 1.0^{d*}	8.1± 0.3^{d}	0.12± 0.1^{d}	6.9± 0.9^{d}
	FOS	50.5± 0.8^{bc}	13.3± 2.0^{e}	0.07± 0.1^{e}	9.0± 1.4^{e}	45.6± 1.9^{e}	9.7± 0.3^{e}	0.1± 0.1^{e}	8.2± 0.2^{e}	49.6± 1.0^{e}	11.8± 0.7^{e}	0.08± 0.1^{e}	9.5± 0.5^{e}

Mean ± standard deviation of three independent replicates for three donors

Different alphabetical letters in the same column are significantly different ($p \leq 0.05$)

This table was derived using this equation Y = Yo + A1e-x/t1; Yo = maximum acetate; t1=time to reach maximum; tau=time at half-life; k = exponential decay constant (derived from the equation); ND=not determined as non-fitting curve. FOS = Fructooligosaccharides; S.A=Stearic acid at 1.5% (w/w); HMT = heat moisture treatment at 20 % moisture at 110° C for 16 hr; S.A+HMT = Combination treatment *=synergistic effect

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Table 4.2.5: Effects of unhydrolyzed residue from unmodified and modified maize starch and maize meal on propionate production from three donors during their 24 h in vitro faecal fermentation.

		Donor 1				Donor 2				Donor 3			
Sample	Treatment	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)
	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	11.2 ± 4.3^{a}	3.0 ± 0.3^{a}	0.33 ± 0.1^{a}	4.7 ± 0.3^{a}	8.9 ± 1.0^{a}	5.1 ± 1.5^{a}	0.2 ± 0.1^{a}	3.7 ± 1.1^{a}	8.4 ± 1.0^{a}	3.1 ± 1.7^{a}	0.32 ± 0.1^{a}	2.6 ± 0.3^{a}
Maize starch	SA	20.9 ± 0.2^{bc}	5.5 ± 0.2^{c}	0.18 ± 0.1^{c}	7.3 ± 0.2^{c}	15.7 ± 1.1^{c}	8.8 ± 1.8^{c}	0.11 ± 0.1^{c}	6.4 ± 1.0^{c}	15.1 ± 1.1^{c}	5.3 ± 0.9^{c}	0.19 ± 0.2^{b}	4.5 ± 1.3^{c}
	HMT	18.2 ± 0.3^{b}	4.5 ± 0.2^{b}	0.22 ± 0.1^{b}	5.8 ± 0.2^{b}	12.1 ± 1.1^{b}	6.5 ± 1.2^{b}	0.15 ± 0.1^{b}	4.5 ± 0.5^{b}	12.9 ± 2.3^{b}	4.9 ± 0.3^{b}	0.20 ± 0.1^{b}	3.2 ± 0.2^{b}
	SAHMT	22.4 ± 1.0^{c}	6.7 ± 0.4^{c}	0.14 ± 0.1^{d}	9.8 ± 0.4^{d}	20.9 ± 1.2^{d}	10.5 ± 1.1^{d}	0.1 ± 0.1^{d}	8.5 ± 0.8^{d}	20.9 ± 1.8^{d}	6.0 ± 0.6^{d}	0.7 ± 0.1^{c}	6.2 ± 0.2^{d}
	FOS	25.3 ± 0.8^{d}	12.8 ± 1.2^{d}	0.08 ± 0.1^{e}	11.3 ± 0.1^{e}	25.4 ± 1.0^{e}	12.2 ± 1.6^{e}	0.08 ± 0.1^{e}	10.2 ± 1.1^{e}	25.5 ± 1.9^{e}	8.5 ± 1.3^{e}	0.1 ± 0.1^{d}	8.1 ± 0.3^{e}
	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	12.2 ± 0.7^{a}	2.5 ± 0.3^{a}	0.4 ± 0.1^{a}	1.8 ± 0.2^{a}	11.6 ± 1.0^{a}	3.1 ± 0.9^{a}	0.32 ± 0.2^{a}	2.5 ± 2.0^{a}	10.6 ± 1.8^{a}	2.5 ± 0.6^{a}	0.4 ± 0.1^{a}	1.8 ± 2.3^{a}
Maize meal	SA	18.4 ± 1.0^{c}	4.7 ± 0.6^{c}	0.21 ± 0.2^{c}	4.6 ± 0.4^{c}	15.9 ± 1.0^{b}	5.8 ± 0.5^{b}	0.17 ± 0.1^{b}	5.0 ± 0.4^{b}	18.4 ± 1.4^{c}	6.9 ± 0.5^{c}	0.14 ± 0.1^{c}	5.4 ± 1.4^{c}
	HMT	15.5 ± 0.5^{b}	3.5 ± 0.9^{b}	0.29 ± 0.1^{b}	3.4 ± 0.2^{b}	14.2 ± 0.5^{b}	5.4 ± 1.2^{b}	0.18 ± 0.1^{b}	4.5 ± 0.8^{b}	14.4 ± 1.5^{b}	4.2 ± 0.6^{b}	0.24 ± 0.1^{b}	3.0 ± 1.2^{b}
	SAHMT	21.1 ± 1.2^{d}	6.0 ± 0.8^{c}	0.17 ± 0.1^{d}	5.3 ± 0.6^{d}	20.9 ± 1.0^{c}	7.4 ± 0.9^{c}	0.14 ± 0.1^{c}	6.0 ± 0.6^{c}	20.9 ± 1.5^{c}	7.7 ± 0.5^{c}	0.13 ± 0.1^{c}	5.8 ± 1.8^{c}
	FOS	25.2 ± 0.4^{e}	12.6 ± 0.2^{d}	0.08 ± 0.1^{e}	6.4 ± 0.3^{e}	25.2 ± 0.8^{d}	11.6 ± 3.6^{d}	0.09 ± 0.1^{d}	7.9 ± 0.5^{b}	27.6 ± 1.8^{d}	9.6 ± 0.5^{d}	0.1 ± 0.1^{d}	7.7 ± 1.7^{d}

Mean ± standard deviation of three independent replicates for three donors.
Different alphabetical letters in the same column are significantly different ($p \leq 0.05$)
This table was derived using the equation $Y = Yo + A_1e^{-x/t1}$; Yo = maximum propionate; t_1=time to reach maximum (hr); tau=time at half-life; K = exponential decay constant (h^{-1}), ND=not determined as non-fitting of curve FOS=Fructooligosaccharides; S.A = Stearic acid was added at 1.5% (w/w); HMT = heat moisture treatment was at 20 % moisture at 110° C for 16 h; S.A+HMT = Combination treatment.

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Table 4.2.6: Effects of unhydrolyzed residue from unmodified and modified maize starch and maize meal on butyrate production from three donors during their 24 h in vitro faecal fermentation.

		Donor 1				Donor 2				Donor 3			
Sample	Treatment	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)	Yo (mM)	time (hr)	K (h^{-1})	Tau (hr)
Maize starch	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	15.8±0.3[a]	3.1±0.4[a]	0.32±0.1[a]	2.3±1.7[a]	13.9±1.1[a]	3.1±2.3[a]	0.32±0.1[a]	1.5±1.6[a]	13.3±0.5[a]	3.4±0.1[a]	0.29±0.1[a]	1.5±0.8[a]
	SA	25.7±0.5[b]	5.8±0.1[c]	0.17±0.1[c]	4.4±0.8[c]	25.1±1.0[c]	7.0±1.1[c]	0.14±0.1[c]	5.0±0.8[c]	30.4±1.0[c]	5.7±0.6[c]	0.18±0.1[c]	3.6±1.1[c]
	HMT	20.6±0.8[c]	4.1±0.8[b]	0.24±0.1[b]	3.4±1.9[b]	18.3±0.4[b]	5.6±0.2[b]	0.18±0.1[b]	3.9±1.6[b]	25.1±0.8[b]	4.2±0.4[b]	0.24±0.1[b]	2.5±0.3[b]
	SAHMT	35.2±1.0[c*]	7.9±0.6[d]	0.12±0.1[d]	6.6±0.4[d]	30.3±0.2[d*]	8.7±0.2[d]	0.11±0.1[d]	6.6±0.8[d]	35.1±1.0[d*]	7.8±2.0[d]	0.13±0.2[d]	6.6±1.4[d]
	FOS	40.3±0.4[d]	10.7±0.6[c]	0.09±0.1[e]	7.3±0.4[e]	35.0±0.9[e]	10.8±0.5[e]	0.09±0.1[e]	8.5±1.7[e]	39.2±1.0[e]	10.8±1.2[e]	0.09±0.1[e]	7.5±0.9[e]
Maize meal	Blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Control	12.3±1.1[a]	2.3±0.3[a]	0.43±0.2[a]	1.2±1.6[a]	12.2±0.6[a]	2.0±3.1[a]	0.50±0.1[a]	2.9±0.1[a]	15.4±0.4[a]	3.1±0.2[a]	0.32±0.1[a]	2.1±0.3[a]
	SA	25.6±0.3[c]	6.0±0.2[c]	0.17±0.1[c]	3.5±0.2[c]	21.3±1.0[c]	5.0±0.9[b]	0.2±0.1[c]	6.0±0.6[c]	25.2±0.9[c]	6.3±0.9[c]	0.16±0.1[c]	5.3±0.6[c]
	HMT	18.6±0.7[b]	4.0±0.7[b]	0.25±0.1[b]	2.8±0.8[b]	16.9±1.0[b]	3.7±0.3[b]	0.27±0.1[b]	4.4±0.2[b]	20.8±1.6[b]	4.1±1.0[b]	0.24±0.1[b]	3.3±1.2[b]
	SAHMT	30.7±1.0[d*]	8.5±0.6[d]	0.11±0.1[d]	7.9±1.8[d]	26.9±1.1[d*]	7.5±0.3[d]	0.13±0.1[d]	8.2±0.3[d]	30.2±2.6[d*]	7.6±0.9[d]	0.13±0.1[d]	6.2±0.6[d]
	FOS	35.3±1.0[e]	10.2±1.1[e]	0.1±0.1[d]	9.7±1.1[e]	35.0±0.4[e]	12.7±0.6[e]	0.08±0.1[e]	10.5±0.5[e]	39.2±0.9[d]	10.2±1.0[e]	0.09±0.1[e]	8.6±0.7[e]

Mean ± standard deviation of three independent replicates for three donors.
Different alphabetical letters in the same column are significantly different ($p \leq 0.05$)
This table was derived using this equation $Y = Yo + A_1e^{-x/t1}$; Yo = maximum butyrate; t_1=time to reach maximum (hr); tau=time at half-life; k = exponential decay constant (derived from the equation), ND=not determined as non-fitting curve.
FOS = Fructooligosaccharides; S.A = Stearic acid was added at 1.5% (w/w); HMT = heat moisture treatment was at 20 % moisture at 110° C for 16 hr; S.A+HMT = Combination treatment. *=synergistic effect

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From the above result (Figures 4.2.4 & 4.2.5) and (Tables 4.2.4 – 4.2.6), the rate and amount of acetate, propionate and butyrate production are dependent on both the treatments and the donors as well. The combination of stearic acid and heat moisture treatment among all the donors in acetate and butyrate showed a synergistic effect (Tables 4.2.4 & 4.2.6) regarding the maize starch and meal indigestible residues. This suggests that the undigestible residues from treated and untreated maize meal and maize starch served as good substrates for *in vitro* fermentation by human faeces.

Similar results from the literature have been reported as in the current research for other resistant starches. Jonathan et al. (2012), used an *in vitro* fermentation system with human faeces as inoculum, to indicate a total SCFA production of 7.6 and 7.7 mM/g organic matter with substrates such as retrograded tapioca starch and retrograded maize starch (i.e., RS3).

In a study by Charrier et al. (2013), the use of high amylose maize starch (RS3) feedings in rats, showed an increase in propionate, acetate, and butyrate, while Kalmokoff et al. (2013) showed an increase in the quantity of propionate with high amylose maize starch (RS 2). Besides the production of organic acids, gas and enzymes have also been used as markers to monitor stimulation of the bacterial activity (Sarbini & Rastall, 2011). Gas production had a positive correlation with SCFA production (Tables 4.2.7 & 4.2.8) and (Figures 4.2.2, 4.2.4 & 4.2.5). While gas production is inherent in the fermentation process, which results in SCFA production, it is also responsible as for the adverse effects of prebiotics in humans (i.e., abdominal discomfort). The ratio of total SCFA production to gas output may therefore be used to estimate tolerability when given *in vivo*. The fermentation of the indigestible residues produced these gases (Louis & Flint, 2009), which are used by the microbial community to help produce SCFA. For instance, Bacteroidetes are part of a community, stabilized by mutual cross-feeding, where other members of the community consume these gases. For example, Archaea produces CH_4 from CO_2 and H_2, while acetogens convert CO_2 into acetate.

Significantly ($p<0.05$) higher concentrations of acetate followed by butyrate were observed in fermentation end products of the indigestible residues of maize starch and maize meal compared to propionate (Tables 4-6). Low propionate production could be due to a non-functional propionyl-

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CoA carboxylase; this makes the gut microbiota (for example *Prevotella* spp) produce a minimal concentration of propionate (Cherrington et al., 1991). This estimated the propionate production rate as lower compared to the reported acetate and butyrate production.

Concerning the maize starch and meal indigestible residues, the combination treatment (SAHMT) of maize starch and maize meal had lowest K value and showed slow fermentation rate for all the donors. This suggests that the complex structure formed from heat moisture treatment in combination with stearic acid addition was not readily fermentable by saccharolytic bacteria in the faeces. This complex structure mostly is the amylose-lipid complexes as shown by DSC (Figure 4.1.1).

In general, the overall effect of the SAHMT of both maize starch and meal was higher production of SCFAs, since more production of SCFAs has a positive impact in our system.

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Table 4.2.7: Correlation coefficient between gas, pH, and short chain fatty acid production of modified and unmodified maize starch.

