University of Reading

Recovery of functional ingredients from sweet corn (*Zea mays*) cobs

Thesis submitted for the requirement for the degree of Doctor of Philosophy in Food and Nutritional Science

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Declaration

I confirm that this is my own work and the use of all materials from other sources have been properly and fully acknowledged.

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Abstract

Sweet corn cob (SCC) is an agricultural lignocellulosic waste generated from the corn processing industry and is available in large amounts. However, the production of high value-added products from SCC is limited, most of it is either discarded or used for the production of biomasses. Hence, research into the comprehensive utilization of SCC as a functional food is of great interest. As such, the aims of the current research were (i) to identify and characterise the functional compounds in SCC, (ii) to examine the effectiveness of enzymatic hydrolysis in the release of ferulic acid (FA) from SCC, (iii) to evaluate the effect of phenolic compounds from SCC on gut microbiota and (iv) to investigate the effect of the incorporation of SCC flour in the baking of rice flour muffin.

Compositional analysis of SCC (Chapter 2) showed that SCC is comprised mainly of cellulose and hemicellulose. In addition, elemental analysis showed that phosphorus, potassium and magnesium are present in SCC at a higher concentration compared to the rest of the minerals being tested. Alkali hydrolysis of the free, esterified and insoluble-bound fractions of SCC showed that the insoluble-bound fraction had the highest amount of total phenolic content, antioxidant capacity assays (TEAC, DPPH and FRAP) and contained the highest amount of FA and *p*-coumaric acid (pCA). More than 80% of the FA and pCA was present in SCC as insoluble-bound form. Supercritical fluid extraction of carotenoid compounds showed that SCC contained the highest amount of β-carotene, followed by zeaxanthin and lutein. Results showed that SCC could be a source of natural colorant, antioxidants and functional ingredients.

Raising concerns over the environmental impact due to the usage of large amounts of chemical solvents has increased the interest of using enzymatic hydrolysis, a more green and sustainable approach in the extraction of bound phenolic compounds, as compared to

conventional solvent extraction. Response surface methodology (Chapter 3) showed that under optimized conditions, the combination of enzymes ferulic acid esterase (FAE) and xylanase (XY) released half the amount of FA, as compared to alkali hydrolysis. Therefore, novel technologies can be further explore to increase the efficiency of enzymatic extraction of FA from SCC.

The therapeutic effect of phenolic compounds depends on their bioactivity, where it is modulated by the gut microbiota depending on their bioavailability. The effect of SCC (high in fibre and bound phenolic acid content) on the gut microbiota ecology, was used to compare against the SCC extract (contains free phenolic acids and xylooligosacchrides) using batch culture fermentation (Chapter 4). SCC extract showed a potential bifidogenic effect by showing a trend of an increase in *Bifidobacterium* and a decrease in pathogenic *Clostridium perfringen,* although the results were not significant. Furthermore, the SCC which is high in fibre content, showed an increase in the production of beneficial short chain fatty acids (SCFA), a key source for the intestinal epithelium and liver.

Incorporation of SCC flour in the baking of rice flour muffin showed improvements in the texture and total ferulic acid content of baked muffin, as compared to control muffin baked with 100% rice flour (Chapter 5). Muffin incorporated with ≤ 20% of SCC flour showed a softer crumb and improvements in terms of height, colour and nutritional value coupled with an increase in fibre and ferulic acid content. Overall, SCC can be a functional food as it can be a source of fibre, phosphorus, potassium, phenolic (ferulic and *p*-coumaric acid) and carotenoid compounds. Enzymatic hydrolysis can be used as an alternative method in the release of the ferulic acid from SCC. In addition, SCC significantly increased the production of beneficial SCFA during colonic fermentation. Furthermore, SCC can be used as an alternative flour in baking to improve the texture and quality of gluten-free rice muffin.

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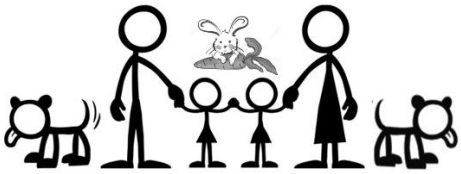

Thank you to all the lecturers and technical staff for your support and advice. Thank you to all my friends in Reading for all the good moments that we shared all these years. Special shout out to all my friends in 4-23, we shared knowledge, laughter, complaints, as well as food.

" We create memories, not just a PhD thesis "

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Chapter 1

Literature Review

1.1 Introduction

According to the Food and Agriculture Organization Corporate Statistical Analsysis (FAOSTAT), the world production of corn reached 1060Mt in 2016. Most corn is used as livestock feed (60 to 70% of global production) and food for human consumption (30 to 40% of global production) (Gwirtz and Garcia-Casal, 2014). Corncobs are a costless agricultural waste that accounts for 27 to 30% of the corn milling industry (Van Doan et al., 2018), and are very rich in cellulose and hemicelluloses (Garg et al., 2008).

Compositional analysis has shown that corn cob contains 41.4% of hemicellulose, 40% of cellulose, 5.8% of lignin, 2.5% of crude protein, 2.1% of starch, 1.8% of ask, 1.1% of water soluble carbohydrate and 0.7% of crude fat (Kaliyan and Morey, 2010). In addition, inorganic elemental analysis has indicated that potassium (10.8 g kg^{-1}), silicon (5.33g kg^{-1}), phosphorus (1.11g kg^{-1}), magnesium (0.55g kg^{-1}), calcium (0.23g kg^{-1}), aluminium (0.18g kg^{-1}), inorganic sulphur (0.14g kg^{-1}), barium (0.11g kg^{-1}) as well as traces of titanium (0.003g kg^{-1}) and strontium (0.002g kg^{-1}) were found in corn cob (Mullen et al., 2010). Furthermore, studies on the phenolic content and antioxidant potential of corn cob have been carried out previously. Sultana et al. (2007) reported that corn cob is a potential source of natural antioxidants that might be useful in the prevention of oxidation of vegetable oils. They further reported that the free radical scavenging ability of corn cob extract was associated with their phenolic content. In addition, Topakas et al. (2007) reported the presence of ferulic and *p*-coumaric acid in corn cob. This showed

that corn cob has the potential to be a functional food and can be an excellent source of insoluble dietary fibre, as well as minerals and phenolic compounds.

The growth in consumer health awareness has increased the demand for natural, safe and health promoting food ingredients. Functional food is defined as 'foods that may provide health benefits beyond basic nutrition' (Bech-Larsen and Grunert, 2003) and plays a role in improving general conditions of the body and risk reduction of some diseases. When the beneficial effect of functional food is due to a component or a series of ingredients present at a lower concentration, they are called functional ingredients (Plaza et al., 2008). Examples of functional ingredients are flavonoids, polyphenolic, carotenoids, probiotics, plant sterols and fatty acids. In recent years, there has been an increase in attention towards phytochemicals such as carotenoids and phenolics due to their anticarcinogenic, antioxidant, antimutagenic and other health benefits (Xia et al., 2018). Therefore, this has led to an increased interest in the use of these natural antioxidants as food ingredients or as food supplements (Rietjens et al., 2002).

Polyphenols are plant secondary metabolites involved in the inhibition of pathogenic activity and defence against ultraviolet (UV) radiation (Beckman, 2000). Some phenolic constituents have been shown to exhibit a strong antioxidant activity (Lu and Foo, 2000) such as ferulic acid (Rice-Evans et al., 1997) and *p*-coumaric acid (Rice-Evans et al., 1996). Natural antioxidants can effectively absorb UV at 100 to 400nm, scavenge free radicals, and chelate transition metals. Thus, they can prevent progressive autoxidative damage and production of off-taste and off-odours (Brewer, 2011). Increasing safety concerns over synthetic antioxidants has led to the increasing demand for a natural antioxidant alternative. In addition, there has been increasing consumer preference for clean label, natural products and reduction in the usage of food additives in food products (Schieber et al., 2001). Therefore, the first working chapter in this thesis

focuses on the proximate analysis and identification of functional ingredients in sweet corn cob (polyphenols and carotenoids) to highlight the potential of sweet corn cob as a source of natural functional food.

The shift towards greener chemistry has led to the increase in using enzymatic hydrolysis instead of chemical hydrolysis, because it has less impact on the environment, lower energy consumption and requires no chemical solvents (Alvira et al., 2010). Most phenolic acids are present in insoluble-bound form, esterified to non-starch polysaccharides such as arabinose and xylose units which can be released via acidic, alkaline, or enzymatic hydrolysis (Yu et al., 2001). Ferulic acid, a phenolic compound widely available in a variety of vegetables and plants (Liu et al., 2018), is often present as insoluble bound form in grains such as corn (Butts-Wilmsmeyer et al., 2018). Torre et al. (2008) reported the use of alkali hydrolysis to cleave the ester linkages in lignin-polysaccharide complex, thus releasing the ferulic and *p*-coumaric acid in corn cob. A combination of enzyme ferulic acid esterase (FAE) and xylanase (XY) have been used to release the insoluble-bound ferulic acid. XY solubilises the cell wall structure by formation of low molecular weight ferulolyted compounds and then, FAE breaks the ester linkage between ferulic acid and the attached sugar (Yu et al., 2002). Therefore, Chapter 3 involves optimization of the concentration, pH and temperature of combination FAE and XY using response surface methodology, to maximise the yield of ferulic acid from SCC.

The gut microbiota plays an important role in the modulation of the bioavailability of dietary polyphenols. It has been reported that about 90 to 95% of total polyphenol intake may reach the intestinal lumen and be subjected to enzymatic activities of the colonic microbial (Anhê et al., 2013). The health benefits derived from the consumption of polyphenol rich foods might be due to the breakdown of original polyphenolic

structures into phenolic metabolites by the colonic microbiota and thus increase their availability for absorption. Recent studies have shown that a polyphenol-rich diet may modulate the gut microbiota by promoting the proliferation of beneficial bacteria and increasing the biodioversity of bacteria in the gut (Viveros et al., 2011, Zhang et al., 2016). Anson et al. (2009b) studied the bioaccessibility of ferulic acid reported that free ferulic acid has a higher bioaccessibility than insoluble-bound ferulic acid. However, there are limited studies on the effect of free and bound phenolic acids, including ferulic acid, on the gut microbiota ecology. Therefore, Chapter 4 focuses on the effect of SCC (comprised mainly of bound ferulic acid) and SCC extract (comprised mainly of free ferulic acid and xylooligosaccharides) on gut microbiota.

The increasing demand for gluten-free products has favoured the design of various gluten-free bakery products that is comparable to the quality characteristic of wheat bakery products (Matos Segura and Rosell, 2011). Coeliac disease is a gluten-induced immunological disorder that affects 1 to 2% of the population in Western countries. At present, approximately 75 to 90% of affected individuals remain unrecognized (Fuchs et al., 2018). A strict lifelong avoidance of gluten ingestion is the only effective therapy for this disease (Shevkani and Singh, 2014). In some countries such as United States and Canada, a gluten free diet is completely devoid of gluten and is based on foods that are naturally gluten free such as corn and rice. However, in countries such as the United Kingdom and Scandinavia, the gluten free diet may include food that has been rendered gluten free (such as wheat starch) but nonetheless contain small amount of prolamin (Thompson, 2001), a compound that is known to be non-tolerated in celiac patients (Silano and De Vincenzi, 1999). Rice flour is one of the most suitable cereals for the development of gluten-free products, however, they are often reported as having a lower volume, poor crumb colour, texture and structure (Matos et al., 2014). Additionally,

milled rice also contains a low level of fibre (Monks et al., 2013) and phenolic compounds including ferulic acid (Zhou et al., 2004). There have been conflicting reports on the effect of baking on free and bound phenolic acid. Holtekjølen et al. (2008) observed a reduction in free phenolic acid in bread containing barley flour after baking and on the other hand, Abdel-Aal and Rabalski (2013) reported an increase in free phenolic acid including ferulic acid in bread, muffin and cookies containing einkorn flour. The effect of baking on free or bound phenolic acid could be due to the baking method as well as the source and nature of the phenolic compounds (Abdel-Aal and Rabalski, 2013). The increase in free ferulic acid might be due to the release of bound ferulic acid during thermal treatment and this can then increase the bioavailability of ferulic acid. Hence, Chapter 5 focuses on exploring the potential of SCC flour as flour used for the baking of a rich in ferulic acid and gluten free rice flour muffin as well as investigating the effect of baking on ferulic acid fractions.

This thesis consists of 6 chapters and the titles of each chapter are as follow:

Chapter 1: Introduction and literature review

Chapter 2: Valorisation of sweet corn (*Zea mays*) cob by extraction of valuable compounds.

Chapter 3: Optimization of enzyme assisted extraction of ferulic acid from sweet corn cob by response surface methodology

Chapter 4: Influence of sweet corn cob on gut microbiota ecology

Chapter 5: Physicochemical properties and ferulic acid content of muffin incorporated with sweet corn cob flour.

Chapter 6: Concluding remark

1.2 Production, utilization and types of corn

Zea mays (corn) is one of the most important cereal crops of the world, followed by rice and wheat. As reported by FAO (2013), the largest producer of corn is the United States, which accounts for up to 40% of the world's harvest, followed by China and Brazil. In 2010, world production of corn reached over 840 million MT and the demand for corn as feed, fuel and food continues to increase (FAO, 2012). Rosegrant et al. (2008) reported that by 2050, the demand for corn will double in the developing world and corn will become the crop having the world's greatest production. Different types of corn are grown throughout the world and they are varied in colours including white, yellow, red and black (**Figure 1.1**). Corn has been used mainly for animal feeding, human consumption and alcohol production (Ranum et al., 2014).

Figure 1.1: Different colours of corn including black, red, yellow and white corn. Source from Narmer (2017)

1.3 Utilization of corn waste

Sweet corn residue, a by-product from the processing of sweet corn for human consumption, accounts for 60-70% of the harvest yield and consist of cobs, discarded kernels, husk leaves and some stalk (Fritz et al., 2001). Various studies on the utilization of corn cob as absorbent (El-Hendawy, 2003), production of bioethanol (Chen et al.,

2007), bio-oil and bio-char (Mullen et al., 2010), as well as xylooligosaccharide (Aachary and Prapulla, 2009) have been carried out. However, research on SCC for its utilization in food is limited. Composition analysis of SCC have been carried out and they reported that SCC consists mainly of cellulose and hemicelluloses (Rivas et al., 2002, Miura et al., 2004, Awosusi et al., 2017, Worasuwannarak et al., 2007). However, information on the bioactive compounds such as phenolic and carotenoid compounds in SCC is scarce.

1.4 Phenolic compounds

Phenolic compounds are secondary metabolites derived from the shikimate, pentose phosphate and phenylpropanoid pathway in plants (Randhir et al., 2004). As one of the most widely occurring groups of phytochemicals, these phenolic compounds play an important role in the physiology and morphology of plants by providing protection against predators and pathogens, helping in growth and reproduction as well as contributing towards the sensory characteristic and colour of fruits and vegetables (Balasundram et al., 2006). Phenolic acids are divided into two subgroups, hydroxycinnamic and hydroxybenzoic acid (**Figure 1.2**). Protocathechuic, gallic, syringic, *p*-hydoxybenzoic, syringic and vanillic belongs to hydroxybenzoic acids group with their common C_6 to C_1 structure, while *p*-coumaric, caffeic, sinaptic and ferulic acid with three-carbon side chain (C_6-C_3) are in the hydroxycinnamic group (Bravo, 1998).

Phenolic compounds have been associated with a wide range of physiological properties including anti-microbial, anti-oxidant, anti-artherogenic, anti-inflammatory, anti-allergenic, anti-thrombotic, vasodilatory and cardioprotective effects (Balasundram et al., 2006). In the recent years, great attention has been given to the beneficial effect of these phenolic compounds in the reduction of diseases such as cardiovascular diseases (Jiménez et al., 2008), cancer and diabetes (Kim and Dale, 2004). Parr and Bolwell (2000) reported that the health benefits of phenolic compounds have showed to be associated

with the consumption of fruits and vegetables, which are rich in phenolic compounds. It was reported that the beneficial effect could be due to the antioxidant effect of phenolic compounds. Therefore, there has been increasing research on fruits and vegetables containing high amounts of phenolic compounds as a natural source of antioxidants.

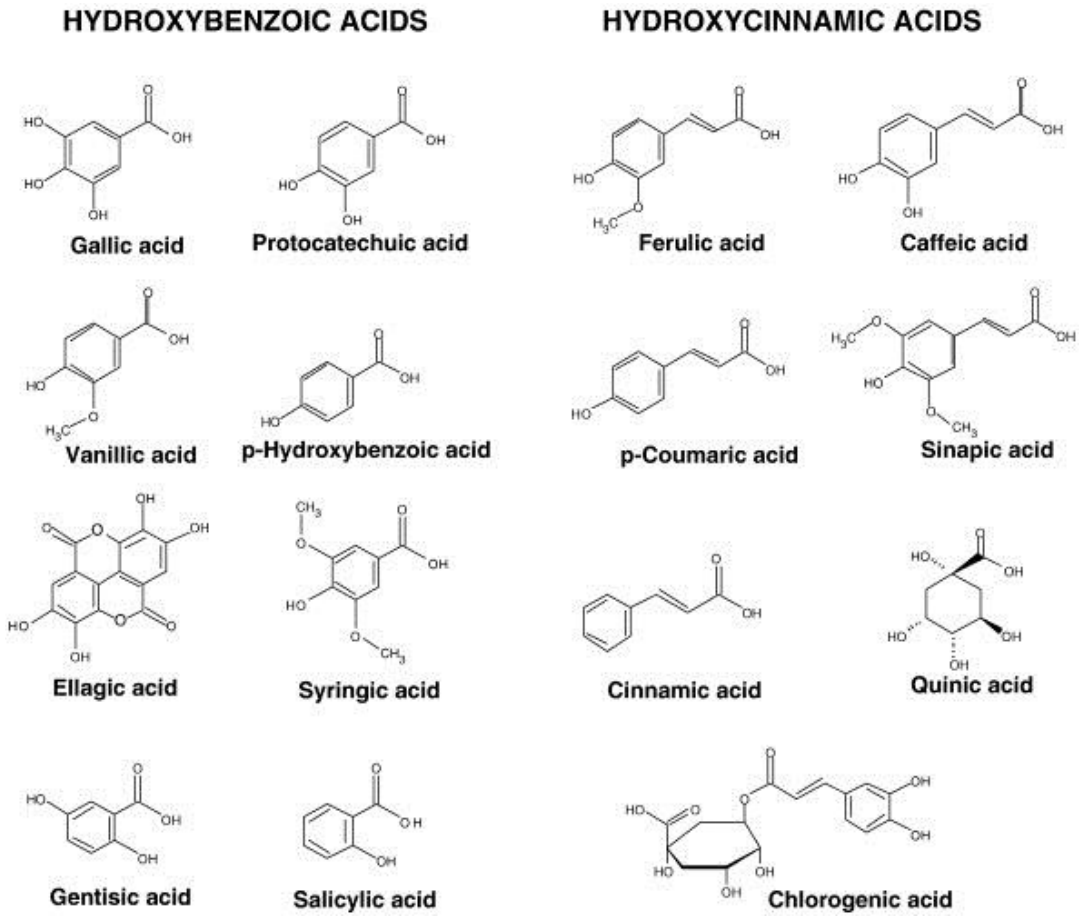

Figure 1.2: Hydroxybenzoic and hydroxycinnamic group of phenolic compounds. Source from Martins et al. (2011)

1.4.1 Ferulic acid

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) (**Figure 1.3**) is a phenolic acid ubiquitous in the plant kingdom. Ferulic acid (FA) is commonly found in vegetables and fruits such as tomatoes, rice bran, as well as sweet corn (Sri Balasubashini et al., 2003)

and exhibits an extensive range of therapeutic effects against diseases such as diabetes, cancer, neurodegenerative and cardiovascular (Srinivasan et al., 2007). The three distinctive structural motifs that can contribute to the free radical scavenging ability of FA are (1) the presence of an electron donating group on the benzene ring for the termination of free radical chain reaction, (2) the carboxylic acid group with an unsaturated C=C double bond providing additional sites for free radicals and protection against lipid peroxidation, and (3) the presence of 3-methoxy and 4-hydroxy groups to stabilized the radical making it sufficiently stable to scavenge reactive oxygen species efficiently (Kanski et al., 2002). The antioxidant activity of FA is due to its ability to form a resonance stabilized phenoxy radical (**Figure 1.4**), due to the presence of its phenolic nucleus and unsaturated side chain (Srinivasan et al., 2007).