Main effects	ΔH	**Gas**			**pH**			**Acetate**			**Propionate**			**Butyrate**		
		Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
ΔH		1.0**	1.0**	0.89	-1.0**	-0.89	-1.0**	0.75	0.64	0.72	0.73	0.69	0.78	0.71	0.63	0.75
Gas D1	1.0**		1.0**	0.91	-1.0**	-0.90	-1.0**	0.79	0.69	0.76	0.77	0.73	0.81	0.75	0.68	0.78
Gas D 2	1.0**	1.0**		0.90	-1.0**	-0.86	-1.0**	0.75	0.65	0.71	0.76	0.76	0.77	0.70	0.63	0.76
Gas D 3	0.89	0.91	0.90		-0.89	-0.78	-0.88	0.94	0.88	0.86	0.96*	0.86	0.91	0.85	0.86	0.96*
pH D1	-1.0**	-1.0**	-1.0**	-0.89		0.89	0.99*	-0.75	-0.65	-0.73	-0.74	-0.70	-0.78	-0.71	-0.64	-0.75
pH D 2	-0.88	-0.90	-0.86	-0.79	0.89		0.81	-0.79	-0.74	-0.88	-0.68	-0.84	-0.88	-0.86	-0.76	-0.74
pH D 3	-1.0**	-1.0**	-1.0**	-0.88	0.99*	0.81		-0.70	-0.58	-0.64	-0.72	-0.62	-0.71	-0.63	-0.56	-0.72
Ace D 1	0.75	0.79	0.75	0.94	-0.75	-0.79	-0.70		0.99*	0.96*	0.97*	0.98*	0.98*	0.97*	0.98*	0.99*
Ace D 2	0.64	0.69	0.65	0.88	-0.65	-0.74	-0.58	0.99*		0.97*	0.95	0.98*	0.97*	0.97*	1.0**	0.97*
Ace D 3	0.72	0.76	0.71	0.86	-0.73	-0.88	-0.64	0.96*	0.97*		0.88	1.0**	0.99*	1.0**	0.98*	0.92
Prop D 1	0.72	0.77	0.76	0.96*	-0.74	-0.68	-0.72	0.97*	0.95	0.88		0.90	0.92	0.88	0.93	0.99*
Prop D 2	0.69	0.73	0.76	0.86	-0.70	-0.84	-0.62	0.98*	0.98*	1.0**	0.90		0.99*	1.0**	0.99*	0.94
Prop D 3	0.78	0.81	0.77	0.91	-0.78	-0.88	-0.71	0.98*	0.97*	0.99**	0.92	0.99*		0.99*	0.98*	0.96*
But D 1	0.71	0.75	0.70	0.85	-0.71	-0.86	-0.63	0.97*	0.97*	1.0**	0.88	1.0**	0.99*		0.98*	0.93
But D 2	0.63	0.68	0.63	0.86	-0.64	-0.76	-0.56	0.98*	1.0**	0.98*	0.93	0.99*	0.98*	0.98*		0.96*

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But D 3	0.75	0.78	0.76	0.96*	-0.75	-0.74	-0.72	0.99**	0.97*	0.92	0.99*	0.94	0.96*	0.93	0.96*	

Table 4.2.8: Correlation coefficient between gas, pH, and short chain fatty acid production of modified and unmodified maize meal.

Main effects	ΔH	**Gas**			**pH**			**Acetate**			**Propionate**			**Butyrate**		
		Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
ΔH		0.97*	0.87	0.90	-0.92	-0.83	-0.74	0.99*	0.99**	0.98*	0.99**	0.98*	0.99*	0.99**	0.99**	0.99**
Gas D1	0.97*		0.84	0.97*	-0.87	-0.93	-0.86	0.97*	0.96*	0.92	0.99*	0.98*	0.99	0.94	0.96*	0.96*
Gas D 2	0.87	0.84		0.73	-0.99*	-0.59	-0.46	0.81	0.87	0.93	0.84	0.94	0.82	0.84	0.88	0.86
Gas D 3	0.90	0.97*	0.73		-0.76	-0.98*	-0.94	0.92	0.89	0.82	0.88	0.90	0.86	0.86	0.89	0.89
pH D1	-0,92	-0.87	-0.99*	-0.76		0.63	0.50	-0.87	-0.91	-0.96*	-0.89	-0.96*	-0.87	-0.89	-0.93	0.90
pH D 2	-0.83	-0.93	-0.59	-0.98*	0.63		0.99*	-0.87	-0.82	-0.73	-0.82	-0.81	-0.80	-0.80	-0.81	-0.83
pH D 3	-0.73	-0.86	-0.46	-0.94	0.50	0.99*		-0.80	-0.73	-0.62	-0.74	-0.71	-0.72	-0.71	-0.72	-0.75
Ace D 1	0.99*	0.97*	0.81	0.92	-0.87	-0.87	-0.80		0.99*	0.96*	1.0**	0.96*	0.99*	0.99*	0.99*	1.0**
Ace D 2	0.99**	0.96*	0.87	0.89	-0.91	-0.82	-0.73	0.99*		0.99*	1.0**	0.98*	0.99*	1.0**	1.0**	1.0**
Ace D 3	0.98*	0.92	0.93	0.82	-0.96*	-0.73	-0.62	0.96*	0.99*		0.98*	0.98*	0.97*	0.98*	0.99*	0.98*
Prop D 1	0.99**	0.99*	0.84	0.88	-0.89	-0.82	-0.74	1.0**	1.0**	0.98*		0.97*	1.0**	1.0**	1.0**	1.0**
Prop D 2	0.98*	0.98*	0.94	0.90	-0.96*	-0.81	-0.71	0.97*	0.98*	0.98*	0.97*		0.95	0.96*	0.98*	0.98*
Prop D 3	0.99*	0.99	0.82	0.86	-0.87	-0.80	-0.72	0.99*	0.99*	0.97*	1.0**	0.95		1.0**	0.99*	0.99*
But D 1	0.99**	0.94	0.84	0.86	-0.89	-0.80	-0.71	0.99*	1.0**	0.98*	1.0**	0.96	1.0**		0.99*	1.0**

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But D 2	0.99**	0.96*	0.88	0.89	-0.93	-0.81	-0.72	0.99*	1.0**	0.99*	1.0**	0.98*	0.99*	0.99*		1.0**
But D 3	0.99**	0.96*	0.86	0.89	0.90	-0.83	-0.75	1.0**	1.0**	0.98*	1.0**	0.98*	0.99*	1.0**	1.0**	

** means significant with $p \leq 0.001$; * means significant with $p \leq 0.05$.

ΔH = Delta H; Gas D1 – D3 = Gas donor 1, Donor 2 and Donor 3; pH D1 – D3= pH Donor 1, Donor 2 and Donor 3; Ace D1 – D3= Acetate Donor 1, Donor 2 and Donor 3; Prop D1 – D3 = Propionate Donor1. Donor 2 and Donor 3; But D1 – D3 = Butyrate Donor1, Donor 2 and Donor 3.

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This slow but ultimately high fermentation of the ALCs could be beneficial since the metabolites produced are evenly distributed and contribute to the energy requirement of the entire colon (Hoover, 2010; Williams et al., 2011). Findings from this study are in agreement with work done by Goni et al. (2000) who reported that fermentability was slow for retrograded starch samples (RS3). They suggested that the fermentation rate of RS3 was associated with the crystallinity level and crystalline type. Wang et al. (2021) also reported that B-type polymorph potato and high-amylose maize starch (RS2) also showed slow and completely fermentable within a 24 h fermentation period. There is limited work on effects of ALC on faecal fermentation. In the current study, there was a significant ($p<0.05$) increase in the production of total SCFAs, acetate and butyrate for both maize starch and maize meal indigestible residues during fermentation of faecal inocula from all three donors. These changes show high fermentability and SCFA production by human faecal inocula of the RS type 5, particularly indigestible residues rich in ALC. The molar ratios obtained for all the substrates confirmed the findings that, in three donors, acetate is by far the dominant SCFA produced during fermentation. Notably, the combination treatment (SAHMT) of maize starch and meal increased the concentrations of butyrate in Donors 2 and 3 similar as the butyrogenic FOS used as control.

4.2.5 Conclusion

Upper GIT indigestible residues from maize meal and maize starch modified with HMT and SA alone and in combination were shown to function as prebiotics, like FOS. Acetate and butyrate appear to be the dominant SCFAs fermentation metabolites of these indigestible residues. Hence, resistant starch type 5 (ALC) could be used to promote health benefits for different ailments. Further studies are required to elucidate the potential of RS 5 as a substrate in *in vivo* studies and its mechanism in the gut needs to be assessed.

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4.3. Microbiota of human faeces used for fermentation of type 5 resistant starch-rich fractions from maize meal and maize starch

Abstract

The effects of indigestible residues of heat moisture treated maize starch and maize meal modified with stearic acid on the microbiota of three human donors were investigated. The indigestible residues were resistant starches predominantly made up of amylose – lipid complexes. A blank was used as the negative control whereas fructooligosaccharides (FOS) was used as the positive control. Three dominant phyla (Firmicutes, Bacteroidetes and Actinobacteria) were initially present in the three donors. After treatments, bacteria with increased relative abundance were the butyrate producers *Roseburia, Ruminococcus, Faecalibacterium* and *Collinsella* spp.; and propionate, and acetate producers such as *Bacteroides, Blautia, Bifidobacterium* and *Parabacteroides* spp. The indigestible residue with the combination treatment of stearic acid followed by heat moisture treatment (SAHMT) of maize meal had higher relative abundance in some of the microbes in Donors 2 and 3 than the other treatments and even FOS compared to Donor 1. Most of the bacteria listed above were stimulated for growth when the indigestible residue with the combination treatment (SAHMT) of maize meal was used as a substrate. This means the combination treatment (SAHMT) of maize meal may be more beneficial to the lower gut than maize starch with the same treatment, or other single treatments.

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4.3.1 Introduction

The human gut microbiome has about 1.3 trillion microorganisms, that are located more specifically in the colon. They are recognized to impact human health. These microbes include mostly bacteria, but also viruses, fungi, and protozoa of which most are commensal with the human intestinal tract (Gill et al. 2006). Most bacterial groups of the microbiota are gram-positive Firmicutes and gram-negative Bacteroidetes, though other important phyla include Actinobacteria which includes bifidobacteria and Proteobacteria. Bacteria have a beneficial effect, such as the production of short-chain fatty acids (SCFA), which provide energy to the colonocytes and body (Vinolo et al. 2011). It also prevents pathogenic bacteria from colonizing of the colon (Clarke et al., 2010), aid in digestion and absorption of nutrients (Maslowski & Mackay, 2011) and stimulates the immune system (Flint, Scott, Louis, & Duncan, 2012; Hooper, Littman, & Macpherson, 2012).

The human microbiome has physiological importance to health and acts like a functional organ of the human body. However, the wrong balance of microbes resulting in an impaired microbiota, also known as dysbiosis (also called dysbacteriosis), can have detrimental effects on human health. Dysbiosis can lead to the production of endotoxins that cause inflammation (Sartor & Mazmanian, 2012) and carcinogens (Zhu, Gao, Wu, & Qin, 2013). Dysbiosis also is associated as a causative factor for cardiovascular diseases (Wang et al. 2011) and obesity (Ridaura et al. 2013; Xu et al. 2013). A dysbiotic microbiota is characterized by a (1) loss in microbial diversity; (2) change in composition, including bloom of pathogenic bacteria and decrease in commensal or potentially beneficial bacteria; and (3) change in metabolic function that ultimately leads to adverse consequences for the host (Schaubeck and Haller, 2015). It is therefore essential to maintain a balance in the colonic microbiota composition for health purposes. Dietary fibre, including resistant starch, in a diet has been recognized to bring about a balance in the colonic microbiota composition and provide beneficial health outcomes (Desai et al. 2016; Sonnenburg et al. 2016). The gut flora in the colon also helps to break down dietary fibre including resistant starch that enter the colon to produce short-chain fatty acids.

Resistant starch (RS) is the portion of starch which is not hydrolyzed *in vitro* after 120 min and *in vivo* continues its passage from the small to the large intestine (Englyst et al. 1992), where it can be fermented by the intestinal microflora (Nugent 2005). RS is subdivided into five classes: RS1

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is the physically protected form of the starch found in whole grains, RS2 represents raw starch granules, RS3 represents retrograded starch (Jiang and Jane 2013), RS4 represents chemically modified starch (Luckett and Wang 2012), and lastly, RS5 is characterized by a lipid component that has complexed with amylose to form a helical structure that contains a fatty acid tail within the central cavity (amylose-lipid complex, ALC) (Hasjim et al. 2013).

In a previous work done, it has been shown that when maize starch and maize meal were heat-moisture-treated (20 % moisture, 110 °C for 16 h) with stearic acid (1.5% w/w starch), about 37 and 38 % of RS were formed as determined by *in vitro* starch digestibility (chapter 1). The RS were found to contain amylose-lipid complexes. The indigestible residue from *in vitro* starch digestibility that contained RS was also found to produce acetic, propionic, and butyric acid as short chain fatty acids during *in vitro* faecal fermentation compared with FOS and the control maize starch and maize meal (Chapter 4.2). This suggests that the indigestible residues containing ALC can function as prebiotics.

Most researchers have investigated the influence of RS types 1-4 on gut health, focused on endpoints such as gas production, SCFAs and gut bacterial composition (Martinez et al. 2010; Upadhyaya and others 2016). Little or no information is available regarding the influence of RS5, specifically from indigestible residues containing ALC on the gut microbiota. The objective of this study was to determine the effect of indigestible residues from heat-moisture modified and unmodified maize starch and maize meal with stearic acid on the human gut microbiota.

4.3.2 Materials and Method

4.3.2.1 Materials

Commercial super fine maize meal was purchased from Rainbow Chicken Limited (RCL) (Pretoria, South Africa). It contained about 15 % moisture, 8 % protein, 1.1% fat content, 0.65 % Ash, dietary fibre of 4.7% and a total starch of approximately 81.3% as-is basis. Commercial maize starch, Amyral® with 12.9 % moisture, 0.64% protein, 0.31% fat content, 0.08 % Ash, dietary fibre content of 0.9 g/100g and total starch content of 95 % as is basis was obtained from Tongaat Hulett® Starch (Edenvale, South Africa). Stearic acid (approximately, 97% G.C. grade) with CAS

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number of 57-11-4 was purchased from Sigma–Aldrich Company (St. Louis, MO, USA). All other reagents used in this study were analytical grade.

4.3.2.2 Methods

4.3.2.2.1: Stearic acid (S.A.) addition to maize meal and maize starch

Stearic acid (1.5% w/w in absolute ethanol) was added to 100 g of each maize starch and maize meal followed by the procedure of D'Silva, Taylor, and Emmambux (2011). The mixture was incubated in the shaking water bath at 50 °C for 30 min with a speed of 120 rpm. After the incubation period, the mixture was then dried in a hot air oven at 40 °C to evaporate the excess solvent. Then residues were stored at 4 °C for further analysis.

4.3.2.2.2. Heat-moisture treatment (HMT)

The maize starch and maize meal were mixed with deionized water to give a desired moisture content of approximately 20% for heat-moisture treatment. The starch slurry was heated above the glass transition temperature, but below the gelatinization temperature (Jacobs & Delcour, 1998), thus a temperature of 110 °C for 16 hr (Kweon et al. 2000) in a hot air oven with and without stearic acid (1.5% w/w solid material) and the undigested residues were used for further studies.

4.3.2.2.3. Production of indigestible residues

Goňi et al. (1997) method was used with slight modification. A sample size of 50 mg undigested residues was used for the upper GIT analysis. One mL of boiling water was added to each sample for dispersion followed by addition of 10 ml HCl–KCl buffer (pH 1.5) and 0.2 mL of a solution containing 1mg of pepsin (Sigma–Aldrich P7000-100G). The samples containing solutions were incubated at 40 °C for 60 min with constant agitation. After incubation period 10 ml of Tris-maleate buffer (pH 6.9) was added to the solutions and adjusted to 25 mL. Aliquots of 0.1 ml were taken at 5 min and then at intervals of 30 min until 120 min and jumped to 180 mins to end digestion. The tubes containing the aliquots taken were placed in boiling water for 15 min to inactivate α-amylase.

Then, 1 mL of 0.4 M sodium–acetate buffer (pH 4.75) and 90 µL of amyloglucosidase with an activity of 64.7U/mg (Megazyme E-AMGDF) was added and incubated at 60 °C for 45 min. The

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solution was centrifuged at 7500 rpm for 5 mins. The supernatant was discarded, and the undigested residue was collected and freeze-dried and stored prior for further analysis.

4.3.2.2.4. Fermentation of indigestible residues

Batch faecal fermentation was performed as the methodology of Lebet et al. (1998) with some minor modifications (Rose, Patterson, & Hamaker, 2010). A 50 mg of each substrate (modified and unmodified maize starch and maize meal) were weighed in three test tubes for triplicate analysis. Faecal samples were obtained from three volunteers who fed on an unspecified and varied diet and had not taken any antibiotics for six months. Faecal samples were collected in plastic bags that were sealed after removing the air and immediately placed inside the anaerobic chamber (10 % H_2, 5 % CO_2, and 85 % N_2; Bactron EZ, SHEL LAB, Cornelius, OR) where all further procedures were performed within 2 hr after collection. Faecal samples were used individually as per donors, and slurry prepared by homogenization with carbonate-phosphate buffer pH 6.8 ± 0.1 in a ratio of 1:3 (w/v) and further strained through four layers of cheesecloth. The samples (indigestible residues from modified and unmodified maize starch and maize meal) with their controls (fructooligosaccharides and blank) were hydrated with 4 ml of carbonate-phosphate buffer pH 6.8 ± 0.1 and then inoculated with 1 ml of the faecal filtrate. The tubes were then sealed and incubated at 37 °C in a shaking water bath. The tubes were finally removed after the incubation period and aliquots of 1 ml of the initial inoculum, 0 and 24 h fermentation time were taken and stored at -80 °C deep freezer for the DNA metagenomics analysis.

4.3.2.2.5 DNA extraction

DNA extraction was carried out from the 1 ml of the stored faecal ferment. Automated DNA extraction was conducted using the QIA cube Connect instrument (Qiagen, Germantown, MD) with the QIAamp Power Faecal Pro DNA kit (Qiagen, Germantown, MD) according to the manufacturer's instruction. The principle is for the DNA to bind the silica-gel membrane and allow the contaminant to pass through. The DNA was purified, and the concentration of DNA was determined using nano drop. The DNA sample (10 µl) was pipetted in a micro-plate for sequencing.

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4.3.2.2.6 16S rRNA gene sequencing

The V4 region of the 16s rRNA gene was amplified for each sample containing DNA extract as described previously (Kozich et al., 2013) by PCR with primers 515F 5'-GTGCCAGCMGCCGCGGTAA and 806R 5'-GGACTACHVHHHTWTCTAAT with a sample-unique 10-mer oligonucleotide barcode. The sequencing was done at the University of Illinois at Chicago (Chicago, IL, USA).

4.3.2.2.7 Bioinformatics

The Illumina-generated sequencing data were processed and analyzed through QIIME2 (q2) platform, including the q2-DADA2 plugin for sequence quality control (Caporaso et al., 2010). Operational taxonomic units (OTUs) were generated using the UCLUST method with a 97% similarity threshold in QIIME, and taxonomic annotations were assigned to each OTU by comparing to the green genes database (version 13.8). Differences in microbial communities of treated samples and controls were evaluated separately in each donor using Shannon Index for alpha diversity. Bacterial relative abundances were visualized at the phylum, and genus levels for each faecal sample through the q2-taxa plugin.

4.3.2.2.8. Statistical analyses

The experimental design was 3*2*2 factorial design (3 donors, stearic acid with 2 levels i.e., 0% and 1.5% and heat moisture treatment with 2 levels i.e., 0% and 20%). Analysis of variance (ANOVA) was used to determine significant differences between donors used with respect to the treatments in terms of the alpha diversity. The data for the donors were analysed separately using SPSS version 22 (USA). Means were compared using the Fischer’s Least Significant Difference Test (LSD) at $P \leq 0.05$.

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4.3.3. Results and discussion

Firmicutes, Bacteroidetes and Actinobacteria were the major phyla found in the faecal inoculum from all three donors (Figure 4.3.1a). Actinobacteria was higher in Donor 2 compared to Donors 1 and 3. Proteobacteria was higher in Donor 3 compared to Donors 1 and 2 (Figure 1a). Similar results were also reported by researchers on the initial inoculum phyla confirming the three phyla (Firmicutes, Bacteroidetes and Actinobacteria) as the major ones in human microbiome with proportions different among individuals (Salonen et al. 2010; Qin et al. 2010; Sender, Fuchs, & Milo, 2016; den Besten et al. 2013).

At the genus level (Fig 1b), *Bacteroides* from phylum Bacteroidetes was initially the most abundant bacteria in Donors 1 and 2 and was in high proportion in Donor 3. Genus *Roseburia* was the most abundant for donor 3, but also present in the other two donors. *Ruminococcus, Blautia* genus from the family Lachnospiraceae and *Faecalibacterium* from family Ruminoccocaceae were also abundant in the donors and these are from phylum Firmicutes. *Bifidobacterium* and *Collinsella* from the phylum Actinobacteria were also abundant in the donors' initial faecal inoculum. The abundance of microbes from these genera are quite common in human faecal microbiomes (Yang et al. 2013; Monro and Mishra 2009). The microbes from genera such as *Roseburia*, *Bacteroides, Bifidobacteria* are well known to ferment dietary fibre and produce acetate, propionate, and butyrate as short chain fatty acid (SCFAs) (Li et al., 2015). The microbes in the initial inoculum are resident commensal bacteria that are known to produce catabolic enzymes for dietary fibre degradation. (Ley et al. 2006; Bach Knudsen, 2015).

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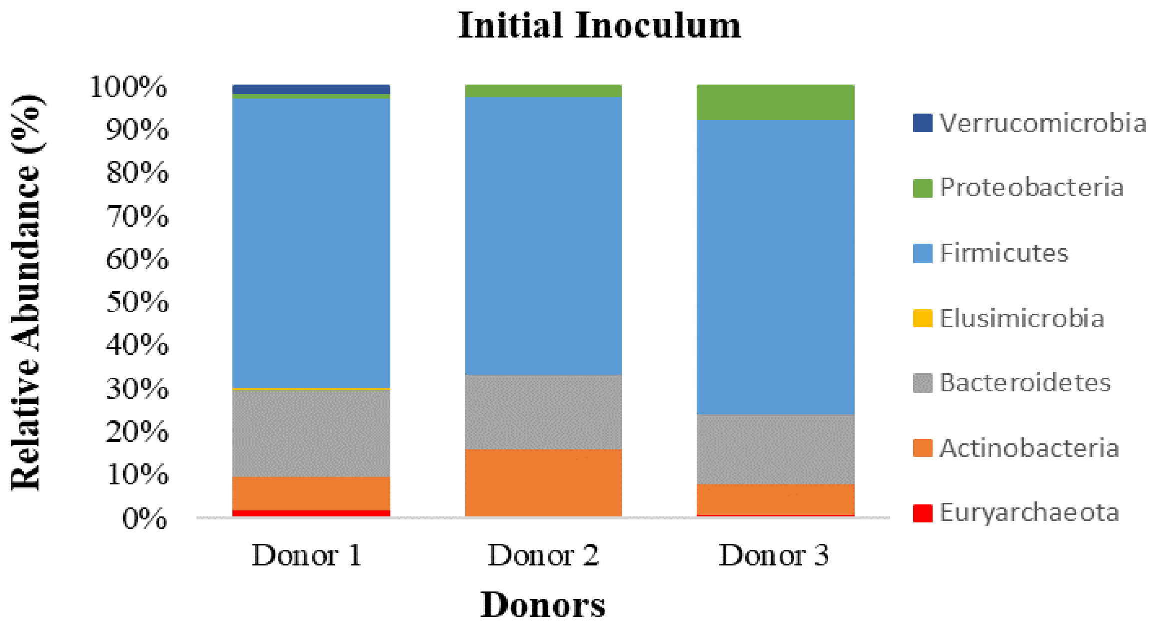
a



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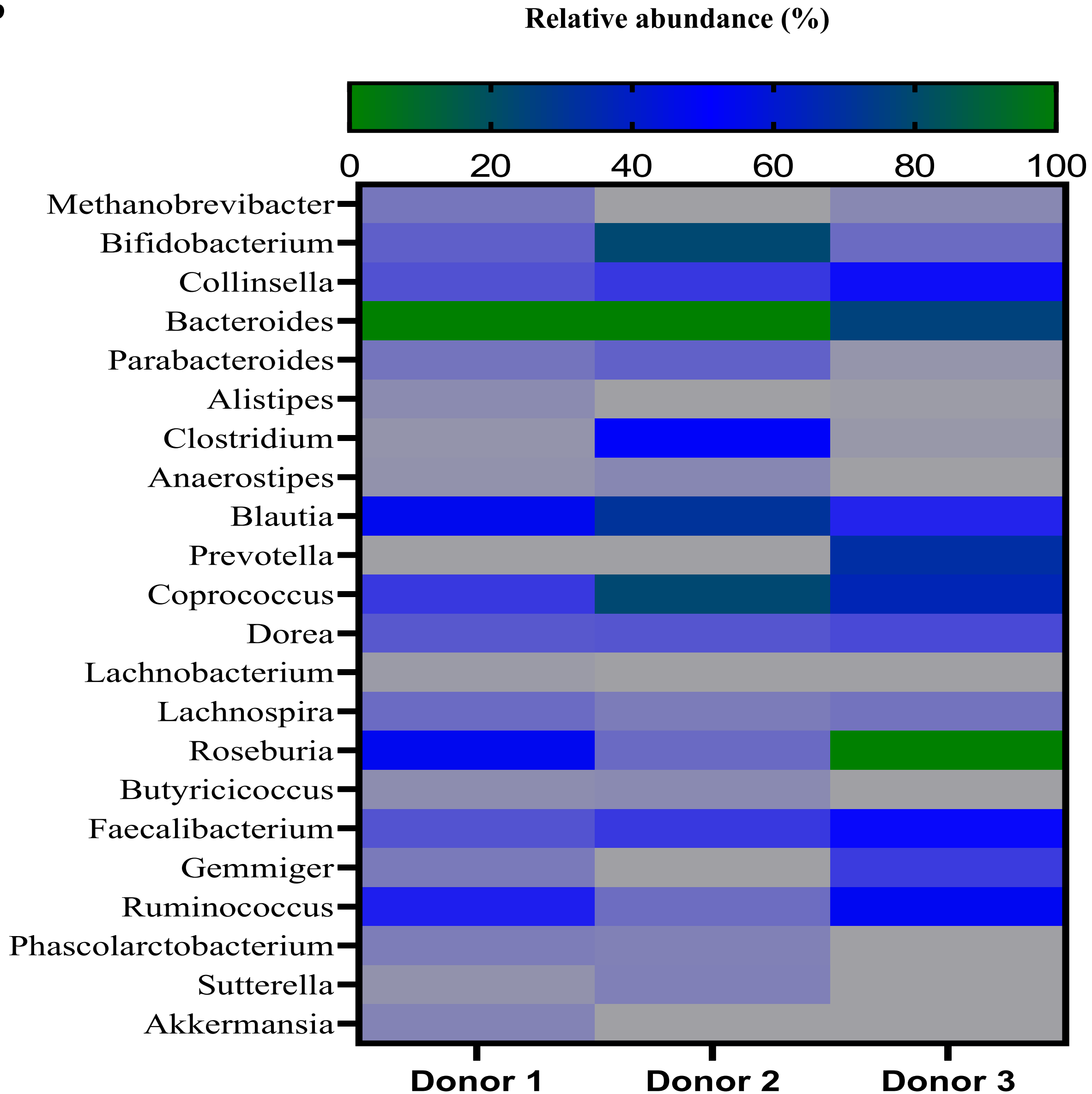

Figure 4.3.1: Initial inoculum of faeces from three donors at the (a) phylum level (b) genus level.

Microbiota composition at the phyla level were affected differently by donor for the indigestible residues from maize starch and maize meal treatments after 24 hr *in vitro* faecal fermentation (Figure 4.3.2 A-C). The phyla Firmicutes, Bacteroidetes and Actinobacteria were still dominant in all the donors when modified and unmodified indigestible residues of maize starch and maize meal were used as substrates during fermentation. Similar phyla results were also reported by Canani et

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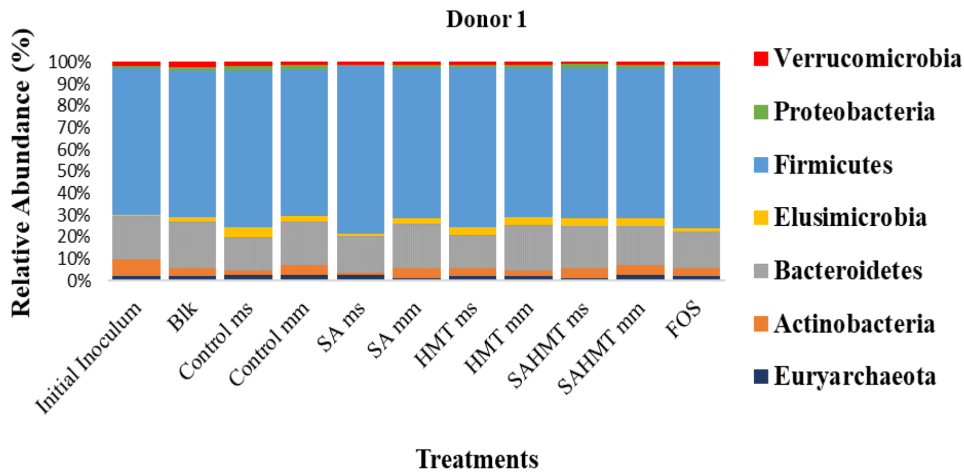
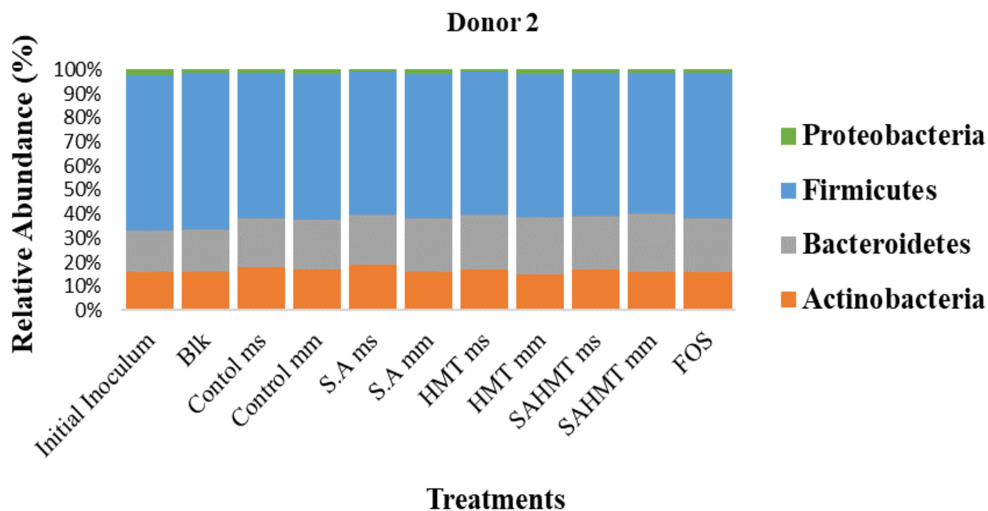
al. (2011) and Lin & Zhang (2017). In Donor 1, the phyla Firmicutes somewhat decreased in comparison with FOS, with maize starch with stearic acid indigestible residues, and Bacteroidetes increased with all the treated indigestible residues. However, this was not observed for Donors 2 and 3 where proportions of the major phyla did not appreciably change between FOS and the ALC treatments. A healthy gut microbiota is dominated by the phyla Firmicutes and Bacteroidetes, followed by the phyla Actinobacteria and Proteobacteria, with occasional observation of the phyla Verrucomicrobia, Elusimicrobia and Euryarchaeota (Benson et al., 2010; Dethlefsen et al., 2008; Dubourg et al., 2013), and this was maintained after fermentation treatments.