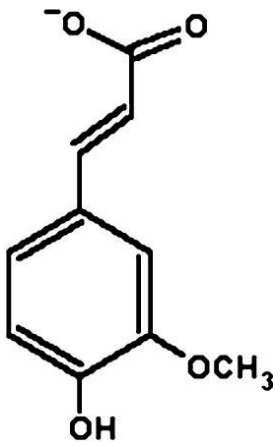

Figure 1.3: Structure of ferulic acid.

Source from Srinivasan et al., (2007)

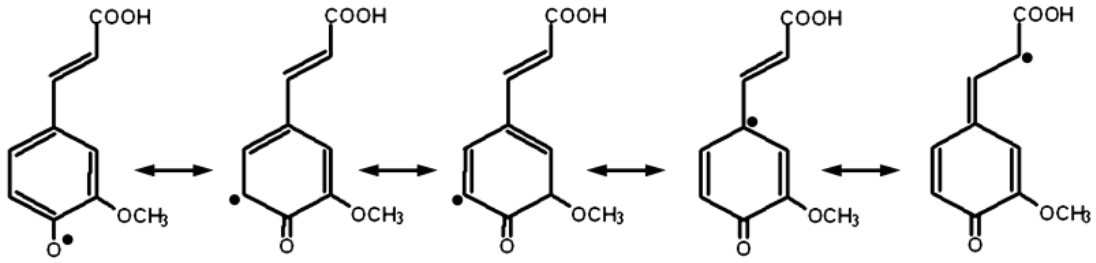

Figure 1.4: Resonance stabilization of ferulic acid radical.

Source from Srinivasan et al. (2007)

1.4.1.1 *Application of ferulic acid in food*

FA is used in the production of vanillin, an important aromatic flavour compound used in the beverages, pharmaceuticals, food and perfume industry. Sakai et al. (1999) reported that the production of vanillin from vanillic acid can be achieved through biotransformation by enzymes secreted by microorganisms including bacteria, yeast and fungi. The possible pathways were (1) the decarboxylation of FA by decarboxylase producing 4-vinylguaiacol and then converting to vanillin; (2) reduction of FA to dihydroferulic acid forming vanillic acid and vanillin; (3) formation of coniferyl alcohol from FA forming vanillic and vanillin.

Besides production of vanillin, the antioxidant and antimicrobial properties of FA also enable them to be used as food preservatives. In a study carried out by Heinonen et al. (1998) comparing the ability of various phenolic compounds to inhibit protein and lipid oxidation, ferulic acid was found to be most efficient as compared to other phenolic compounds such as catechin, epicatechin, propyl gallate, malvidin, caffeic acid, delphinidin, quercetin, and rutin. They further reported that ferulic acid is less affected by pH as compared to gallic, chlorogenic and caffeic acid and therefore was most efficient in the inhibition of lipid and protein oxidation. Ferulic acid has been used to cross-link

with polysaccharide to increase the viscosity and form gels of some polysaccharides such as arabinoxylan and pectin. This helps the low viscosity, low molecular weight and poor gel formation capacity of these polysaccharides to make new gels in food processing (Figueroa-Espinoza et al., 1999).

1.4.1.2 *Extraction of ferulic acid*

Extraction of FA from food by-products such as brewer's spent grain (Mussatto et al., 2007), sugar beet pulp (Oosterveld et al., 2000), flax shives, wheat and corn bran (Buranov and Mazza, 2009) have been carried out previously. FA can be absorbed and easily metabolized in the human body. However, FA seldom occurs in free form in plants and is often conjugated with mono and oligosaccharides, lipids, polyamines and polysaccharides. The bioaccessibility of FA is determined by the percentage of free FA (Anson et al., 2009b). They found that limited bioavailability of FA is attributed to the low bioaccessibility of FA bound to the indigestible polysaccharide of the cell wall. Ferulic acid residue can form an ester linkage between the carboxylic group of FA and the primary alcohol on the C5 carbon of the arabinose side chain of arabinoxylan (Hartley and Ford, 1989), as well as ether linkage to lignin monomers (Scalbert et al., 1985) (**Figure 1.5**).

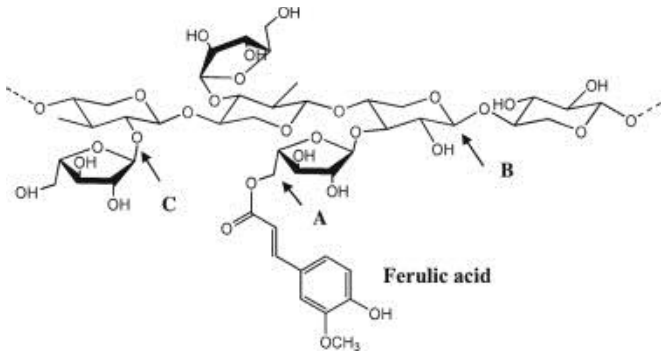

Figure 1.5: Structure of ferulic acid esterified to (A) arabinoxylan, (B) xylan backbone and (C) arabinose.

Source from Buanafina (2009)

The bound ferulic acid can be released by acid hydrolysis or alkaline hydrolysis (Lam et al., 1994). Acid hydrolysis breaks the glycosidic bond and solubilizes sugars (Fazary and Ju, 2007) while alkaline hydrolysis breaks ester linkages between phenolics to the cell wall (Moussa-Ayoub et al., 2011). Bonoli et al. (2004) reported that alkali hydrolysis is more reliable in the extraction of hydroxycinnamic acid, as compared to acid hydrolysis. However, chemical hydrolysis offers several disadvantages including the usage of large amounts of solvent and subsequent solvent disposal problems (Alinia et al., 2010), leading to an increase in environmental pollution.

1.5 Carotenoid compounds

Carotenoids, a class of flavonoids, are isoprenoids found ubiquitously in microorganism and plants. They are essential components of the photosystem and responsible for the yellow-to-red colouration of flower, vegetables and fruits (Tanaka et al., 2008). Carotenoids have a 40-carbon skeleton of isoprene units and more than 600 different carotenoids have been identified (Liu, 2007). The long series of conjugated double bonds forming the central part of a carotenoid molecule (**Figure 1.6**) give them their chemical reactivity, shape and light-absorbing properties (Liu, 2007).

Britton (1995) reported that carotenoids are essential for photosynthesis, production and reproduction in plants, as well as acting as antioxidants in lipid environments through their ability to react with free radicals, forming less reactive free radical products. Lutein and zeaxanthin are the major carotenoids in the macular region of the retina in humans and they have been reported to protect the eye from free radicals and near-to-UV blue light (Wenzel et al., 2003), while α-carotene, β-carotene, and β-cryptoxanthin have provitamin A activity. Dietary intakes of lutein and zeaxanthin have shown to reduce the risk of cataracts and age-related macular degeneration (Landrum and Bone, 2001). Studies on carotenoid content in corn kernels have been reported by Scott

and Eldridge (2005), Hu and Xu (2011), Xu et al. (2010) and Kopsell et al. (2009). α-carotene, β-carotene, α-cryptoxanthin, β-cryptoxanthin, zeaxanthin and lutein have been reported to be present in corn kernels.

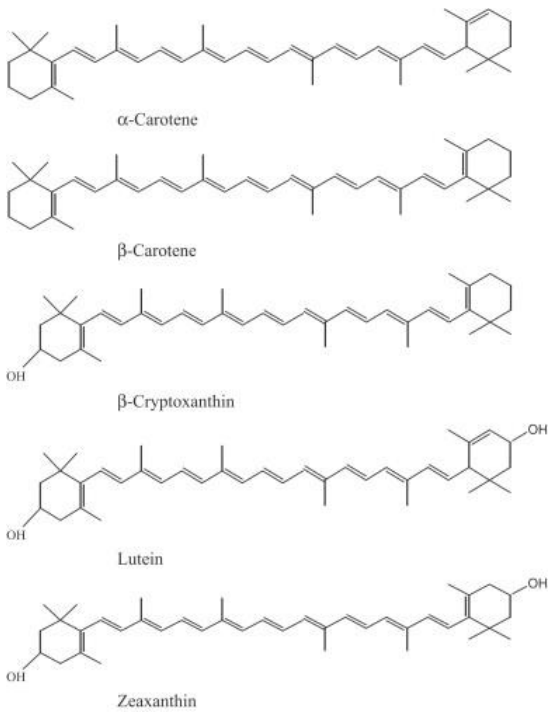

Figure 1.6: Chemical structure of carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein and zeaxanthin).

Source from Liu (2007)

1.5.1 Extraction of carotenoids

Most carotenoids available on the market are derived from chemical synthesis and thus cannot meet the consumers' need for natural carotenoids (Gu et al., 2008). Therefore, researchers shifted attention from chemical synthesis to extraction of carotenoids from biological sources such as *Rhodobacter sphaeroides* (Chen et al., 2006), carotenoid-rich food such as carrots (Sun and Temelli, 2006) or food by-products. Extraction of natural

carotenoids from food by-products have been carried out using tomato pomace (Vági et al., 2007), pomegranate waste (Goula et al., 2017), carrot pulp (Chen and Tang, 1998) and shrimp waste (Sachindra and Mahendrakar, 2005).