A



B



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C

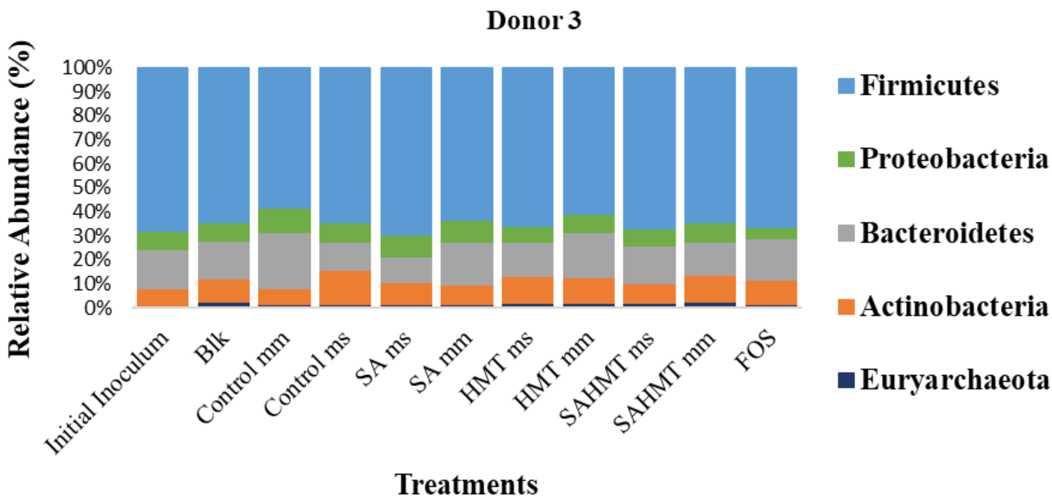

Figure 4.3.2: Effects of indigestible residue of the individual treatments from maize starch and maize meal at the phylum level on microbiota composition after 24 hr *in vitro* faecal fermentation.

Key: Blk = Blank, MS = Maize starch, MM = Maize meal, SA = Stearic acid at 1.5% (w/w), HMT = Heat moisture treatment at 20% moisture, SAHMT = Combination treatment.

Several microbes were identified but only a few were selected for discussion based on their relative abundance and health implications. These bacteria were as follows; three of them are major butyrate producing microbes: *Roseburia, Ruminococcus* and *Faecalibacterium* from the phylum Firmicutes, acetate and propionate producing bacteria such as *Bacteroides* and *Parabacteroides* spp., from the phylum Bacteroidetes and *Blautia spp*. from the phylum Firmicutes, and *Bifidobacterium spp*. from the phylum Actinobacteria. *Collinsella spp* is also from the phylum Actinobacteria which is also known to produce butyrate. The selected microbes play a significant role in the development of a healthy gut microbiome.

Effects of indigestible residues of RS5 modified and unmodified maize starch and maize meal on selected genera after 24 h of faecal fermentation is shown in Table 4.3.1 and Figure 4.3.3. The genus *Roseburia* had higher relative abundance for donor 2 compared to donors 1 & 3 (Figure 4.3.3). *R. faecis and R. inulinivorans* were detected in both modified and unmodified maize starch and maize meal treatments (Figure 4.3.3). Roseburia spp were able to ferment both maize starch and meal treated with stearic acid alone, HMT alone and the combination treatment (SAHMT).

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From Table 4.3.1, the genus *Roseburia* increased in maize starch (MS) added with stearic acid (SA) alone and HMT alone increased two-fold compared to the control maize starch and maize meal (Control ms & mm) and three-fold for FOS. In Donor 2, the relative abundance of the genus *Roseburia* in the combination treatment of maize starch (SAHMT ms) was two times lower compared to the control maize starch and maize meal, SA mm, HMT ms, FOS and fermentation at 0 h (initial inoculum), whereas in Donor 3, the relative abundance of the genus *Roseburia* increased as two-fold of the combination treatment of maize meal (SAHMT mm) compared to the control ms & mm as well as the 0 h fermentation. Report from Susan et al. (2002) showed similar results when working on RS4 (chemical modification). They suggested that fermentation of RS4 promoted an increased production of butyrate by the genus *Roseburia*.

The genus *Blautia* was detected in all the treated and untreated maize starch and meal with relatively minimal abundance (Figure 4.3.3). *B. producta* and *B. obeum* was high in abundance when SA mm was used a substrate compared to the other indigestible residues in Donor 3 (Figure 4.3.3). The genus *Blautia* was high for the indigestible residues compared to control ms and SA ms in Donor 1 (Table 4.3.1). In Donors 2 and 3, the combination treatment of maize meal (SAHMT mm) was greater in abundance compared to the control mm and even FOS (Table 4.3.1). The higher abundance of the genus *Blautia* observed after 24 h of fermentation from residues of SAHMT mm in Donors 2 & 3 could be attributed to the physical form of the dietary fibers as resistant starch (Goñi, Martín-Carrón, and Calixto, 2000) (i.e., amylose-lipid complexes) and their role in fermentation of the ALC. The genus *Blautia* plays an important role in nutrient assimilation and the defense of the colonic epithelial cells against disease such as diabetes type-2 and obesity. It is also known as an acetate-producing microbe, which helps in the reduction of gluconeogenesis (Nyholm and Graf, 2012; McFall-Ngai et al, 2013).

The abundance of *Ruminococcus* in Donor 1 increased during faecal fermentation for blank, SA ms and HMT ms compared to FOS and the other indigestible residues after 24 hours of fermentation (Table 4.3.1). However, in Donor 2, the abundance of *Ruminococcus spp*. in 0 h (initial inoculum) and 24 h fermentation for the blank was almost three-fold higher than even FOS and the other indigestible residues. Maize starch and maize meal treated with stearic acid alone, heat moisture treatment alone and their combination treatment were higher compared to their respective control maize starch & maize meal (Table 4.3.1). On the other hand, Donor 3 showed

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two-fold increase for *Ruminococcus* from faecal fermentation of indigestible residue of maize meal modified with HMT followed by stearic acid (SAHMT). Many anaerobic bacteria are known to degrade starch, but *Ruminococcus bromii* (Figure 4.3.3) has been identified as a key primary degrader of resistant starches (Ze, Duncan, Louis, Flint, 2012). *R. bromii* was abundant in Donor 2 compared to Donors 1 and 3 (Figure 4.3.3).

In the genus *Faecalibacterium* Donors 2 and 3, the abundance of *Faecalibacterium* was predominant in the combination treatment (SAHMT) of maize meal compared to the control maize starch and maize meal, 0 h (initial inoculum) and the blank after 24 h of faecal fermentation as well as FOS and the other modified indigestible residues. *F. prausnitzii* was identified (Figure 4.3.3) but its abundance was not prominent. The 0 h (initial inoculum) and the fermentation that happened in the blank at 24 h doubled its abundance compared to the indigestible residues of maize starch and maize meal for Donor 1. *F. prausnitzii* can improve gut barrier function (Stenman, Burcelin, and Lahtinen 2015).

Considering the genus *Bacteroides*, regarding Donors 2 and 3, after 24 h of faecal fermentation of the indigestible residues of maize meal treated with stearic acid followed by heat moisture treatment, the abundance of *Bacteroides* was two-fold compared to the control maize starch and meal, 0 h (initial inoculum) and the blank after 24 h of fermentation. This also suggests that members of the genus *Bacteroides* are well equipped with machinery to degrade indigestible carbohydrates such as resistant starch (Koropatkin et al., 2012; Flint et al., 2012: Yang et al., 2013). The 0 h (initial inoculum) and blank after 24 h fermentation had higher abundance compared to fermentation of the indigestible residues for Donor 1. Stearic acid alone, heat moisture treatment alone and the combination treatment of maize meal was higher compared to its respective maize starch in Donor 1. The abundance of *Bacteroides* spp. was reduced seven-fold in control maize starch after 24 h of fermentation compared to the 0 h (initial inoculum) and the blank. Members of the genus *Bacteroides* are known to produce succinate, acetate, and propionate (Krieg et al., 2012; Jakobsson et al., 2014) to promote the health to individuals. *Bacteroides ovatus* which was detected in all the samples of treated and untreated maize starch and maize meal was higher in abundance in Donors 3 and 1 compared to Donor 2 whereas *B. plebeius* was more abundant in Donor 2 compared to Donor 1 and 3 (Figure 4.3.3).

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The results observed with regards to *Parabacteroides* for Donor 1 indicate that the 0 h (initial inoculum) was high in abundance compared to the indigestible residues and even FOS. Low genus count of *Parabacteroides* was also observed by Swidsinski et al. (2005) and Joossens et al. (2011) in treatments on inulin and corn bran. After 24 h of faecal fermentation, the increase observed in the combination treatment (SAHMT) of maize meal was twice the value of 0 h (initial inoculum) fermentation of donor 2 and 5 times than 0 h (initial inoculum) fermentation for Donor 3 (Table 4.3.1). The combination treatment of maize meal was also higher compared to control maize starch. The increase observed in SAHMT mm might be due to preference exhibited by the members of the genus *Parabacteroides*. Interestingly, species of the genus *Parabacteroides* produce acetate and propionate of which 90 percent of the propionate is transported to the liver through the portal vein and a substantial portion of this is used for gluconeogenesis and suppressing cholesterol synthesis (Pereira & Gibson, 2002). Furthermore, some reports suggest that *P. distasonis* which was identified in both modified and unmodified maize starch and meal (Figure 4.3.3) could even have the potential to serve as a probiotic to promote digestive health in humans based on microbiome or animal studies (Parker et al., 2020).

In both Donors 2 & 3, the abundance of the *Bifidobacteria* spp. increased when indigestible residues of the combination treatment (SAHMT) of maize meal was fermented after 24 h compared to other indigestible residues, 0 h and the blank and even FOS (Table 4.3.1). The results obtained show that *Bifidobacteria* spp. in 0 h fermentation had higher count and was three-fold higher than even FOS and the other indigestible residues in Donor 1. The blank after 24 h fermentation also decreased two-fold in the *Bifidobacteria* spp.

Several studies have reported improvements in colon regularity following ingestion of fermented milk products that contain *Bifidobacterium* (Marteau et al. 2002; Guyonnet et al. 2007; Meance et al. 2011). Bifidobacteria are commonly found in the faeces of infants and have been shown to colonize the colonic epithelium of the infant and protect it from pathogenic bacteria (Bezirtzoglou et al., 2006; Kumarswamy et al., 2012; Zivkovic et al., 2011). The genus *Bifidobacteria* contains the enzyme fructose-6-phosphate phosphoketolase to degrade resistant starch to produce lactate through the process known as the bifidus pathway which is the marker for the genus *Bifidobacteria* (Lawrence, 2011). The lactate produced is further acted on by the butyrate-producing bacteria to produce butyrate (Goni et al. 2000) which help to minimize the risk of obesity and type 2 diabetes

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in some individuals. *Bifidobacteria* also help in the production of B vitamins and prevention of infection caused by other bacteria such as *E. coli.*

After 24 h of fermentation, the relative abundance of *Collinsella spp.* observed in Donors 2 & 3 doubled in the indigestible residue of the combination treatment (SAHMT) (Table 4.3.1) of maize meal compared to control maize starch and meal as well as the initial inoculum and FOS and other modified indigestible residues. The combination treatment (SAHMT) of maize meal stimulated the growth of *Collinsella spp.* in Donors 2 & 3 after 24 h faecal fermentation compared to Donor 1. The abundance of the genus *Collinsella* was higher in the 0 h fermentation (initial inoculum) compared to the control maize starch and meal after 24 h and the other modified indigestible residues and FOS as well in Donor 1. *Collinsella spp.* are known to produce butyrate to provide nutrition to the epithelial cells of the colon (Lagkouvardos, Overmann, and Clavel, 2017). *C. aerofaciens* was identified in both modified and unmodified maize starch and maize meal (Figure 4.3.3). *C. aerofaciens* is a butyrate-producing intestinal microbe which plays an important role in colonic health and serves as an energy source for epithelial cells. Previous studies have suggested that butyrate-producing bacteria could alleviate inflammatory bowel disease, type 2 diabetes, and obesity (Geirnaert et al., 2015; Jia et al., 2017).

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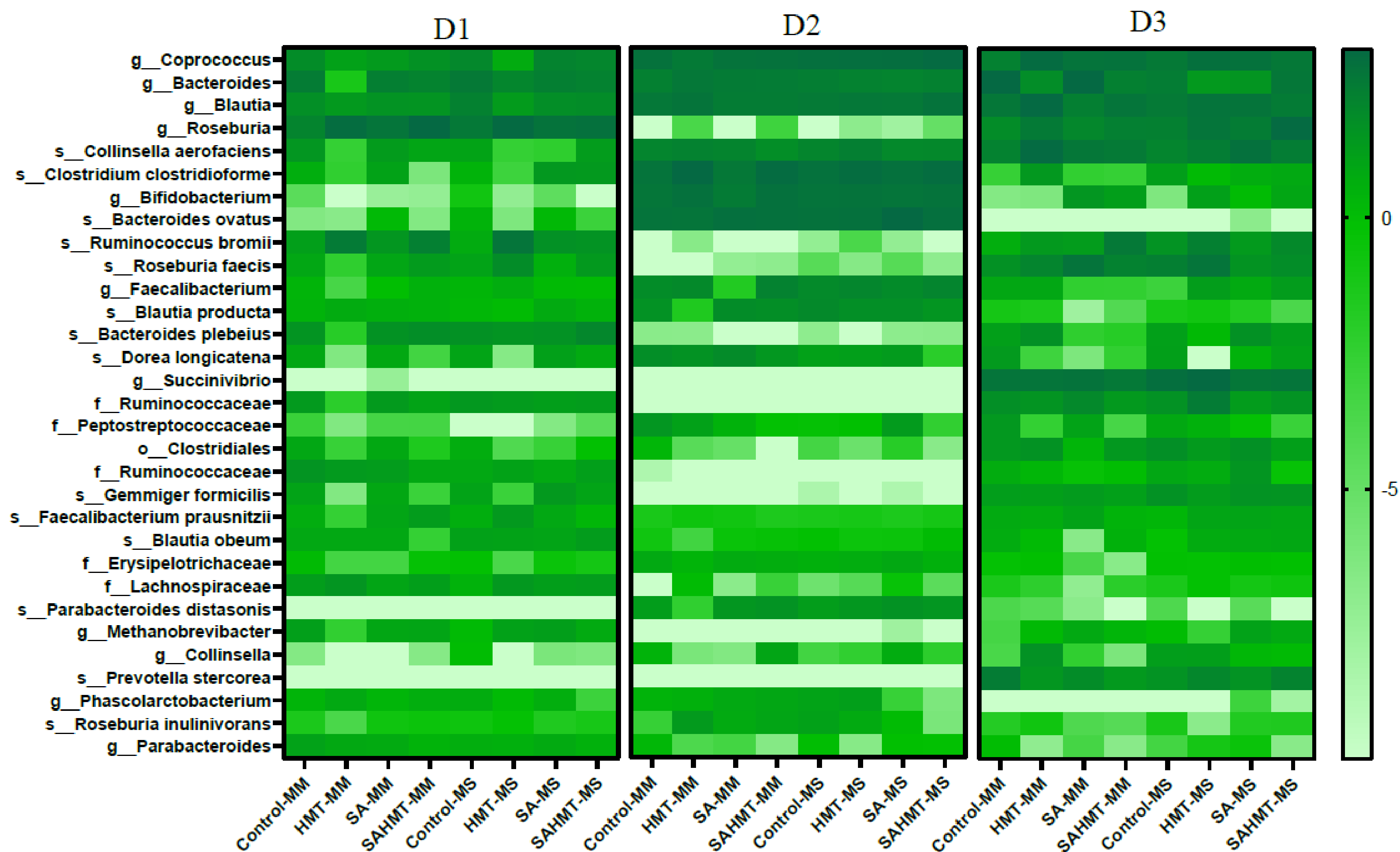

Figure 4.3.3: Heatmap analysis of 16S rRNA gene sequencing of the modified and unmodified maize starch and meal ferment after 24 h

Key: MS = Maize starch, MM = Maize meal, SA = Stearic acid at 1.5% (w/w), HMT = Heat moisture treatment at 20% moisture, SAHMT = Combination treatment.

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Table 4.3.1: Effects of indigestible residues of modified and unmodified maize starch and maize meal on selected genus after 24 h of faecal fermentation

Selected microbe at the genus level	Donor	Relative abundance at 0 h	Relative abundance at 24 h									
		Initial inoculum	Blank	FOS	Control ms	Control mm	SA ms	SA mm	HMT ms	HMT mm	SAHMT ms	SAHMT mm
Roseburia	**1**	8.9±0.1 [+]	9.0±0.1 [+]	14.7±0.1 [++]	4.5±0.1 [–]	5.9±0.1 [–]	11.1±0.2 [+]	7.3±0.1 [+]	14.6±0.2 [++]	7.9±0.1 [+]	8.5±0.1 [+]	7.6±0.1 [+]
	2	10.6±0.1 [+]	9.2±0.1 [+]	11.0±0.1 [+]	9.5±0.1 [+]	6.1±0.1 [–]	8.6±0.1 [+]	11.7±0.2 [+]	11.5±0.1 [+]	8.0±0.1 [+]	4.0±0.1 [–]	9.8±0.1 [+]
	3	8.5±0.1 [+]	3.0±0.1 [– –]	14.7±0.1 [++]	9.0±0.1 [+]	6.6±0.1 [–]	7.8±0.1 [+]	9.2±0.1 [+]	6.6±0.1 [–]	7.2±0.1 [+]	10.9±0.1 [+]	16.6±0.1 [++]
Blautia	**1**	15±0.1 [++]	11.6±0.1 [+]	12±0.1 [+]	2.0±0.1 [– –]	8.6±0.1 [+]	5.2±0.1 [–]	10.2±0.1 [+]	7.0±0.1 [+]	11.0±0.1 [+]	8.7±0.1 [+]	8.8±0.1 [+]
	2	8.1±0.1 [+]	6.2±0.1 [–]	6.7±0.1 [–]	7.6±0.1 [+]	10.1±0.1 [+]	9.2±0.1 [+]	11.3±0.1 [+]	8.4±0.1 [+]	8.3±0.1 [+]	8.3±0.1 [+]	15.9±0.1 [++]
	3	2.5±0.1 [– –]	4.5±0.1 [–]	10±0.1 [+]	9.4±0.1 [+]	8.0±0.1 [+]	5.7±0.1 [–]	6.2±0.1 [–]	4.1±0.1 [–]	2.0±0.1 [– –]	6.8±0.1 [–]	16.8±0.1 [++]
Ruminococcus	**1**	13.9±0.1 [++]	14.3±0.1 [++]	10.6±0.1 [+]	6.8±0.1 [–]	8.2±0.1 [+]	13.5±0.2 [++]	8.0±0.1 [+]	12.9±0.1 [+]	9.3±0.1 [+]	6.4±0.1 [–]	7.0±0.1 [+]
	2	15.1±0.1 [++]	14.8±0.1 [++]	5.7±0.1 [–]	1.4±0.1 [– –]	6.1±0.1 [–]	6.4±0.1 [–]	11.4±0.1 [+]	9.8±0.1 [+]	9.4±0.1 [+]	10.5±0.1 [+]	9.6±0.1 [+]
	3	8.7±0.1 [+]	11.7±0.1 [+]	7.8±0.1 [+]	7.0±0.1 [+]	6.2±0.1 [–]	8.2±0.1 [+]	9.2±0.1 [+]	9.5±0.1 [+]	5.3±0.1 [–]	10.5±0.1 [+]	16.1±0.1 [++]
Faecalibacterium	**1**	15.9±0.1 [++]	14.6±0.1 [++]	11.5±0.1 [+]	2.5±0.1 [– –]	9.3±0.1 [+]	9.4±0.2 [+]	10.7±0.1 [+]	9.4±0.1 [+]	11.8±0.1 [+]	7.4±0.1 [+]	8.8±0.1 [+]
	2	10.1±0.1 [+]	9.5±0.1 [+]	6.7±0.1 [–]	8.3±0.1 [+]	10.1±0.2 [+]	10.8±0.1 [+]	11.3±0.1 [+]	11.5±0.1 [+]	8.0±0.1 [+]	9.5±0.1 [+]	16.3±0.2 [++]
	3	8.2±0.1 [+]	5.6±0.1 [–]	8.2±0.1 [+]	5.6±0.1 [–]	7.2±0.1 [+]	5.5±0.5 [–]	7.0±0.1 [+]	2.9±0.1 [– –]	5.6±0.1 [–]	7.6±0.1 [+]	16.6±0.1 [++]

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Bacteroides	**1**	16.6±0.1 [++]	15.1±0.1 [++]	9.7±0.1 [+]	2.0±0.1 [– –]	8.7±0.1 [+]	5.7±0.1 [–]	8.7±0.1 [+]	6.8±0.1 [–]	11.7±0.1 [+]	7.5±0.1 [+]	7.6±0.1 [+]
	2	8.7±0.1 [+]	8.1±0.1 [+]	5.2±0.1 [–]	6.8±0.1 [–]	9.5±0.2 [+]	8.5±0.1 [+]	10.9±0.1 [+]	9.0±0.1 [+]	9.0±0.1 [+]	7.5±0.1 [+]	16.9±0.2 [++]
	3	6.6±0.1 [–]	11.2±0.1 [+]	12.4±0.1 [+]	5.8±0.1 [–]	12.9±0.2 [+]	3.8±0.1 [– –]	11.2±0.1 [+]	5.6±0.1 [–]	7.4±0.1 [+]	8.1±0.1 [+]	14.9±0.1 [++]
Parabacteroides	**1**	16.3±0.2 [++]	9.9±0.1 [+]	7.9±0.1 [+]	2.3±0.1 [– –]	12.0±0.2 [+]	5.5±0.1 [–]	10.1±0.1 [+]	6.1±0.1 [–]	13.8±0.2 [++]	6.5±0.1 [–]	8.7±0.1 [+]
	2	7.4±0.1 [+]	5.5±0.1 [–]	12.9±0.1 [+]	3.8±0.1 [– –]	9.7±0.1 [+]	7.8±0.1 [+]	10.2±0.1 [+]	8.9±0.1 [+]	9.0±0.1 [+]	8.0±0.1 [+]	16.8±0.1 [++]
	3	3.1±0.1 [– –]	11.3±0.1 [+]	8.8±0.1 [+]	3.3±0.1 [– –]	13.4±0.2 [+]	4.6±0.1 [–]	12.0±0.2 [+]	1.9±0.1 [– –]	6.6±0.1 [–]	3.8±0.1 [– –]	17.2±0.1 [++]
Bifidobacteria	**1**	17.1±0.2 [++]	4.3±0.1 [– –]	6.7±0.1 [–]	4.4±0.1 [– –]	4.4±0.1 [– –]	5.7±0.1 [–]	5.5±0.1 [–]	5.1±0.1 [–]	4.4±0.1 [– –]	4.4±0.1 [– –]	6.0±0.1 [–]
	2	10.6±0.1 [+]	10.6±0.1 [+]	8.1±0.1 [+]	6.7±0.1 [–]	8.6±0.1 [+]	8.3±0.1 [+]	9.8±0.1 [+]	8.6±0.1 [+]	6.8±0.1 [–]	7.1±0.1 [+]	16.9±0.1 [++]
	3	5.6±0.1 [–]	7.0±0.1 [+]	5.3±0.1 [–]	8.4±0.1 [+]	6.1±0.1 [–]	7.7±0.1 [+]	8.5±0.1 [+]	8.4±0.1 [+]	8.1±0.1 [+]	9.1±0.1 [+]	17.9±0.1 [++]
Collinsella	**1**	16.7±0.2 [++]	11.0±0.1 [+]	9.1±0.1 [+]	1.6±0.1 [– –]	9.9±0.1 [+]	2.1±0.1 [– –]	7.9±0.1 [+]	7.6±0.1 [+]	6.2±0.1 [–]	7.5±0.1 [+]	7.5±0.1 [+]
	2	8.2±0.1 [+]	7.3±0.1 [+]	11.4±0.1 [+]	9.3±0.1 [+]	13.2±0.1 [+]	13.4±0.2 [+]	11.8±0.1 [+]	10.2±0.2 [+]	10.0±0.1 [+]	10.0±0.1 [+]	16.4±0.1 [++]
	3	2.5±0.1 [– –]	6.6±0.1 [–]	9.3±0.1 [+]	10.9±0.1 [+]	4.8±0.1 [–]	6.0±0.1 [–]	8.3±0.1 [+]	6.4±0.1 [–]	6.2±0.1 [–]	6.9±0.1 [–]	17.1±0.2 [++]

Mean ± standard deviation of three independent replicates for three donors.
Different alphabetical letters in the same column are significantly different ($p \leq 0.05$)
MS = Maize starch, MM = Maize meal, SA = Stearic acid, HMT = Heat moisture treatment, SAHMT = Combination treatment,
FOS = Fructooligosaccharides
++ = value increased over the negative control more than 50% ; + = value increased less than 50%; - = value decreased less than 50%

– – = value decreased more than 50% within a row; 7.0 = considered midpoint.

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In addition to the above genera, *Methanobrevibacter* from the family Methanobacteriaceae and phylum Euryarchaeota is another genus in the microbiota (Figure 4.3.1b). The genus was detected in all the donors. It is the major producer of methane (CH_4) in man which result from combining CO_2 and H_2 produced from other bacteria. The methane produced decreased the amount of gas production in the gut (Garcia, Patel and Ollivier, 2000). Large amount of gas production can result in gastrointestinal discomfort (Ahmad et al, 2000).

The gut microbiota have a hierarchical preference for the prebiotic at its disposal (Rogers et al., 2013). This hierarchical preference in the colon could be the grounds for the high competitiveness of the community. Indeed, there were specific strains of microbes that were stimulated for growth by specific treatments of indigestible residues of both maize starch and maize meal. From the data, the combination treatment of maize meal (SAHMT) stimulated the growth of some microbes especially in Donors 2 & 3. The identified bacteria (*Roseburia, Blautia*, *Ruminococcus, Faecalibacterium, Bacteroides, Parabacteroides, Bifidobacterium* and *Collinsella*) play an important role in the gut microbiota. *Roseburia, Ruminococcus, Faecalibacterium* and *Collinsella* have been suggested to ameliorate metabolic syndrome associated with obesity and type – 2 diabetes (Fabersani et al., 2019; Yang et al., 2017). The reason being that metabolic syndrome is associated with dysbiosis in the gut microbiome. The above-mentioned microbes add up to balance the microbes in the gut microbiome. The above-mentioned microbes are butyrate-producing microbes and the butyrate produced can increase glucose tolerance and insulin sensitivity to prevent obesity and type – 2 diabetes (Ahmad et al., 2000).

α-Diversity (evenness and/or richness) is another indicator of gut health and in this study different communities were measured using Shannon Index calculator. Richness is about the number of species in gut microbiota, and evenness also talks about how common each available species is in gut microbiota. A community (donors microbiome) that has a high Shannon Index value shows that a high number of species is detected, and their abundances are similar. A reduction in Shannon index value can be attributed to a microbe in a microbiota unable to utilize available substrates and unable to cross-feed (Magurran and Ramnarine, 2004).

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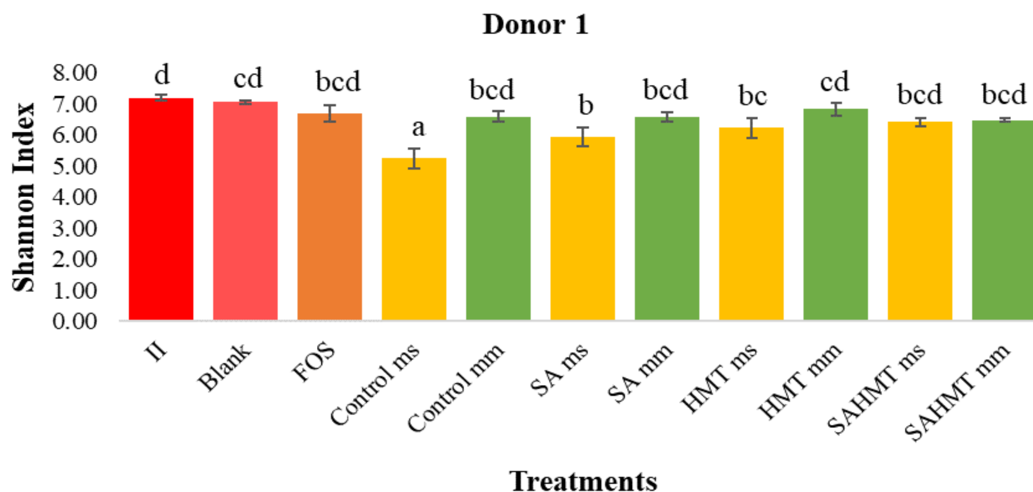
With regards to the indigestible residues, the combination treatment (SAHMT) of both maize starch and meal had the highest α-diversity value compared to the control maize starch. There was a reduction in species diversity in Donor 1 compared to Donor 2 and 3 (Figure 4.3.4). In Donor 2, the combination treatment (SAHMT) of maize meal was significantly higher compared to control maize starch and meal, FOS, and combination treatment (SAHMT) of maize starch as well as the blank respectively (Figure 4.3.4B). Also, for Donor 3, the combination treatment of maize meal (SAHMT mm) was significantly higher compared to control maize starch and maize meal, FOS, and the other treated indigestible residues as well as blank (Figure 4.3.4C). This implies that the microbes to degrade the indigestible residue of the combination treatment of maize meal (SAHMT mm) were diverse. This also confirms the results obtained in Donors 2 & 3 about the diversity of the eight selected different genera discussed above. In Donor 1, the initial α-diversity value was greatest compared to the treated and untreated indigestible residues. The treated indigestible residues of maize meal had highest Shannon Index value compared to maize starch. This means that the diversity of species available to feed on the maize meal was higher compared to maize starch. This result was consistent with previous *in vivo* and *in vitro* studies, which indicated that RS substrate increased the community diversity of gut microbiota (Barouei et al., 2017; Deehan et al., 2020). This is a desirable result of a microbial community as the stability of α-diversity is important to ensure that the microbial functions are maintained under dietary perturbations.