In the past, carotenoids have been mainly extracted using organic solvents such as acetone (Aravantinos-Zafiris et al., 1992), ethanol, ethyl acetate or hexane (Amaya Guerra et al., 1997). In addition, the recovery of carotenoids has been carried out using crude oil (Sachindra and Mahendrakar, 2005), bio-solvent such as *d-limonene* (Chemat-Djenni et al., 2010), ultrasonic, grinding and HCl-assisted extraction (Gu et al., 2008). The increasing consumer demand for natural products combined with the development of supercritical fluid technology has led to the increase in using supercritical carbon dioxide for the recovery of natural bioactives (Sun and Temelli, 2006) such as carotenoids. The use of carbon dioxide is neither toxic nor flammable, as well as being available at low cost and high purity. In addition, due to its moderate critical temperature, carbon dioxide suits to be used to extract thermally labile and reactive compounds (Vági et al., 2007).

1.5.2 Application of carotenoids in food

Carotenoids have been used in the food, feed and neutraceutical industries (Jaswir et al., 2011). Currently, carotenoids are used as natural nutrient supplements (Bone et al., 2018), food colorants (Breithaupt, 2004), as well as nutraceutical for cosmetic (Sathasivam and Ki, 2018) and pharmaceutical (Okonogi and Riangjanapatee, 2015) purposes. Nevertheless, their major food use is as a food colouring agent. It is estimated that the global carotenoid market will reach US$1.2 billion by 2018 (Naziri et al., 2014). Food manufacturers uses natural and synthetic food colorants to gain consumer's attention. The market demands over healthy products and functional food, have considered the use of chemical products as bad and the use of natural additives can provide the final product with a healthy value. Furthermore, consumers often raise

complaints against the use of food colorants due to the belief that they only have a cosmetic value and are associated with health damage (Mattea et al., 2009). Thus, there is a trend towards natural food colours since various studies have showed a possible correlation between the consumption of carotenoids with disease prevention (Olmedilla et al., 2001).

1.6 Enzymatic hydrolysis of ferulic acid from sweet corn cob

Enzymatic hydrolysis is a potential alternative to conventional solvent-based extraction methods (Puri et al., 2012) and is an effective and specific method for the release of bound phenolics (Acosta-Estrada et al., 2014). As the pressure on pharmaceutical and food industry to identify a more sustainable route for the extraction of new compounds increases, enzymatic hydrolysis has received increased attention as it is 'greener' or more eco-friendly (Meyer, 2010). Zheng et al. (2009) reported that the use of carbohydrate-hydrolyzing enzymes such as amylases, glucanases, cellulases, hemicellulases and pectinases is effective in the release of polyphenols (**Figure 1.7**). They reported that the ability of enzymes to disintegrate the plant cell walls matrix can facilitate the extraction of polyphenol.

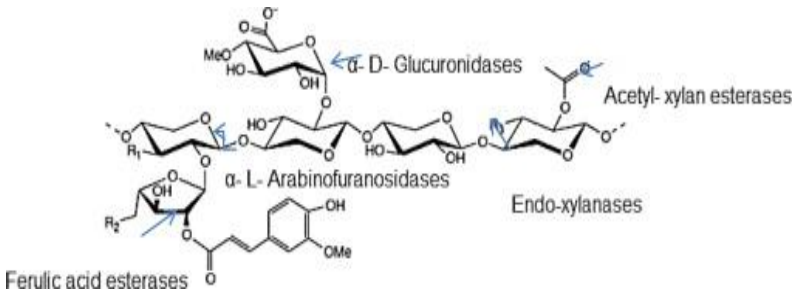

Figure 1.7: Chemical structure of xylan showing the xylan backbone, substituents and the corresponding sites for enzymatic hydrolysis.

Source from Shallom and Shoham (2003)

1.6.1 Combination of ferulic acid esterase and xylanase for the release of ferulic acid

The combination of auxiliary enzymes such as ferulic acid esterase (FAE), cellulases, and hemicellulases is necessary to break the lignocellulosic composition structure of biomasses (Selig et al., 2008). FAE has been reported to show a synergistic effect with other hydrolytic cell wall degrading enzymes in the degradation of cell wall (Faulds and Williamson, 1999) for examples arabinose and arabinofuranosidases in sugar beet pulp (Kroon et al., 1996) as well as xylanases in oat hulls (Yu et al., 2002) and wheat (Bartolomé et al., 2002). FAE plays an important role in the degradation of the plant cell wall structure by hydrolyzing the ferulate ester groups involved in the cross linking between hemicelluloses (Faulds and Williamson, 1994) while xylanase depolymerize the plant cell wall component xylan, resulting in the conversion of the polymeric substance into xylose and xylooligosaccharides (Subramaniyan and Prema, 2002).

Faulds and Williamson (1995a) reported a maximum release of 95% of ferulic acid from wheat bran was achieved by the combined action of FAE from *Aspergillus niger* and xylanase from *Trichoderma viride*. In addition, Yu et al. (2002) reported that in the absence of *Trichoderma xylanase,* only little amounts of ferulic acid were released from oat hull. In the presence of *Trichoderma xylanase*, a significant release of ferulic acid by *Aspergilllus* FAE was observed. The release of ferulic acid from barley and wheat cell walls by FAE is effective only in the presence of xylanase (Bartolome and Gomez-Cordoves, 1999). These studies clearly showed a synergistic interaction between FAE and xylanase. The efficiency of the release of free ferulic acid from complex cell wall involves a two-step process. First, a specific cell wall degrading enzyme such as xylanase is required to solubilise part of the cell wall structure forming low molecular weight ferulolyted compounds. Then, the ferulic acid esterase can act on these ferulolyted compounds, releasing the ferulic acid (Faulds and Williamson, 1995b). The initial

enzymatic hydrolysis alters the physical properties of the cell wall making it more accessible to further enzymatic attack (Yu et al., 2005).

1.6.2 Optimization of enzymatic hydrolysis via response surface methodology

Several factors including time, temperature, pH, and enzyme to substrate binding can influence enzymatic activity (Liaset et al., 2000) and thus optimization of these factors is important to maximise the yield and rate of hydrolysis. The conventional method for optimization involves changing one independent variable at one time while maintaining other variables at a fixed level. This method is extremely time consuming and expensive when a large number of variables are involved (Kunamneni and Singh, 2005). In addition, this one-variable-at-a-time method does not take into account the interaction between variables (Bezerra et al., 2008). To overcome this, experimental factorial design and response methodology can be used to optimize enzymatic hydrolysis.

Response surface methodology (RSM) is a statistical approach for the modelling and optimization of multiple variables. This method generates a mathematical model by combination of mathematics with statistics to describe the process, analyse the effect of independent variables and thus optimize the processing operation (Baş and Boyacı, 2007). Multivariate experiments are designed to lower the number of experiments needed for the optimization and to precisely collect these results as compared to a traditional full factorial designs (Tan et al., 2009). RSM has been used in the past few years to optimize the conditions for enzyme reaction (Lee et al., 2006b), composition of fermentation process (Ambati and Ayyanna, 2001), extraction of bioactive compounds (Yuan et al., 2015) and food processing methods (Mendes et al., 2001). The optimization of enzymatic extraction of phenolic compounds via RSM has been reported previously by Chen et al. (2011), Sun et al. (2011) and Tchabo et al. (2015).

1.7 Effect of phenolic compounds on gut microbiota

1.7.1 Gut microbiota and health

It is estimated that 500 – 1000 different species of microbiota inhabit the gastrointestinal tract, which makes up to about 10^{11} or 10^{12} cells/g of faeces (Cardona et al., 2013). *Bacteroides* are the most common bacteria present in the gut microbiota, which constitute around 30% of all bacteria in the gut, followed by *Clostridium, Provotella, Eubacterium, Ruminococcus, Fusobacterium, Peptococcus* and *Bifidobacterium*. *Lactobacillus* and *Escherichia* are also present but at a lower amount (Beaugerie and Petit, 2004). Clostridial cluster IV and XIVa of *Firmicutes* including species of *Eubacterium, Roseburia, Faecalibacterium* and *Coprococcus* are reported to inhibit the growth of pathogenic bacteria and increase the production of SCFA (Nicholson et al., 2012) while *Clostridium perfringens* is an important pathogen associated with the onset of inflammatory bowel disease and progression of colonic cancer (Guarner and Malagelada, 2003). In addition, probiotic strains comprised of bifidobacter and lactobacilli often associated with clinical effects including immunomodulation, prevention of diarrhoea and modulation of intestinal microflora (Saarela et al., 2000). Gut microbiota helps in maximising the absorption of nutrients and energy, they are also essential in the maintenance of body health status (Power et al., 2013). Intestinal disorder such as inflammatory bowel disease are often associated with microbial infections and an imbalance in the composition of gut microbiota (De Cruz et al., 2012).

1.7.2 Microbial production of short chain fatty acids (SCFA)

The fermentation of non-digestible substrates and endogenous mucus by gut microbiota not only stimulate the bacterial growth, but will also produce SCFAs (acetate, butyrate and propionate) and gasses (Wong et al., 2006). They further reported that lactate, succinate, ethanol, valerate, formate, isobutyrate, isovalerate, 2-methyl-butyrate and

caproate are also the end products of bacteria fermentation. Acetate contributes to more than half of the total SCFA detected in the feces (Louis et al., 2007) and is a product of carbohydrate fermentation by gut bacteria.

The metabolic pathway that contributes to the formation of acetate, butyrate and propionate in the human gut is shown in **Figure 1.8**. There are three different pathway used by the colonic bacteria for the formation of propionate: the propanediol, succinate and lactate pathway and normally involve the *Firmicutes* and *Bacteroidetes*. Additionally, in the production of butyrate, the butyryl-CoA:acetate CoA-transferase pathway is used by the majority of the gut butyrate-producers, including *Eubacterium, Roseburia* and *Faecalibacterium* (Louis et al., 2007). After the production, these SCFA will then be absorbed and used via different biosynthetic routes by the host (Den Besten et al., 2013). Acetate is the primary substrate for cholesterol synthesis, and butyrate is the preferential energy source use by epithelial cells. In vivo research on propionate supplementation showed that propionate was shown to reduce cholesterol level (Chen et al., 1984).

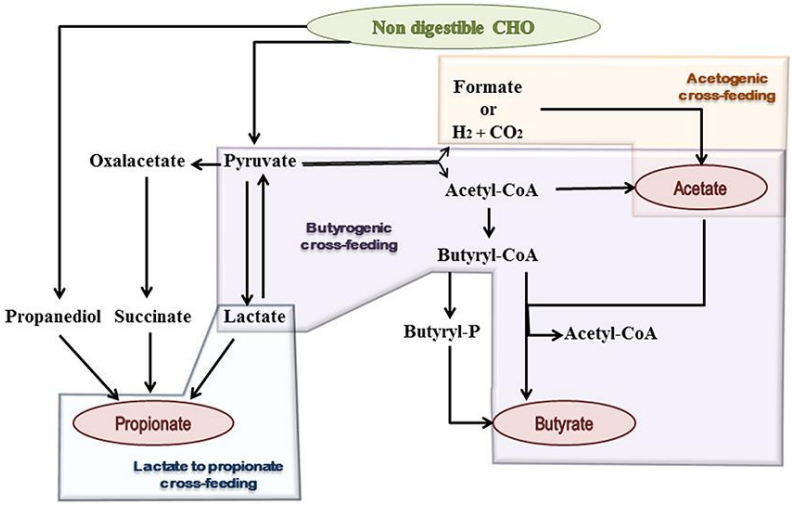

Figure 1.8: Metabolic pathway of the production of acetate, propionate and butyrate in the human gut.

Source from Ríos-Covián et al. (2016)

Acetate has been reported to be responsible for the ability of *Bifidobacteria* to inhibit enteropathogens (Fukuda et al., 2011). There has been increasing interest in the metabolism of butyrate and propionate due to their relationship with some diseases. For example, Arrieta et al. (2015) found reduced levels of propionate producers in children at risk of asthma and Machiels et al. (2014) reported a low level of butyrate producer in patients diagnosed with ulcerative colitis.

1.7.3 The interaction of polyphenol and gut microbiota

Recent evidence has showed that compositional change in gut microbiota, referred to as dysbiosis, is associated with diseases such as diabetes (Burcelin et al., 2011), cardiovascular diseases (Stock, 2013), non-alcoholic fatty liver disease (Dumas et al., 2006), obesity (Delzenne et al., 2011), and colorectal cancer (Wang et al., 2012). Therefore, the potential health benefits of polyphenols including anti-oxidant, anti-cancer, anti-inflammatory and anti-microbial properties have received great research interest.

Most polyphenols passes through the small intestine without being absorbed, thus reaching the gut microbiota which colonises the colon (Scalbert and Williamson, 2000). The absorption of polyphenols in the small intestine depends on the polymerization and degree of structural complexity, where polyphenols with low molecular weight such as monomeric and dimeric structure can be absorbed into the small intestine (Appeldoorn et al., 2009), while oligomeric and polymeric polyphenols such as hydrolysable or condensed tannins with molecular weights close to 40,000Da will reach the colon (Manach et al., 2005). It has been estimated that the absorption of polyphenol in the small intestine is only 5 to 10% of the total polyphenol intake, and the remaining 90 to 95% of polyphenols may accumulate in the large intestinal lumen (Clifford, 2004). The gut microbiota will then breakdown the original polyphenolic structures into low-molecular-weight phenolic metabolites to be absorbed, resulting in the potential health benefits

following the consumption of polyphenol-rich food (Cardona et al., 2013). Additionally, polyphenols can also modulate the composition of the gut microbiota through the stimulation of beneficial bacteria and inhibition of pathogenic bacteria (Ozdal et al., 2016).

A human intervention study indicated that consumption of wild blueberry drink significantly increased the number of *Bifidobacterium*, suggesting the effect of polyphenol on the modulation of intestinal microbiota composition (Vendrame et al., 2011). Furthermore, consumption of red wine polyphenol also showed a significant increase in the bacterial number of *Prevotella, Enterococcus, Bacteroides, Bifidobacterium, Eggerthella lenta, Blautia cocoides-E. rectale* and *Bacteroides uniformis* group, while the quantity of *Lactobacillus* spp was unaltered (Queipo-Ortuño et al., 2012). On the other hand, Lee et al. (2006a) found that the growth of pathogenic bacteria such as *Clostridium difficile, Clostridium perfringens* and *Bacteroides* spp. was significantly repressed by tea phenolics, while *Bifidobacterium* and *Lactobacillus* were affected less.

1.7.4 Impact of ferulic acid on gut microbiota

The free and some conjugated phenolic acids are reported to be readily absorbed in the human large and small intestine (Manach et al., 2005) while the insoluble matrix of bound phenolic acid hinders the access of necessary enzyme such as ferulic acid esterase and xylanase which limits its bioavailability (Zhao et al., 2005). During colonic fermentation, the degradation of cell wall polymers and the release of ferulic acid is due to the action of several hydrolytic enzymes. It has been reported that xylanase has the most important hydrolytic activity for the degradation of wheat bran fibre (Bartolome et al., 1995), and the release of ferulic acid through the cleaving of ferulic acid-sugar linkage is contributed by the action of ferulic acid esterase (Faulds and Williamson, 1995b).

The bioavailability of phenolic compounds such as ferulic acid is determined by its bioaccessibility (Anson et al., 2009a). Upon consumption of wheat bran, little or no feruloyl groups were solubilized by the enzymes and chemical secretion of the upper gut, and the release of ferulic acid happened in the presence of human intestinal microflora (Akin et al., 1993). Bioavailability is defined as the amount or proportion of antioxidant that is digested, absorbed and utilized in normal metabolism while bioaccessibility is defined as the amount of ingested nutrient that is available for the absorption in the gut (Hedrén et al., 2002). Anson et al. (2009b) reported that the free FA have higher bioavailability as compared to FA embedded in the indigestible polysaccharide of plant cell wall. Extensive research on the beneficial effect of FA has been investigated *in vitro* and in rodents. For example, in a human trial (20 volunteers) carried out by Turner (2015), they reported that flatbread containing higher amounts of free FA (4.74mg/flatbread) can improve acute endothelium dependent vasodilation as compared to control flatbread (0.16mg free FA/flatbread). Sudheer et al. (2007) reported that 10 to 150 μM of FA is showed to contract nicotine induced glutathione depletion and lipid peroxidation in lymphocytes and FA at 250 to 500μM of FA is shown to reduce protein and lipid peroxidation in peripheral blood monocuclear cells and neuronal cells (Barone et al., 2009). However, most of the studies use 100% free FA at a dosage that is over the estimated human intake. The human consumption of FA is estimated to be about 80-165mg FA/meal which equates to approximately 1 to 2mg/kg of body weight (Barone et al., 2009). Moreover, the major source of FA in the human daily diet is through the consumption of whole grain products and the amount of free FA is limited to 1 to 4% of the total FA (Anson et al., 2009b).

1.8 Incorporation of bioactive ingredients from food by-products into food

Food waste has long been considered as a matter of minimization, treatment and prevention due to the environmental impact caused by its disposal. The term 'food by-products' is often used by scientists to introduce the potential of food waste to be used as a substrate for the recovery of functional compounds and to develop into new products with a market value. Valorisation of these by-products for edible purposes is a challenging field of research (Galanakis, 2012). Nevertheless, there have been increasing publications in the utilization of these by-products in edible food. For example, Oreopoulou and Tzia (2007) explored the use of phenols and carotenoids from fruit by-products as natural food or beverage preservatives due to their ability to extend the shelf-life of products by delaying the formation of off flavour and rancidity. In addition, Madureira et al. (2010) reported that cheese processing whey is an abundant source of proteins and lactose, and can therefore be used for the delivery of oligopeptides and monosaccharides in soft drinks and nutritional supplements. Furthermore, protein hydrolyzates from fish by-products have also been proposed by Kristinsson and Rasco (2000) to use as seafood flavours for soups or surimi.

In the bakery industry, there have been increasing publications in the incorporation of food by-products to increase the functional properties or nutritional values of the products. Majzoobi et al. (2011) reported the use of tomato pomace as a good and cheap source of hydrocolloids to improve the quality of flatbread. In addition, Mildner-Szkudlarz et al. (2013) incorporated white grape pomace into wheat biscuits to increase the total dietary fibre and total phenolic content. Furthermore, in the baking of gluten-free bakery products, carrot (Majzoobi et al., 2016) and orange (O'Shea et al., 2015) pomace have been reported to improve the quality of gluten free cake and bread. Incorporation of carrot pomace reduced the cake density, cohesiveness and hardness,

while increasing the sensory scores. In addition, incorporation of orange pomace flour can help to improve the total dietary fibre intake of a coeliac patient. Furthermore, Phimolsiripol et al. (2012) improved the dietary fibre content of rice based gluten free bread using rice bran.

1.8.1 Use of alternative flour in gluten-free bakery product

Currently, many gluten-free products available on the market are of low quality, due to its lack in flavour and poor mouthfeel (Gallagher et al., 2003). Furthermore, gluten-free products are frequently made up of refined flour or starch and are not enriched/fortified. Thus, the levels of nutrients might not be the same as the gluten-containing counterparts that they are intended to replace. Therefore, ambiguity still remains whether or not the lifelong adherence to gluten-free diet ensures a nutritionally balanced diet in coeliac patients.

Rice flour has received increasing attention as a substitution for wheat flour in the production of gluten free products due to its white colour, bland taste, hypoallergenic and digestibility properties (Rosell et al., 2007). Different types of hydrocolloids are added to most starch or rice based gluten free products. For example Arendt et al. (2002) reported that a combination of rice flour with high fat powder produces biscuit of comparable quality to wheat biscuits and Kang et al. (1997) showed that locust bean gum, hydroxypropylmethylcellulose (HPMC), guar gum, xanthan gum, agar and carrageenan have successfully formed rice bread. However, these gluten-free bakery products containing gums as gluten replacements are often lacking in fibres and nutrients. Therefore, there has been an increase in the research exploring the potential of alternative gluten free flour. Schober et al. (2003) reported that the combination of brown rice and soya flour along with corn and potato starch produces gluten-free biscuits comparable to wheat biscuit, and gives the best overall acceptability in sensory testing. In addition, the

use of pseudocereals, a minor cereal that is less common and grown in small regions of the world (Saturni et al., 2010), such as quinoa white flour (Elgeti et al., 2014), amaranth, buckwheat flour (Alvarez-Jubete et al., 2009), as well as chickpea, carob germ and soya (Miñarro et al., 2012) flour in the formulation of gluten-free baking have been reported previously. Furthermore, it has been reported that cereals and pseudocereals can be a potential food source of polyphenols (Saturni et al., 2010). For example, buckwheat is one of the best grain sources of polyphenol compounds (Gallardo et al., 2006) such as gallic, p-hydroxybenzoic, ferulic and p-coumaric acid (Hung and Morita, 2008) and their use in gluten-free crackers has showed to increase the total phenolic content and antioxidant activity as compared to wheat crackers (Sedej et al., 2011).