A



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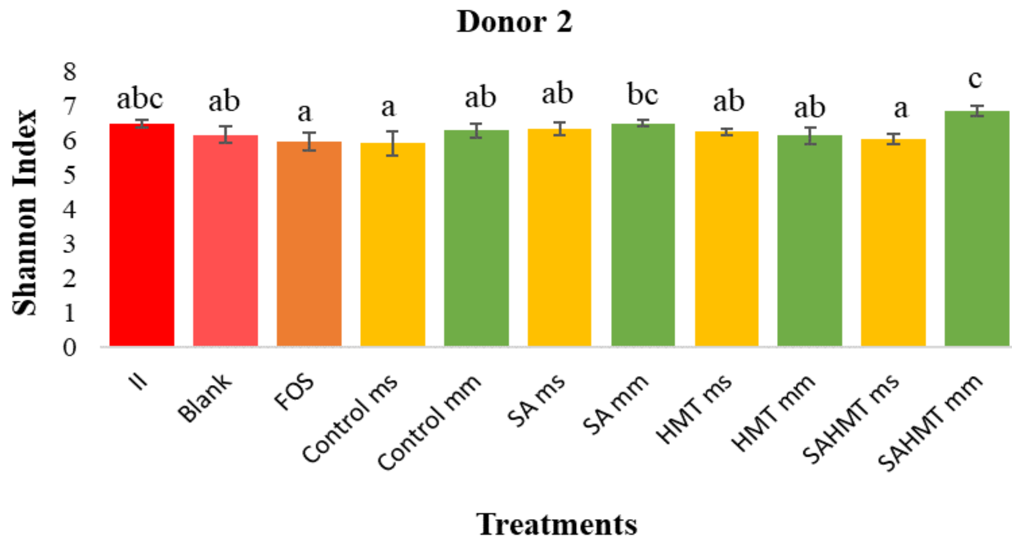
B



C

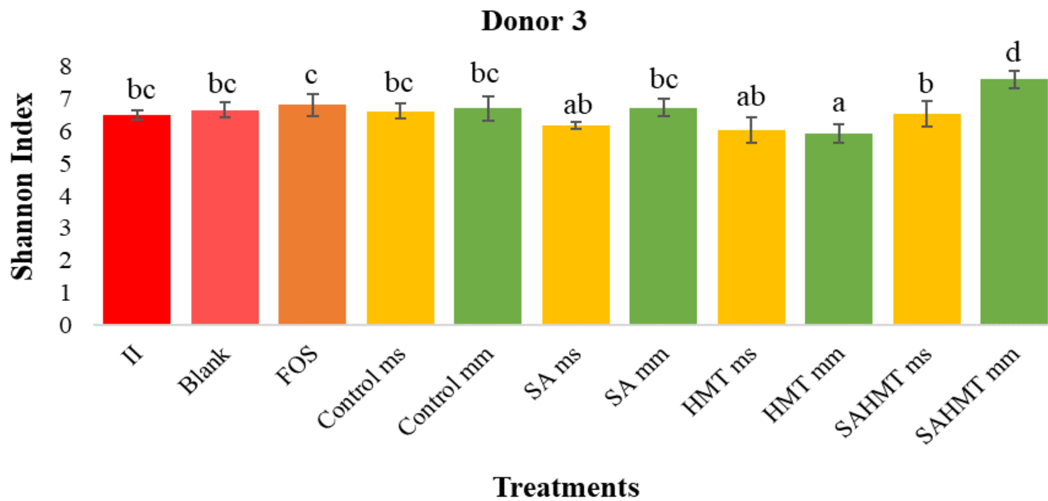


Figure 4.3.4: Changes in alpha-diversity of the faecal microbiota communities (A=donor 1; B=donor 2; C=donor 3) during *in vitro* fermentation of indigestible residues of maize starch and maize meal, as measured by Shannon's Index Calculator.

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4.3.4. Conclusions

Indigestible residues from maize meal and maize starch modified with HMT and SA alone and in combination appear to function as prebiotics, like fructo-oligosaccharides. Firmicutes, Bacteroidetes and Actinobacteria are the three most dominant phyla present in the fermentation of the treated indigestible residues from the faeces of the three donors. The combination treatment of maize meal (SAHMT mm) of the treated indigestible residues which is resistant starch type 5 (RS 5) from amylose-lipid complexes (ALC) stimulates the growth potentially beneficial bacteria including *Roseburia, Blautia, Faecalibacterium, Ruminococcus, Bacteroides, Parabacteroides, Bifidobacteria* and *Collinsella* that have predominant abundance in the three donors. RS 5 shows a higher Shannon Index value in two of the three donors for the SAHMT maize meal treatment, indicating that it promotes higher alpha diversity in the gut microbiota. Overall, the results obtained from the RS5 from ALC suggest a positive promotion of gut health that could lead to amelioration of metabolic syndrome diseases. Further studies are required to elucidate the potential of RS 5 in *in vivo* investigations and its clinical benefits needs to be assessed.

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4.3.5 Reference

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5.0 General Discussion

This segment initially provides an evaluation of some of the methodologies used in this research. Subsequently, the main outcomes in this study are examined. This will comprise of the effect of digestion on maize starch and maize meal with added stearic acid alone, and heat-moisture treatment alone and their combination treatment. A proposed mechanism is presented for how the undigested enzyme-resistant portions of the modified and unmodified maize starch and maize meal behave in an in vitro fecal microbiota fermentation, and finally a look at the gut microbes that degrade the indigestible residues and its potential implication on health.

5.1 Methodology

5.1.1. Integration of stearic acid into starch

Stearic acid (1.5% w/w) was dissolved in absolute ethanol and then added to maize starch and maize meal to form a homogeneous mixture. Ethanol was used because it is an organic solvent that has adequate non-polar characteristics to allow the dissolution of stearic acid and promote its diffusion into the micropores of the starch granules of maize starch and maize meal. This facilitates interaction with amylose to form amylose-lipid complexes during pasting. Confocal laser scanning microscopy revealed that stearic acid can be disseminated into starch granules by adding the stearic acid-ethanol mixture (D'Silva et al. 2011).

5.1.2. Heat moisture treatment of maize starch and maize meal

Starch granules were incubated at room temperature for 24 h at moisture of 20 % (w/w) and temperature at 110 °C for 16 h in an oven (Chung et al., 2009b). A 20 % moisture at 110 °C were the conditions used for the heat-moisture treatment in this study (Chung et al., 2009b). At these conditions the temperature was below the starch gelatinisation temperature, since low moisture content increases the gelatinisation temperature of starch above 81°C (Chung et al., 2009b). Starch granules were sprayed with water to 20 % moisture content (w/w) and thoroughly mixed with a spatula. The mixing was done to enable even distribution of the moisture in the starch granule. Containers were filled with the hydrated starch leaving limited headspace and sealed with lids. The heat-moisture treatment method of Zeleznak & Hoseney (1987) was modified slightly by

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equilibrating moisture in sealed containers for 24 h with agitation (as the containers were left on a shaking tray overnight) before placing in an oven at 110 °C. This enabled uniform hydration of the starch granules to promote interaction of the starch polymer chains during heat-moisture treatment.

5.1.3. Analytical methods

5.1.3.1. Pasting and gelling properties of maize starch and maize meal

The rheometer used was like the Rapid Visco Analyser RVA, as they both provide a similar curve. The rheometer provides a more general capability for rheological evaluation with increased sensitivity compared to the RVA (Nyakabau et al., 2013). The canister used in the RVA is bigger in size compared to the rheometer, hence a smaller sample size was used. Like the RVA, there was minimal evaporation during pasting that may affect the viscosity. The pasting properties showed relative viscosity changes between the treatments rather than measuring absolute viscosity.

The pastes of both maize starch and maize meal were poured into plastic containers and allowed to cool overnight in a room under controlled temperature (21°C) and relative humidity (46.6% - 47.5%). Gelling properties were determined using a texture analyzer. Gel firmness was measured using a small cylindrical probe to perform a penetration test at a chosen distance into the sample. Firmness is the force required to penetrate the starch gel. The penetration test was performed instead of a compression test, since most of the samples did not gel.

5.1.3.2. Thermal properties of maize starch and maize meal using differential scanning calorimeter (DSC) and Wide-angle X-ray diffractometer (XRD)

Differential scanning calorimetry is widely used to study the thermal behaviour of starches and gelatinisation (Yu & Christie, 2001). Maize starch and maize meal samples were hydrated, and moisture was equilibrated overnight before running the DSC since starch hydration influences the gelatinization temperature (Biliaderis *et al*., 1980). A ratio of 1:3 solid-to-water content was used; water was used as a plasticizer in the process. A plasticizer is a lubricant that enhances starch chain mobility. The water enables the melting of the crystallites to occur at lower temperatures (Resio

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& Suarez, 2001). The pan hermetic seal allows DSC to be carried out up to a temperature of 140 °C.

Wide angle X-ray diffraction scattering was used to analyse added stearic acid followed by heat-moisture treatment to promote the development of V-amylose complexes before and after pasting. The freeze-dried samples of maize starch and maize meal were ground to a fine powder to prevent lumps, because this could distort the penetration of the X-ray waves through the sample and result in noisy X-ray patterns (Scrivener et al., 2016). The samples were equilibrated for 5 d in a desiccator at 95 % projected relative humidity at room temperature (~25 °C) to minimize variations in the results due to moisture content variability (Svergun & Koch, 2003). XRD cannot distinguish between the different types of amylose-lipid complexes but can distinguish between the different sizes of the complexes (Lalush et al., 2005).

5.1.3.3. Starch Digestibility

Starch is hydrolyzed by amylolytic enzymes to generate glucose in plants, animals, and humans for the supply of energy. Starch is successively hydrolysed in the mouth and small intestine to oligosaccharides by salivary and pancreatic alpha-amylase, respectively, before being digested to glucose by the alpha-glucosidases and absorbed by the small intestine epithelial cells. The final products of starch hydrolysis by alpha-amylase are maltose, maltotriose and the alpha-limit dextrins containing branched points impervious to alpha-amylase. The products of alpha amylase digestion are again hydrolyzed to glucose by the collective activity of two brush border exo-hydrolase double-headed enzymes, maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) (Nichols et al., 2003).

The most common approach used for upper gastrointestinal digestion are Englyst et al. (1992) and Goni et al. (1997). In this study, the Goni et al., (1997) procedure was used. This procedure includes the use of alpha-amylase and fungal amyloglucosidase to assimilate starch, while pepsin is used to digest protein. The use of alpha amylase and amyloglucosidase concurrently simulates the process of starch hydrolysis in the small intestine where a larger percentage of starch is digested (Hasjim et al., 2010). *In vitro* methods, in general, have several limitations, including lack of consideration of gastric emptying rate, digesta viscosity, and transit time through the gastrointestinal tract. *In vivo* investigations have indicated that these factors have impact on the

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rate of starch digestion (Turnbull et al., 2005). However, the Goni et al. (1997) method was well correlated ($R^2 = 0.952$) with *in vivo* studies (Goni et al., 1997) for some food materials, though maize meal porridge was not included in the study.

Glyceamic index (GI) was estimated on maize meal and maize starch with and without the addition of stearic acid alone, heat moisture treatment alone and their combination treatment. Goni et al. (1997) proposed a first order rate equation that was used to predict the estimated glycaemic index. In this technique, the glycaemic index was computed from the hydrolysis index. The GI values were based on the glycaemic effect compared with a standard food (Venter et al., 2003). The hydrolysis index was determined as the area under the curve of the test food as a percentage of the subsequent area under the curve of the reference sample. The Goni et al. (1997) GI forecast model has been stated to give good GI estimates (Germaine et al., 2008).

5.1.3.4. In vitro faecal fermentation of indigestible residue

Indigestible residues of modified and unmodified maize starch and maize meal were used for the faecal fermentations. Undigested carbohydrates are considered as dietary fibre and are either fermented in the colon or act as a bulking agent (Amine et al., 2003). In vitro faecal fermentation cultivates a multifaceted intestinal microbiota under meticulous environmental requirements for transporting microbial modulation and metabolic rate investigations (Payne et al. 2012). A summary of fermentation models and their limitations is represented in Table 5.1.1 below.

Table 5.1.1: Different faecal fermentation models

Fermentation Model	Methodology	How well it correlates with in vivo	Limitation	Reference
Batch fermentation model: A closed system used to grow bacteria strains in a control reaction	Operates under anaerobic condition. It is used to study the effect of substrates on	It affects the *in vivo* relevance for a longer time. In vivo data shows a weak positive	Studies proceed over a short period of time. Restricted by substrate depletion and	Venema, (2015). Aura et al., (2014)

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inoculated with faecal suspension.	biodiversity of microbiota and metabolites produced.	correlation (R^2 = 0.65) with the batch fermentation model.	accretion of the final products of microbial metabolism.	
Dynamic Fermentation model: It has well defined pH's of 5.5, 6.2 and 6.8.	Consist of three vessels of increasing size: Vessel 1: has high accessibility of substrate indicating rapid bacterial development. Vessel 2: Involves using protein, complex carbohydrate as substrates. Vessel 3: Represents neutral pH, it has low availability of substrate with slow bacterial proliferation rate representing distal colon.	In vivo data has positive correlation (R^2 = 0.814) with the dynamic fermentation.	Its limitation is retention time in the vessel and the defined pH used in the fermentation process.	Sivieri et al. (2014)

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Inoculation of gut fermentation model: Substrates are usually inoculated with liquid faecal suspension from either individuals or pooled stools.	Bacterial strains and culture conditions.	Fermentation model positively correlates (R^2 = 0.75) with most *in vivo* studies	The inoculation and colonization of *in vitro* fermentation systems affects the duplicability of the project.	Payne et al., (2012) Macfarlane et al.,(1992)
Host – gut microbiota interaction	It uses an interplay between the gut microbiota and the host occurs mainly at the gastrointestinal mucosal barrier. The host movement impacts the conformation and abundance of gut microbiota, whereas the gut microbiota in turn modulates the metabolic activities of the host.	*In vivo* studies show positive correlation with host – gut microbiota interaction	Its limitation is by stimulating the host functionality.	Cinquin et al., (2004) Venema and Van den Abbeele (2013)

In the current study, the model that was used was the batch fermentation model. The batch fermentation model used yielded short chain fatty acids from the indigestible residues used as

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substrates. Short chain fatty acids were calculated from gas chromatography of supernatants of faecal slurries mixed with the standards. The SCFA values are an indicator of the fibre present in the sample .