The predominant phenolic acids present in rice are ferulic and *p*-coumaric acid (Kuroda et al., 1995). Gorinstein et al. (2007) compared the polyphenol contents of some cereals and pseudocereals reported that rice (95µg g^{-1} DW) showed lowest total phenolic content when compared to rice bran (293µg g^{-1} DW), buckwheat (290µg g^{-1} DW), quinoa, (250µg g^{-1} DW), *A. cruentus* (160µg g^{-1} DW), *A. hypochrondriacus* (154µg g^{-1} DW), *A. hybridus* (150µg g^{-1} DW) and soybean (120µg g^{-1} DW). They also reported that rice contained the lowest antioxidant capacity as compared to the rest of the cereal and pseudocereal tested. Zhou et al. (2004) reported that milled rice contains low levels of ferulic acid (61 – 84mg kg^{-1}) as compared to brown rice (255 – 362mg kg^{-1}). These phenolic compounds are concentrated in the bran layers and are lost with the separation of rice bran during the processing of milled rice (Tian et al., 2004). They further reported that the soluble and insoluble-bound ferulic acid in white rice (0.07 and 5.26 mg/100g of flour, respectively) is lower as compared to brown rice (0.32 and 15.19mg/100g flour, respectively).

1.8.2 Effect of baking on free and bound phenolic acids

It has been reported by Duodu (2011) that the processing of pulses or cereal may give positive or negative effects on the content of phenolic compounds, which can then affect their bioactive properties and potential health benefits. Furthermore, Anson et al. (2009b) investigated the bioaccesibility of ferulic acid using an *in vitro* system simulating the upper gastrointestinal transit and digestion reported that the bioaccesibility of ferulic acid is dependent on the percentage of free ferulic acid available. The structure of phenolic acid, whether in free or bound form, would affect their behaviour during processing and hence affect their bioavailability for absorption (Abdel-Aal and Rabalski, 2013). They reported an increase of free phenolic acid and reduction in bound ferulic acid in flat bread, cookies and muffins baked with einkorn wholegrain flour. However, conflicting results have been reported by Holtekjølen et al. (2008) where the amount of free phenolics decreased and an increase in bound phenolics was observed during the baking of bread containing barley flour. The effect of baking on free and bound phenolic compounds might be due to the baking method as well as the source and nature of the phenolic compounds (Abdel-Aal and Rabalski, 2013).

Changes in phenolic acid during thermal processing such as baking could be due to polymerization and oxidation of phenolics, depolymerisation of high molecular weight phenolics, thermal degradation, production of Maillard reaction products and release of bound phenolics from the food matrix (Duodu, 2011). Cheng et al. (2006) reported that heat stress can cause an increase in the free phenolics in wheat as a result from the degradation of conjugated polyphenolic compounds. These changes are subjected to many factors including structure of food matrix, source and nature of bioactive compounds, as well as types of thermal processing.

1.8.3 Impact of incorporation of food by-products on the physical properties of baked products

Incorporation of food by-products into the formulation caused changes in the physical properties of the baked products. Addition of apple pomace into gluten free bread has been reported to give crumbs with a less cohesive and resilient texture, as well as specific volumes (Rocha Parra et al., 2015). In addition, Majzoobi et al. (2016) reported that the addition of carrot pomace powder improves the cake density, cohesiveness and hardness of gluten-free sponge cake. However, Šarić et al. (2016) reported that gluten free cookies added with raspberry and blueberry pomace increased the hardness of cookies, due to the increase of dietary fibre content from the added pomaces. Singh et al. (2016) reported that gluten-free rice muffin added with black carrot dietary fibre shows decreases in specific volume and firmness. The change in the physical properties seems to be dependent on the types of bakery product, nature and the level of incorporation of the by-product.

1.9 Hypothesis and Objectives

The hypothesis of this research is sweet corn cob contains valuable phytochemicals and nutrients that are extractable, which can be further processed into food product for human consumption, rather than remaining as lignocellulosic waste.

Four objectives have been set to test this hypothesis:

(i) to investigate the proximate, minerals and phytochemical composition of SCC.

Chapter 2: Valorisation of sweet corn (Zea mays) cob by extraction of valuable compounds.

(ii) to investigate the effect of extraction parameters (enzyme concentration, pH, and temperature) on the yield of ferulic acid from SCC.

Chapter 3: Optimization of enzyme assisted extraction of ferulic acid from sweet corn cob by response surface methodology

iii) to evaluate the possible prebiotic effect of SCC (containing fibres and insoluble phenolic compounds) and SCC extract (containing free phenolic compounds) on the human gut microbiota by batch culture fermentation.

Chapter 4: Influence of sweet corn cob on gut microbiota ecology

(iv) to produce ferulic acid-rich GF rice muffin incorporated with varying level of SCC flour and to evaluate the physicochemical properties of these muffins

Chapter 5: Physicochemical properties and ferulic acid content of muffin incorporated with sweet corn cob flour.

Chapter 2

Valorization of sweet corn (*Zea mays*) cob by extraction of valuable compounds

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The results from this chapter have been presented at:

(i) Nursten Symposium 2016, Reading, UK.

Award: Fourth runner up

(ii) SCI Young Researchers in Agri-Food 2016, Reading, UK.

Award: 1st Prize- flash poster presentation

(iii) Processing technologies for the recovery of added value components from food processing waste organized by FoodWasteNet, Reading, UK.

(iv) Food Chemistry conference 2016 – Poster presentation, Amsterdam

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Abstract

The main objective of this study was to investigate the proximate, mineral and phytochemical compositions of sweet corn cob (SCC), which is often neglected and regarded as agricultural waste. Compositional analysis showed that more than 60% of SCC was composed of insoluble dietary fibre, with cellulose being the major constituent. Results also showed that SCC can be a good source of non-essential protein and minerals (phosphorus, potassium and manganese). SCC had a total phenolic content of 6.74 g GAE kg^{-1} DW, of which bound phenolics were predominant. The bound phenolics fraction showed the highest antioxidant capacity in all three antioxidant capacity assays (TEAC, FRAP and DPPH) and contained the highest amount of ferulic and *p*-coumaric acid. The main carotenoids present in SCC were β-carotene, zeaxanthin and lutein. This investigation shows that SCC can be a promising source of natural colorant (carotenoids), antioxidants (phenolics) and nutritional supplements (proteins and phytochemicals).

Keywords: sweet corn cob; composition analysis; minerals; carotenoid; phenolic; supercritical fluid extraction; antioxidant activity; ferulic acid.

2.1 Introduction

Corn is one of the most important cereal crops globally (Shiferaw et al., 2011). Kim and Dale (2004) reported that the world annual production is about 520 x 10^9 kg and North America (42%), Asia (26%), Europe (12%) and South America (9%) are the main producers. Most sweet corns are processed into frozen corn kernels, canned corn kernels or corn cobettes, resulting in the production of large amounts of by products, which includes the corn silks, husks and cobs. For every 100kg of corn grain obtained, approximately 18kg of corncobs are produced, most of which is used as animal feed, or remain unused as lignocellulosic waste (Torre et al., 2008). Previous studies on corncobs have focused on the production of bioethanol (Chen et al., 2007), bio-oil and bio-char (Mullen et al., 2010), solid biofuel (Ioannidou et al., 2009) and xylooligosacharides (Yang et al., 2005)

As the world population increases, it is essential that alternative sources of nutrients and proteins are explored to overcome the world food shortage. Plant waste contains compounds that have the potential to be used as food ingredients or as nutritional supplements, examples of which are pectin from apple pomace or citrus peel, phenolic compounds from potato peels, as well as lycopene from tomatoes and other red fruits (Mirabella et al., 2014). Proximate and nutritional analysis of fruits and vegetables play an important role in assessing their nutritional significance. Mineral components such as potassium, calcium, sodium, magnesium, phosphorus, iodine and iron are crucial for human nutrition (Erkan and Özden, 2007). In recent years, there has been continuous research in the functional properties of plant phytochemicals. Amongst them, extensive research has focused on ferulic and *p*-coumaric acid as they are widely distributed in the plant kingdom and have been used as feedstocks for the production of vanillin, or as antioxidants in food preservation (Kumar and Pruthi, 2014). Various studies have found that SCC may also be a good source of phenolic compounds and different extraction approaches have been tested including alkaline hydrolysis (Ares et al., 2016,

Torre et al., 2008), ultrasonic extraction (Lai et al., 2012), surfactant-based cloud-point extraction (Dhamole et al., 2014) as well as enzymatic hydrolysis (Pérez-Rodríguez et al., 2017) showing the presence of ferulic and *p*-coumaric acid as well as carotenoids.

The main objective of this study was to investigate the proximate, minerals and phytochemical composition of SCC. In addition, the potential of supercritical fluid extraction to extract carotenoids from of SCC was also assessed.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Ferulic acid (>99%), *p*-coumaric acid (>99%), gallic acid (97.5%) and ascorbic acid were purchased from Sigma Aldrich. β-carotene (>98%), lutein (>95%) and zeaxanthin (>98%) were obtained from Extrasynthese Company, Genay, France. All other reagents and chemicals used in this experiment were of analytical grade.

2.2.2 Sample Preparation

The preliminary studies on the extraction of free phenolics (Section 2.2.4) were carried out using sweet corn purchased from Sainsbury Supermarket (Reading, UK) in January 2015. The SCC used for the rest of the experiments was harvested in Senegal in December 2015 and was provided by Barfoots of Botley Company Ltd (UK). Corn kernels were removed manually from the cobs and discarded. The SCC were then chopped into 5cm pieces in length, placed in the blast freezer (-18°C, 1 hour) and then freeze dried (Christ Gamma 2-16) until constant weight was achieved. The dried samples were finely ground in a mill (Apex Comminuting Mill), sieved through a 150 mesh screen (<0.1mm particle size), thoroughly mixed and stored in the freezer (-80°C) until further analysis.

2.2.3 Proximate composition and minerals analysis of SCC

Moisture content was determined by using the Mettler Toledo halogen moisture analyser (Model: HE73). Ground SCC was analysed for proximate composition by AOAC method for protein (979.09), lipid (963.15) and ash (923.03) (AOAC, 2005). Structural carbohydrates and lignin (Klason and acid-soluble) were determined by the NREL procedure (Sluiter et al., 2008). Starch content was determined by amyloglucosidase method using Megazyme total starch assay kit (Li et al., 2001). Analysis of free amino acids content was carried out in accordance to Elmore et al. (2005). Minerals including calcium, magnesium, iron, zinc, copper, manganese, sodium and potassium were extracted from the samples by dry ashing method and determined by atomic absorption spectrophotometer (novAA® 350) as described in AOAC 985.35 (AOAC, 2005). Phosphorus was determined spectrophotometrically as described in AOAC 995.11 (AOAC, 2005).

2.2.4 Optimisation of extraction time and solvent of free phenolic compounds in SCC

SCC powder (5g) was extracted with 50mL of solvent using a platform shaker (Heldolph Multi Reax) at 1200rpm at room temperature. The extracts were centrifuged at 5000rpm for 15 minutes (Sigma 3K10) and filtered prior to the total phenolic (Section 2.2.6) and antioxidant capacity (Section 2.2.8) assay.

Firstly, SCC powder was extracted using 80% methanol for 1, 2 or 3 hours. Secondly, two different solvent systems, namely ethanol and methanol were selected for the extraction of free phenolics from SCC using a series of extraction solvents of 10, 20, 50, 80 and 100% (%v/v; water/ethanol or methanol), with the optimized extraction time in the previous step.

2.2.5 Extraction of free, esterified and insoluble-bound phenolic compounds in SCC

The extraction of free, esterified and insoluble-bound phenolic compounds in SCC was carried out according to the method described by Sosulski et al. (1982). The free phenolic fraction was extracted based on the optimized conditions in Section 2.2.4.

2.2.6 Determination of total phenolic content

The phenolic content was determined using the Folin-Ciocalteu method as described by Singleton and Rossi (1965). The absorbance was measured at 760nm with a spectrophotometer (CE1021, Cecil), and the phenolic content was expressed as g gallic acid equivalents per kg of dry weight (g GAE/kg DW). The calibration curve was established using gallic acid (50-1000mg/L) as the standard sample (R^2= 0.9993).

2.2.7 HPLC analysis of phenolic compounds of SCC

The analysis of phenolic compounds was carried out using HP Agilent 1050 liquid chromatography, equipped with a DAD detector. The separation of the phenolic compounds was performed using a Zorbax SB-C18 column (2.1 x 15mm, 1.8 micron). The mobile phase was (A) formic acid/HPLC water (0.1:100 v/v) and (B) formic acid/acetonitrile (0.1/100 v/v). Solvent B was increased to 25% (0- 25 min), followed by 90% B for 30 minutes and then a final wash of 100% B for 10 minutes. The injection volume was 5µL with a flow rate of 0.2mL/min. Identification was carried out by comparing the retention time to the corresponding standards at 280nm and the amount of individual phenolic compounds (ferulic acid and *p*-coumaric acid) was calculated using an external calibration curve (R^2 = 0.9998 for both ferulic and *p*-coumaric acid).

2.2.8 Determination of antioxidant activities

The trolox equivalent antioxidant capacity (TEAC) assay and 2,2- diphenyl-1-picryhydrazyl (DPPH) radical assay were conducted as described by Li et al. (2008) and Zhao

et al. (2014), respectively. The standard curve for TEAC (R^2 = 0.987) and DPPH (R^2 = 0.989) assay were constructed using Trolox at different concentrations (50 – 1000µmol for TEAC and 1 – 170µmol for DPPH). The results were expressed as mmol trolox equivalent (TE)/kg sample.

The reducing ability of the extracts was determined using the FRAP assay according to Benzie and Strain (1996) with slight modifications. A total of 10µL of standard, blank (water) or sample was added to each well in a 96-well microtiter plate (Cellstar®). Then, 300µL of FRAP reagent was added and the absorbance of the reaction mixture was read at 595nm using a GENio Pro™ microplate reader with Magellan software. The standard curve (R^2= 0.9997) was constructed using ascorbic acid solution (50-1000µmol). Results were expressed as mmol ascorbic acid (AA)/kg DW.

2.2.9 Extraction and identification of carotenoid compounds

2.2.9.1 Conventional extraction

This extraction was based on the method described by Gorocica-Buenfil et al. (2007). The extracts were re-dissolved in ethanol and were stored at -18ºC until HPLC analysis.

2.2.9.2 Supercritical fluid extraction (SFE)

Supercritical fluid extraction was carried out in a SFE unit (SciMed, UK), according to Goto et al. (2015) with slight modification. SCC powder (5g) was loaded into the extraction vessel. The extraction pressure (350 bars) was controlled and the temperature was maintained at 60°C throughout the extraction. Carbon dioxide was fed into the extraction vessel at 15g/min. During the extraction (1 hour), a flow of 15% ethanol was pumped into the system to act as a co-solvent.

2.2.9.3 HPLC analysis of carotenoid compounds in SCC

The analysis of carotenoids present in SCC was performed using a YMC-C30 silica-based reversed-phase column (250 x 4.6 mm) coupled with a 1260 DAD detector (Agilent

Technologies, UK). The mobile phases were (A) methanol/MTBE/water (82:16:2 v/v/v) and (B) methanol/MTBE/water (23:75:2 v/v/v). The analysis followed a gradient program for the mobile phases of 0 min 0% B, 45 min 50% B, 55 min 100% B, 60 min 100% B. Identification was carried out at 450nm by comparing the retention time to the corresponding standards and external standard method was used to quantify the amount of individual carotenoids (lutein, zeaxanthin and β-carotene).

2.2.10 Statistical Analysis

Analyses were carried out in triplicate unless otherwise stated. Values shown in tables and graphs are presented as means ± standard deviation. The data was analysed by using Minitab statistical software (version 16.1.0). Differences among treatments were determined using a one way ANOVA and Fisher test. Differences were considered as significant, when $p \leq 0.05$.

2.3 Results and discussion

2.3.1 Proximate composition of SCC

Results of the proximate composition of SCC are presented in **Table 2.1**. Miura et al. (2004) reported a lower value of protein (5%), lipid (1%) and ash (2%) content of SCC harvested from Northeastern China. The variation might be due to differences in the source of corn cob as the chemical composition of crops has been seen to vary with climatic conditions, crop cultivar as well as with the soil of the area (Iqbal et al., 2006). More than 60% of the SCC was composed of insoluble dietary fibre, with cellulose being the major constituent, followed by hemicellulose, as previously reported by Miura et al. (2004), Awosusi et al. (2017) and Worasuwannarak et al. (2007). These results suggest that SCC can be a good source of insoluble dietary fibre.

To date, SCC has mainly been used as a carbohydrate source but it may also be a good source of protein and minerals. There is an increasing demand for more insight on the potential of plant-based protein (Iqbal et al., 2006). A total of 18 free amino acids were identified in SCC (**Table 2.1**) with serine and glutamine as the predominant ones followed by alanine, proline, aspartic and glutamic acid. Overall, the free amino acid content accounted for 0.7% of the composition in SCC, although free arginine was not measured. SCC that is high in non-essential amino acids can be used to compliment other plant protein sources to increase the overall protein quality of the mixture. Young and Pellett (1994) reported an improvement on the protein quality of corn and soy flour, when used in combination.

The total mineral content measured in this study was 2.18%, which correlated well with the ash content value (3.01%) in SCC. Phosphorus (10.12±0.06mg g^{-1} DW) was the most abundant mineral, followed by potassium (9.62±0.21mg g^{-1} DW) and magnesium (1.67±0.10mg g^{-1} DW). Calcium, lead, zinc, manganese, copper and iron were also present in SCC but in lower amounts. The quantity of all minerals were higher in the SCC analysed in the present study compared to those reported previously by Abubakar et al. (2016), Anukam et al. (2017) and Awosusi et al. (2017). The minerals and trace elements content may vary in plants due to the influence of environmental conditions such as presence of light, water availability elevated CO_2, elevated ozone levels and agricultural technologies (Nour et al., 2014).

Table 2.1: Composition, minerals and amino acid content analysis of sweet corn cob.

Component (%w/w based on dry matter)[ab]	
Protein	6.70 ± 0.06
Lipid	7.18 ± 0.08
Ash	3.04 ± 0.05
Lignin	
Klason Lignin	1.03 ± 0.00
Acid Soluble lignin	3.08 ± 0.00
Cellulose	40.40 ± 1.73
Hemicellulose	
Galactose + Xylose + Mannose[c]	19.12 ± 0.80
Arabinose[c]	4.45 ± 0.06
Starch	3.21 ± 0.08
Minerals content (mg g^{-1} DW)[ab]	
Iron	0.01±0.00[d]
Copper	0.01±0.00[d]
Zinc	0.04±0.00[d]
Lead	0.07±0.00[d]
Calcium	0.21±0.06[d]
Magnesium	1.67±0.10[c]
Potassium	9.62±0.21[b]
Phosphorus	10.12±0.06[a]
Manganase	0.08±0.12[d]

Amino acid composition (mg g^{-1} DW)[ab]	
Non-essential amino acids :	
Serine	1.36 ± 0.13[a]
Glutamine	1.16 ± 0.24[a]
Glutamic	1.09 ± 0.08[b]
Alanine	0.90 ± 0.06[c]
Aspartic	0.86 ± 0.09[c]
Proline	0.69 ± 0.06[d]
Asparagine	0.35 ± 0.07[e]
Tyrosine	0.08 ± 0.04[fg]
Glycine	0.04 ± 0.01[fg]
Ornitine	0.04 ± 0.00[fg]
Essential amino acids :	
Lysine	0.15 ± 0.02[f]
Tryptophan	0.13 ± 0.01[fg]
Histidine	0.12 ± 0.00[fg]
Threonine	0.09 ± 0.02[fg]
Leucine	0.07 ± 0.01[fg]
Phenylalanine	0.05 ± 0.01[fg]
Valine	0.05 ± 0.01[fg]
Isoleucine	0.02 ± 0.01[g]

[a] *All content based on the freeze-dried sweet corn cob*

[b] *Values presented as mean ± standard deviation*

[c] *Presented as polymers, contributing to hemicellulose content*

2.3.2 Phenolic composition and antioxidant activity of SCC

2.3.2.1 Optimisation of extraction of free phenolic compounds in SCC

Hydroalcoholic solutions have been widely used in the extraction of phenolic compounds. In this research, extraction of free phenolics from SCC using methanol and ethanol as extraction solvents were compared. Extraction time (one to three hours) had no significant effect (p>0.05) on the total phenolic content and FRAP of SCC extract (data not shown). By taking into consideration the economic and practical aspects as well as optimising the recovery of phenolic compounds and antioxidant capacity, one hour extraction time was chosen as the best extraction time for free phenolic and antioxidant compounds from SCC.