5.1.3.5. Metagenomic gene sequencing

Metagenomics is a molecular tool used to investigate DNA obtained from environmental samples, to examine the community of micro-organisms present, without the obligation of obtaining pure cultures (Ghosh et al., 2018). Table 5.1.2 gives a summary of different methods used for this metagenomics and their limitations as well.

Table 5.1.2: Different methods used for metagenomics

Method	Principle	Limitations	Reference
16S rRNA Sequencing	Purify the DNA from the faeces. Amplify the 16s rRNA gene. Sequence PCR product Compare sequence to database. Identify the bacterial species.	Results are relative rather than quantitative. It can be biased due to varying PCR amplification frequencies and incomplete reference databases used for sequence analysis. Does not determine cause and effect relationships. Results are usually based on the genus	Kong and Segre, (2012) Cogen et al., (2010)

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		level instead of species level.	
18S rRNA Sequencing	DNA sequencing encodes small subunits rRNA of eukaryotic ribosomes. It also entails conservative and variable regions. DNA sequencing indicates the species differences among eukaryotic organisms.	Intense sequencing methods have become an effective way to study microbial community diversity than traditional cloning. Sequencing errors can result in incorrect variety especially due to the incidence of singletons	Kunin et al (2010) Quince et al., (2011).
Shotgun metagenomic sequencing	Extraction of DNA from the cell. The DNA is then fragmented into smaller components which are independently sequenced.	It is expensive to sequence large numbers of metagenomics without access to sequencing. Biases in functional profiling	(Makki et al, 2018)
Internal transcribed spacer (ITS)	Can endure more mutations in the evolutionary process	ITS is recommended for universal DNA	Schoch et al., (2012)

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	because of fewer natural selection burden. It exhibits broad sequence polymorphism in most eukaryotes. ITS fragments are small and are easy to analyse. They have been used extensively in phylogenetic analysis of dissimilar fungi.	barcode markers for fungi. ITS is also limited in intraspecific variation	

In the current study, the method used in the metagenomics was 16s rRNA gene sequencing. The method is good for faecal ferments and identify microbes at a refined level. 16s rRNA amplicon gene sequencing cannot be well identified at the species level. The data obtained from using 16s rRNA amplicon gene sequencing clearly showed that the indigestible residues from modified and unmodified maize starch and maize meal have prebiotic potential and could be beneficial for the health needs of consumers.

5.2 Nutrition and functional properties of modified and unmodified maize starch and maize meal before and after pasting.

Starch occurs as granules in the maize endosperm. It is made up of 20 – 30 % amylose and 70 – 80 % amylopectin. In these studies, maize starch and maize meal were modified with stearic acid

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alone (1.5 %, w/w), heat-moisture treatment at 20 % moisture and their combination treatment. Amylose can form single helix or double helical structures (Chung & Liu, 2009). The helical cavity of amylose is considered hydrophobic, while the outside of amylose helix contains hydrophilic hydroxyl groups. The latter stabilizes the amylose helical form by intra-molecular hydrogen bonds outside the helix. Amylopectin is an extremely branched polymer of alpha D-glucopyranosyl units linked via alpha (1-4) glycosidic linkages, and alpha (1-6) glycosidic linkages at branch points (Parker and Ring 2001).

Both separate and amalgamation of added stearic acid and heat-moisture treatment for both maize starch and maize meal showed molecular changes of both amylose and amylopectin. Heat-moisture treatment may induce chain interactions such as amylose-amylopectin A-side chain interactions (AM-AP), amylose-amylose interaction (AM-AM), amylopectin side chain interacting with another amylopectin side chain (AP-AP) and finally, endogenous free fatty acids interact with the amylose (AM-Lipid) (Chung et al., 2009).

DSC analysis of pasted heat-moisture treated maize starch and maize meal with stearic acid showed type I and type II amylose-lipid complexes (ALC) (Research Chapter 4.1, Figure 4.1.4 a & b) and XRD results also showed the formation of ALC increased in crystallinity (Research Chapter 4.1, Figure 4.1.4 c & d). The increase in crystallinity could be due to interaction of amylopectin-amylopectin (AP-AP) side chain, endogenous lipids complexing with the amylose (Lipid-AM), amylose with amylose (AM-AM) interaction, and/or A-side-chain amylopectin with amylose (AP-AM). These interactions are via H bonds except for formation of ALC. The reduction in digestibility observed in Research Chapter 4.1 (Figure 4.1.2a & b) was likely due to the stated interaction that increased the relative crystallinity of the maize starch and maize meal observed in both DSC and XRD (Research Chapter 4.1, Tables 4.1.4 & 4.1.5). These structures resisted the digestion in the upper gastrointestinal tract possibly because the alpha (1,4) glycosidic bonds were not accessible to alpha-amylase.

According to Tan and Kong (2020), ALCs inhibit enzyme-substrate interaction, thereby reducing starch digestibility and increasing the resistant starch component. Jun-chao & Su-ling (2011) also reported that the rise in RS content could be because of improved interactions between amylose and amylopectin side chains. Chung et al (2014) reported that heat-moisture treated (HMT) maize

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starch pastes with added lauric acid had more amylose-lauric acid complexes which reduced RDS and increased RS. HMT maize starch forms highly ordered structures with higher relative crystallinity that hinder the action of alpha amylase (Brumovsky and Thompson, 2001). Summary of the functional and nutritional properties of maize starch and maize meal after pasting is represented in Table 5.2.1 below.

Therefore, indigestible residues of the modified maize starch and maize meal with the following agents of modification, stearic acid alone, HMT alone and the combination treatment, would enter the colon to be fermented by the colonic microbiota and to yield SCFA.

Table 5.2.1: Summary of the functional and nutritional properties of the modified maize starch and maize meal samples after pasting.

Sample	Addition of stearic acid (1.5 % w/w) (S.A)	Heat-moisture treatment (20 %) (HMT)	Combination treatment (SAHMT)
Maize starch and maize meal	Compared to control: Reduced peak viscosity speculated to be due to the lipid surrounding the granules. Reduced digestibility. Reduced EGI and RDS, increased SDS and RS. Increased relative crystallinity, increased enthalpy of gelatinisation,	Compared to control: Reduced digestibility Reduced EGI and RDS, increased SDS and RS due to more compact molecular organization. These could be. (i) Separate segments of amylose-lipid complexes are entangled with free amylopectin and amylose chains.	Compared to control: Reduced viscosity due to the complex formation Reduced digestibility. Reduced EGI and RDS, increased SDS and RS. DSC showed the formation of ALC type I and II. Crystallinity increased compared to HMT and S.A and the control due

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		(ii) Amylopectin entangled with amylose chains to give a more compact organisation.	V-amylose complex formation.

5.3 Fermentation in the colon

Principal component analysis (PCA) was used to appreciate the connection between the treatments (stearic acid alone, heat moisture treatment alone, the combination treatment and fructooligosaccharides) of both maize starch and maize meal and the measured variables of the *in vitro* colonic fermentation, as well as to elucidate the relationship between the variables (Figure 5.2.1). The first 2 principal components accounted for about 96.9 % for maize starch and 97.2 % for maize meal of the total variation. Principal component 1 accounted for 91.7 %, whereas principal component 2 accounted for about 5.2 % for maize starch. In maize meal, the principal component 1 contributed 94.3 % and principal component 2 contributed 2.9 % (Figure 5.1).

Principal component 1 was responsible for the variation, and this suggests that the combining effects of heat-moisture treatment with stearic acid on faecal fermentability was similar to fructooligosaccharides in relation to the other treatments. This suggests that the combination treatment has a prebiotic potential like FOS.

The similarity between SAHMT and FOS in both maize meal and maize starch suggest that the bacteria fermenting the two residues were similar. From the microbial analysis, most of the microbes that preferred SAHMT also preferred FOS,

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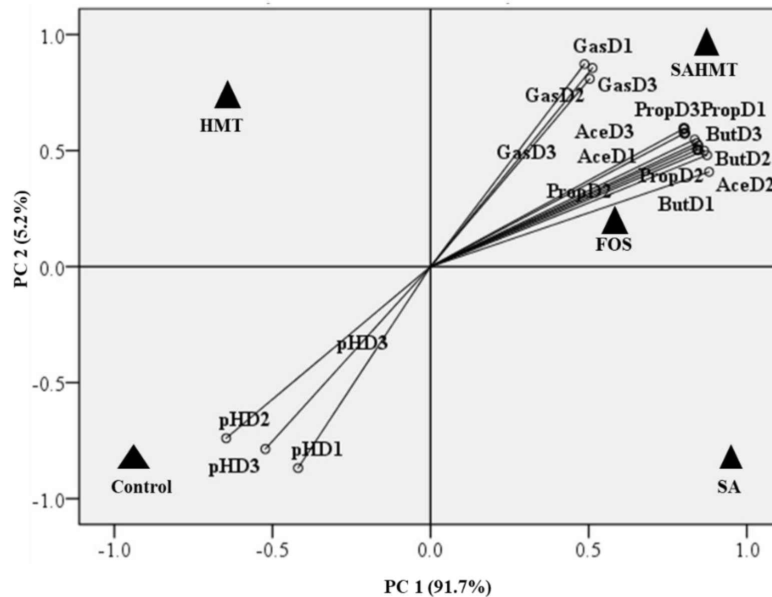
A. Maize starch

B. Maize meal



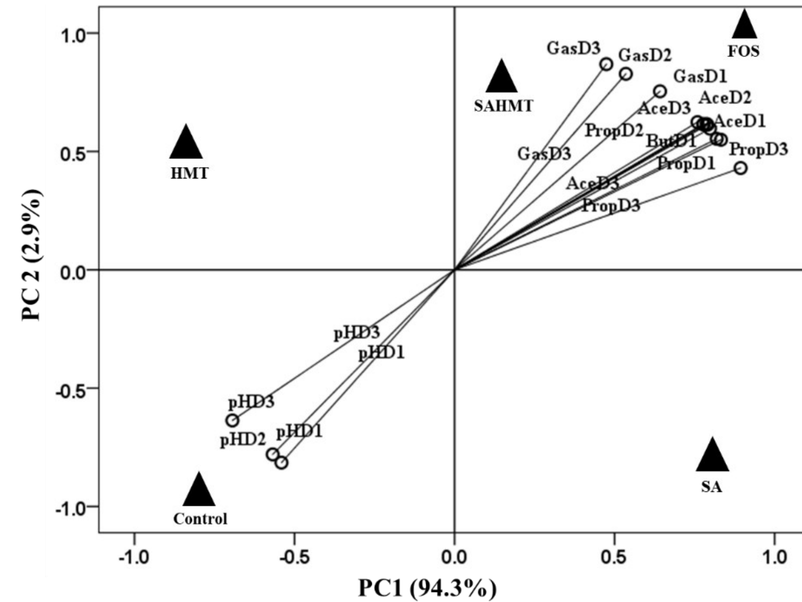


Figure 5.2.1: Principal component analysis of SCFA, gases and pH from unmodified and modified (A) maize starch and (B) maize meal. Acetate Donor 1 (Ace D1), acetate Donor 2 (Ace D2), acetate Donor 3 (Ace D3), propionate Donor 1 (Prop D1), propionate Donor 2 (Prop D2), propionate Donor 3 (Prop D3), Butyrate Donor 1 (But D1), butyrate Donor 2 (But D2) and butyrate Donor 3 (But D3), gas Donor 1 (Gas D1), gas Donor 2 (Gas D2), gas Donor 3 (Gas D3), pH Donor 1 (pH D1), pH Donor 2 (pH D2) and pH Donor 3 (pH D3)

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Fermentation in the colon occurs when food in the upper gastrointestinal tract is not digested and enters the colon. The colonic microflora act on the food to produce SCFAs (Bach Knudsen, 2015). Moreover, SCFAs (butyric acid, acetic acid, and propionic acid) perform an important role in the health of individuals (Hinnebusch et al., 2002; Tedelind et al., 2007). The ratio of acetic acid, propionic acid, and butyric acid is strongly determined by the type of the fermented fibre/carbohydrate (Wong et al., 2006). In the current study, acetate was the dominant metabolite followed by butyrate, and then propionate.

In this study, the indigestible residues from modified and unmodified maize starch and maize meal were used as substrates for faecal fermentation to produce gases and SCFA under the condition of reduced luminal pH. In research chapter two and three, it was established that these indigestible residues are considered as resistant starch, since it escaped upper gastrointestinal tract digestion. The resistant starch (RS) was mostly RS type 5, made up of amylose–lipid complex (ALC) formation and most likely some RS 3 as retrograded starch depending on the treatments.

Amylose-lipid complex as resistant starch type 5 is a highly fermentable fibre, which is also classified as dietary fiber (Baxter et al. 2019). The amylose-lipid complex was not as rapidly fermented as fructooligosaccharides, the positive control (reference material). Amylose-lipid complex help shape the microbial composition of the gut. The major SCFAs (acetate, propionate, and butyrate) are absorbed by colonic epithelial cells (Wu et al. 2018) and promote water and mineral absorption (Baxter et al. 2019). SCFAs, particularly propionate, are efficient in regulation of fat and cholesterol synthesis in the liver and controlling body weight gain by triggering glucagon-like peptide-1 secretion and increasing satiety (Maljaars and others, 2008). Acetate also helps to regulate the pH of the gut, controls appetite and nourishes butyrate-producing bacteria, as well as providing protection against pathogenic bacteria. Butyrate also serves as an energy source for colonocytes and prevents leaky gut (Rosenbaum, Knight, & Leibel, 2015). With these benefits listed, the SCFAs play an important role in terms of gut health.

The concentrations of SCFA produced during fermentation of indigestible residues, especially the combination treatment was similar to FOS (reference material), though its rate was noticeably slower. Fermentation by anaerobic bacteria is usually determined by chemical structure, chain length and the solubility/insolubility of the sample (Gidley, 2013; Hopkins et al. 2003). The

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chemical structure of the resistant starch indigestible residue from both maize starch and maize meal indicates more crystalline structure (research chapter 1) and formation of amylose-lipid complex, molecular rearrangement of the side chains as well as insolubility nature of the sample. Slow fermentation rates have been reported for wheat and maize arabinoxylans with complex branched xylan structure (Rumpagaporn et al., 2015). Fermentability of RS 3 samples were delayed, and this was due to the fermentation rate of RS 3 which was associated with the crystalline nature of the retrograded starch (Goñi, et al. 2000). Resistant starch with increased crystallinity exhibited slow fermentability and produced more butyrate and less propionate, indicating that the physical form of the resistant starch affects fermentation rate and its resulting end products (Mikkelsen et al., 2011). In the current study, the combination treatment (SAHMT) had a slow fermentation rate. This is perhaps because of the crystalline nature of the sample, as well as the V-amylose complex formation.

5.4 Potential health benefit of modified maize meal

From Table 5.2.2, the amount of resistant starch available in untreated maize meal is 5.4g. When the maize meal is treated with stearic acid, resistant starch increased to 22.4 g, for HMT was 20.4 g, and for the combination treatment was 25.8 g. Resistant starch in the combination treatment was about fivefold higher than the untreated maize meal, and HMT alone and SA alone were fourfold higher. Thus, consumption of 100 g of treated maize meal with stearic acid followed by heat-moisture treatment will markedly increase the quantity of RS that the consumer gets. Resistant starches have a lot of potential health benefits, such as improved insulin sensitivity, reduced blood sugar levels, and increased satiety and lower cholesterol and triglyceride concentrations (Lunn and Buttris, 2007). Consumption of maize meal could help to ameliorate diet-related diseases like type – 2 diabetes and obesity in Southern African countries.

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Table 5.2.2: Consumption of 100 g of maize meal and its related resistant starch

Sample	R S (%)	RS per 100 g of maize meal
Control	8	5.4g
S. A	33	22.4g
HMT	30	20.4g
SAHMT	38	25.8g

S.A = stearic acid; HMT = heat moisture treatment; SAHMT = combination treatment

RS = Resistant starch

This data is based on research chapter 4.1 where the general RS was quantified.

The indigestible residue from the combination treatment from maize meal would behave as dietary fibre from a physiological point of view. It was shown to be fermentable to induce the development of beneficial bacteria and prevent the growth of possible pathogenic microorganisms. These properties together with other beneficial physiological effects like prebiotic effect, effects related to SCFA production, improved mineral absorption and reduction in the risk of colon cancer would inform nutritionists to formulate foods rich in SAHMT. There are several potential benefits derived from the consumption of food rich in SAHMT. These are:

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a. Prebiotic effects.

A prebiotic is a non-digestible food ingredient that has a positive effect on the consumer by stimulating the growth and/or activity of one or a small number of bacteria in the colon, improving the consumer's health. (Gibson, Probert, Van Loo, and Roberfroids, 2004). It is widely acknowledged that the bacterial community found in the human gastrointestinal tract has a significant influence on intestinal function and human health. (Boehm, Jelinek, Knol, M'Rabet, Stahl, Vos, and Garssen, 2004). These communities consist of diverse kinds of species and their numerical functions are very wide and different. Majority of these bacteria live in the colon and are primarily anaerobic. Dietary having SAHMT avoids enzymatic hydrolysis in the small intestine and enters the colon with its structure intact. SAHMT are resistant to gastric acidity, human enzyme hydrolysis, and intestinal absorption, as well as being fermented by the gut microflora and selectively stimulating the growth and/or activity of bacteria that contribute to the consumer's welfare and health, confer on SAHMT their property as prebiotics.

b. Effects related to the production of SCFAs.

SCFA is produced by the fermentation of SAHMT in the colon. All the SCFAs are quickly absorbed in the large intestine and metabolized by various tissues, including butyrate by the colonic epithelium, part of propionate and acetate by the liver, and part of acetate by muscle and other peripheral tissues. Research conducted by Swennen et al. (2006) indicates that absorption of SCFAs impacts consumers metabolism. Acetate and propionate impact carbohydrate and lipid metabolism (Brandt, 2001). Propionate decreases hepatic gluconeogenesis and hinders urea development in the liver (Yao, Muir, & Gibson, 2016). SCFAs lower the pH effectively, this indorses helps the avoidance of pathogenic bacteria before their metabolites may be pre-carcinogenic (Brandt, 2001). Butyrate is a significant source of energy, as well as a regulator of cell growth and differentiation in the intestinal mucous (Brandt, 2001).

c. Mineral absorption effect

Consumption of SAHMT will have positive impact on minerals such as calcium, magnesium, iron and zinc that will be absorbed. As SAHMT is a dietary fibre, it binds to or sequesters the minerals mention in above, decreasing their absorption in the small intestine and their arrival in the colon. The bound or sequestered minerals are released and become available for absorption during the

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fermentation of the dietary fibre in the colon. In addition, the high concentrations of the SCFAs from the colonic fermentation of SAHMT reduces the colonic pH and increases the solubility of the minerals, particularly of calcium and magnesium (Bosscher and others 2006). Consumption of SAHMT in diet also has a positive impact on bioavailability of Ca and Mg. High dietary calcium absorption raises peak bone mass, which may delay the onset of osteoporosis-related fractures in later life (Bosscher and others 2006).

d. Decreased risk of colon cancer.

Colonic fermentation of SAHMT produces mainly beneficial bacteria by reducing the colonic pH. This makes pathogenic bacteria which are carcinogenic not to thrive in the colon. The bacteria that promotes health limits the growth of dangerous bacteria, lowering the synthesis of carcinogenic chemicals and bacteria enzymes that contribute to colon carcinogenesis (Martin, Jimenez, and Motilva, 2001). Furthermore, butyrate increases normal cell growth while inhibiting the proliferation of malignant cells.

e. Regulation of lipid metabolism

It is worth noting that fermentation of SAHMT has the potential to decrease the level of serum lipids which thus leads to the reduction of the risk of diabetes and obesity. SCFAs are produced in the colon during this process. Cholesterogenesis and lipogenesis pathways are subdued by propionate (Murphy et al., 2010). Fermentation pattern of SAHMT especially, the proportion between acetic acid and propionic acid that goes to the liver may act as a possible lipid reduction characteristic of SAHMT.

f. Regulation of glucose metabolism

Fermentation of SAHMT may influence plasma glycaemia and insulinemia through their outcome on the production of SCFAs. Propionic acid decreases gluconeogenesis and supports hepatic glycolysis. These fatty acids incidentally impact on the hepatic metabolism of glucose. The high ratio of acetate to propionate reduced glycemia in diabetic hyperglycemia. There is incidental proof for a dropped gluconeogenesis by the liver (Barrera et al., 2011). SCFAs especially acetate and butyrate may affect plasma-glucose level by growing the hormone Glucagon-like peptide-1 (GLP-1) which implicitly regulates blood glucose levels by increasing the discharge of insulin and

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decreasing the release of glucagon by the pancreas (Barrera et al., 2011). This makes SCFA to positively disturb glucose metabolism by stabilizing plasma glucose levels and improving glucose management. The SCFAs produced also increase the gut hormone Peptide YY (PYY) which is known as satiety hormone. This is done through the activation of free fatty acid receptors 2 and 3 (Ffar 2 and Ffar 3). The increase in the hormone affects the blood glucose levels thereby affecting glucose metabolism.

5.5 Prebiotic potential of Amylose-lipid complexes (ALCs)

Prebiotics are simply considered as feed for the probiotics. Prebiotics are typically thought of as carbohydrates with a short chain length (Belobrajdic, King, Christophersen, Bird, 2012), nonetheless some polysaccharides have also exhibited prebiotic potential such as resistant starch type 5 (RS 5) from amylose-lipid complex. Prebiotics differ from colonic meals in that the latter act as general fuel for the endogenous colonic bacteria, giving energy, metabolic substrates, and necessary micronutrients to the host.

This current study depicts how ALCs highly available in the indigestible residues can be considered as prebiotics. This can only be achieved if the following conditions are fulfilled: (a) ability to resist gastric acid and hydrolysis of amylases and glucoamylases, (b) the ability to be fermented by the bacteria in the gut and (c) ability to selectively stimulate the growth of one or a limited number of bacteria in the gut.

a. Ability to resist gastric acid and hydrolysis of alpha amylase and amyloglucosidase.

Amylose-lipid complex which is considered as resistant starch type 5 (RS 5) escaped assimilation in the small intestine. The presence of lipid in starch molecules may slow the enzymatic hydrolysis of the starch. The resistance to amylase and glucoamylase hydrolysis by amylose–lipid complexes limits starch swelling and minimizes enzymatic hydrolysis of starch granules. (Jiang et al., 2010). In research chapter 1 of the current study, *in vitro* digestibility was used to mimic the mammalian upper digestive tract. The results show that most portions of the maize starch and meal escape digestion. The undigested portion was further analysed using DSC and was identified as ALC formation (Research chapter 2, Figure 2.1). The undigested portion which was determined as ALC

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blocked the enzyme site for attacking the alpha 1,4 glycosidic bonds, thus resisting enzymatic hydrolysis. Cui and Oats (1999) reported that the developmentof amylose-lipid complex lowers the digestibility of starch and modulates the reduction of glycaemic response. Work done by Mapengo and Emmambux, (2020) also reported reduction in digestibility of maize meal and they concluded that, this was due to the formation of ALC.

b. Ability to be fermented by the gut microbiota

The starch that managed to avoid digestion in the upper gastrointestinal system finally makes its way to the colon. The RS 5 that enters the colon to be qualified to be considered as prebiotics, it must be used as a substrate for prevailing microbes in the gut to ferment. According to Salminen et al. (1998), two types of fermentation exist in the gut, namely saccharolytic and proteolytic fermentation. Since RS 5 from ALC is from carbohydrate with a lipid molecule, the favourable saccharolytic bacteria will act on it as soon as it enters the colon to produce SCFAs (acetate, propionate, and butyrate) and gases (carbon dioxide, hydrogen, and methane) as by product. The ALC (RS 5) served as a good substrate for the saccharolytic bacteria. The type of substrate available in the colon will determine the site of fermentation as well as the concentrations of the SCFAs produced (Wong, de Souza, Kendall, Emam, and Jenkins, 2006). In research chapter 2, the SCFAs produced were quantified to know the concentrations of the individual SCFAs and to ascertain the fermentation rate. It was shown that the indigestible residues containing ALC were a fermentable substrate with the evidence of producing SCFAs (acetate, propionate, and butyrate) and its fermentation rate was slow. The slow fermentation rate was attributed to the chemical structure of the ALC which makes it not easily accessible to the gut microbiota. In an anaerobic environment, gut microorganisms were thought to be nearly incapable of breaking down free fatty acids (Candido and others, 2018). The slow fermentation rate indicates that absorption of the SCFAs produced will be at the distal colon.

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c. Ability to selectively stimulate the growth of one or limited number of gut bacteria

In addition to the above, amylose-lipid complex as demonstrated by the fermentation of dietary fiber, must be fermented by a diverse range of gut microbiota, including pathogens. (Gibson & Roberfroid, 1995). These require quantifiable microbiological analysis of main colonic bacterial genera and its benefit to the consumer (Sarbini et al., 2013). From Research Chapter 3, the microbes observed from the colonic fermentation were analysed based on their genera to determine which of the major beneficial gut bacteria were stimulated for growth. The obtained results prove that the major beneficial gut bacteria such as *Roseburia, Bacteroides, Bifidobacteria, Clostridia, Ruminococcus* were identified and stimulated for growth. Tools such as molecular-based microbiological procedures which was established and recognized as a reliable tool and the production of SCFAs, gases, reduced luminal pH and enzymes were used to examine stimulation of the bacteria activity (Sarbini & Rastall, 2011). These tools were used to design the study so that the ALC may be considered as a prebiotic. It is note-worthy that ALC promoted the growth of most of the acetate and butyrate and propionate producing microbes in the gut. To fully prove the prebiotic nature ALCs, a strategic human clinical study must be conducted.

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6.0. CONCLUSION AND RECOMMENDATION

Heat moisture treatment of both maize starch and maize meal with stearic acid cause an interaction between starch polymers and form amylose-lipid complexes. This observed interaction changes functional, nutritional properties, and health benefits associated with the combination treatment. The change in the functional properties is observed during pasting at 91℃ for 35 min, where there is a decrease in final viscosity, decreased breakdown viscosity, non-gelling tendencies, and an increase in enthalpy of gelatinization. Both amylose and amylopectin appear to be transformed at molecular level during heat-moisture treatment. Incorporation of stearic acid followed by heat-moisture treatment promotes amylose-lipid complexes. The DSC and XRD results from the study show that stearic acid addition to both maize starch and maize meal followed by heat-moisture treatment induces the development of amylose-lipid complexes (Type I, IIa and IIb). The combined effects of amylopectin-amylopectin, amylose-amylopectin interaction and amylose-lipid complex formation increase relative crystallinity, lowered breakdown viscosity, non-gelling tendencies, and a rise in enthalpy of gelatinization.

Stearic acid in combination with heat-moisture treatment promotes the formation of amylose-lipid complex which lowers starch hydrolysis by limiting access of alpha amylase to alpha 1,4 glycosidic bonds for starch hydrolysis in maize meal and maize starch, hence reduces the estimated glycemic index and increases the resistant starch content of these modified samples. The RS produced were classified under RS5. The heat moisture treatment with added stearic acid produces the highest resistant starch containing both V type amylose lipid complexes and some type III due to amylose-stearic acid interaction and amylopectin-amylopectin, respectively.

The indigestible residues from in vitro upper gastrointestinal tract (GIT) digestion of modified and unmodified maize meal and maize starch have prebiotic effects. Residues from the combination treatment of heat-moisture treatment of maize meal and maize starch with stearic acid with higher resistant starch containing amylose-lipid complexes have higher SCFAs (butyrate, acetate, and propionate) production. Thus, the indigestible residues, rich in RS5 derived ALC serve as prebiotics for the gut microbes to utilize. Some of the microbes identified are stimulated for growth due to the use of indigestible residues as a substrate. Firmicutes, Bacteroidetes and Actinobacteria are the major phyla detected to have utilized the indigestible residues used.

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It is therefore proposed that since the incorporation of stearic acid in combination with heat-moisture treatment to maize starch and maize meal is a 'clean label' starch, consumers who prefer food additives without synthetic chemicals will accept it. In addition to the above, it is recommended that ALCs which act as a prebiotic for most gut microbiota should be looked into and investigated deliberately.

Future studies can be done on the structure and digestibility of stearic acid addition in combination with heat-moisture treatment of maize meal using high-performance anion exchange chromatography (HPAEC) and Size Exclusion Chromatography (SEC). The use of HPAEC coupled with SEC would help to determine precise and thorough characterization of starch multi-scale structure of the maize meal. The HPAEC would be used to ascertain the molecular structure of amylose and amylopectin, such as the molecular size and distribution, the degree of polymerization (DP), amylose content, and degree of branching (DB). The SEC would be used to determine the weight chain length distribution (CLD), (logX) of separate chains from debranched starch as a function of DP (X). A bimodal peak of amylopectin branches is an indication of long and short chains of the amylopectin. It is important to note that HPAEC and SEC are good devices to characterize the starch molecular chain length distribution to draw a more comprehensive conclusion on the *in vitro* starch digestion.

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7.0 PUBLICATION FROM THIS RESEARCH

Asare, I. K., Mapengo, C. R., & Emmambux, M. N. (2021). In Vitro Starch Digestion and Physicochemical Properties of Maize Starch and Maize Meal Modified by Heat-Moisture Treatment and Stearic Acid. Starch-Stärke, 73(3-4), 2000128.

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