Ethanol extracts showed the highest total phenolic content and FRAP (**Figure 2.1A and B**) compared to methanol extracts. The total phenolic content and FRAP content increased when the concentration of ethanol increased from 0% to 50%, and decreased from 80% to 100%. Methanol extracts of SCC showed the same pattern in both total phenolic content and FRAP assay to the ethanol extracts. The mixture of water and ethanol are commonly used for the extraction of plant phenolic due to the wider range of phenolic constituents that can dissolve in the aqueous ethanol mixtures as compared to mono component solvent system (Alothman et al., 2009). Considering the yield, safety, economic and practical advantages in using ethanol as an extraction solvent, concentration of 50% ethanol for 1 hour were chosen as the best solvent and time conditions for the extraction of free phenolics from SCC.

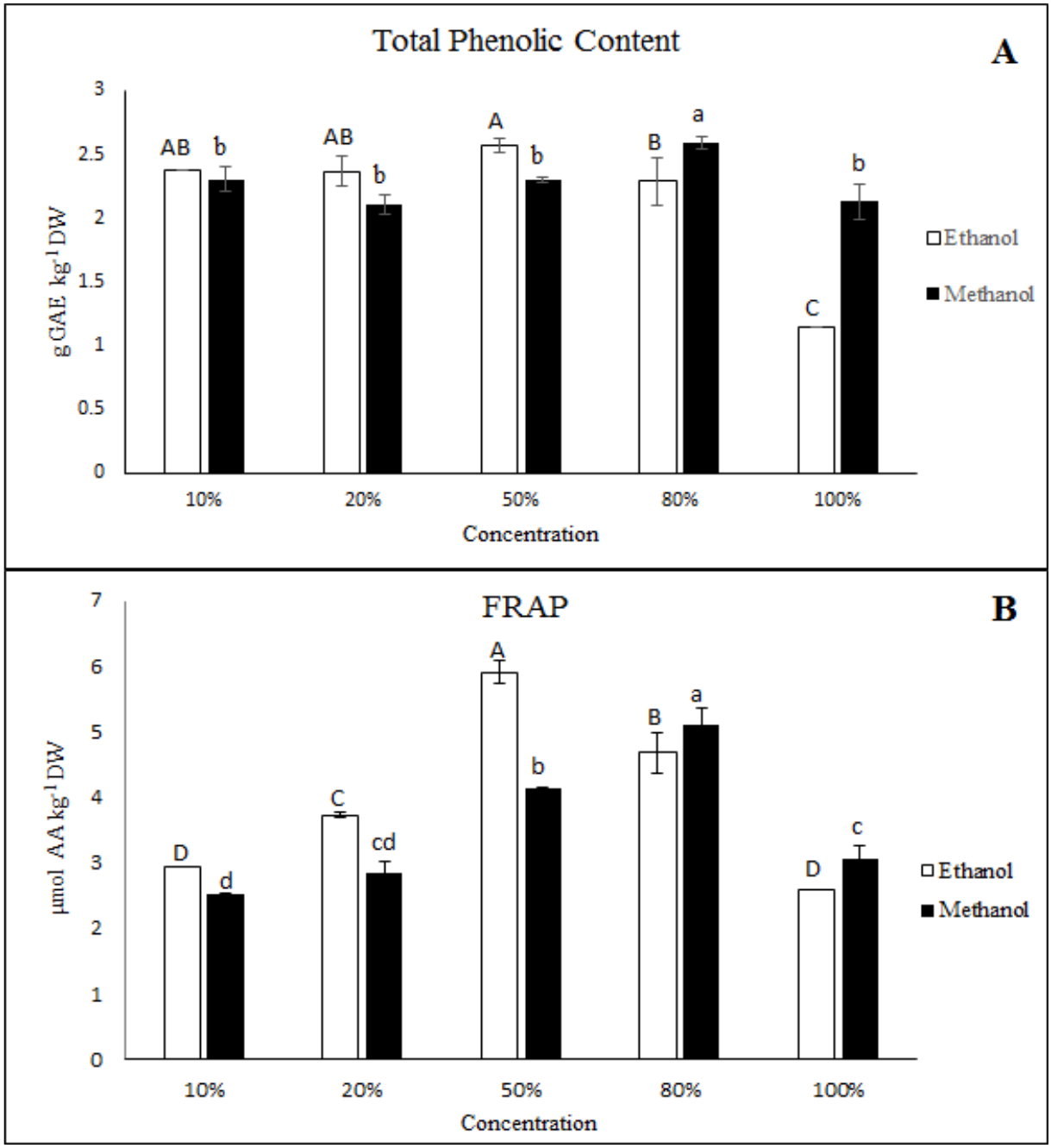

Figure 2.1: Effect of different concentration of ethanol and methanol on (A) total phenolic content and (B) ferric reducing antioxidant power (FRAP) of sweet corn cob.

Values are presented in means ± standard deviation of duplicate samples; concentration of same solvent with different letters are significant different ($p<0.05$).

2.3.2.2 Total Phenolic content and antioxidant activity of SCC

Phenolic compounds in SCC were present in free, esterified and insoluble-bound forms (**Figure 2.2A**). The highest level of phenolic compounds were present in the insoluble-bound form (5.41 ± 0.27 g GAE kg^{-1} of sample) followed by the free phenolic (0.9g ± 0.08 GAE kg^{-1}) and esterified phenolic (0.43g ± 0.05 GAE kg^{-1}) fractions. **Table 2.2** showed that in all antioxidant assays, the highest activity was found in the insoluble bound fraction. The overall relationship between antioxidant activity and total phenolic content of SCC (**Table 2.3**) was a positive and highly significant correlation ($p \leqslant 0.01$), suggesting that the phenolic compounds are the most important contributors towards the antioxidant activity of SCC.

Table 2.2: Trolox equivalent antioxidant capacity (TEAC), DPPH radical scavenging capacity, ferric reducing antioxidant potential (FRAP) of free, esterified and insoluble bound fractions of sweet corn cob

Fractions	**TEAC (mmol TE kg^{-1})**	**DPPH (mmol TE kg^{-1})**	**FRAP (mmol AA kg^{-1})**
Free phenolics	9.54 ± 1.03^{b}	1.74 ± 0.13^{b}	1.83 ± 0.21^{b}
Esterified phenolics	4.69 ± 0.59^{b}	0.43 ± 0.18^{c}	1.07 ± 0.11^{b}
Insoluble-bound phenolics	131.23 ± 23.87^{a}	3.68 ± 0.30^{a}	10.86 ± 0.56^{a}

**Values are presented in means ± standard deviation of triplicate samples; mean values within the same column with different letters are significantly different ($p<0.05$).*

Table 2.3: Pearson's correlation coefficient between total phenolic content (TPC), trolox equivalent antioxidant capacity (TEAC), DPPH radical scavenging capacity, ferric reducing antioxidant potential (FRAP), ferulic acid and p-coumaric acid content of sweet corn cob[a].

	TPC	**TEAC**	**DPPH**	**FRAP**
ABTS	0.981			
DPPH	0.936	0.885		
FRAP	0.993	0.981	0.932	
Ferulic acid content	0.995	0.984	0.900	0.990
***p*-coumaric acid content**	0.996	0.984	0.902	0.990

[a] *95% confidence level*

**All correlations are significant at $p \leq 0.01$ level*

2.3.2.3 Quantification of ferulic and *p*-coumaric acid in SCC

The total amount of ferulic and *p*-coumaric acid present in all three fractions of SCC (**Figure 2.2B**) was 3.06 ± 0.19 and 4.23 ± 0.25g kg^{-1} DW, respectively. Ferulic and *p*-coumaric acid in the insoluble-bound phenolic fraction of SCC was highest, with 2.96 and 4.08 g kg^{-1}, respectively. Free and esterified phenolic fractions had significantly lower ($p<0.05$) ferulic and *p*-coumaric acid content as compared to the insoluble-bound fraction. Strong correlation was found between ferulic and *p*-coumaric acid content of SCC for all three antioxidant assays (**Table 2.3**) carried out in this study. This showed that both ferulic and *p*-coumaric acid content contributed towards the antioxidant activity of SCC. Dewanto et al. (2002) reported that the presence of ferulic acid in sweet corn kernels was highest in the insoluble-bound fraction (4.2g $100g^{-1}$), followed by soluble conjugated fraction (0.096g $100g^{-1}$) and free fraction (0.0105g $100g^{-1}$). Furthermore, previous investigations have shown that the ferulic acid and *p*-coumaric acid content in yellow corn grain ranged from 0.006 to 1.80g kg^{-1} and 0.00012 to 0.00050 g kg^{-}

[1], respectively (Assabgui et al., 1993, Hu and Xu, 2011). This research showed that all three phenolic fractions contain a higher amount of ferulic acid as compared to sweet corn kernels suggesting that SCC can be a good source ferulic acid and *p*-coumaric acid.

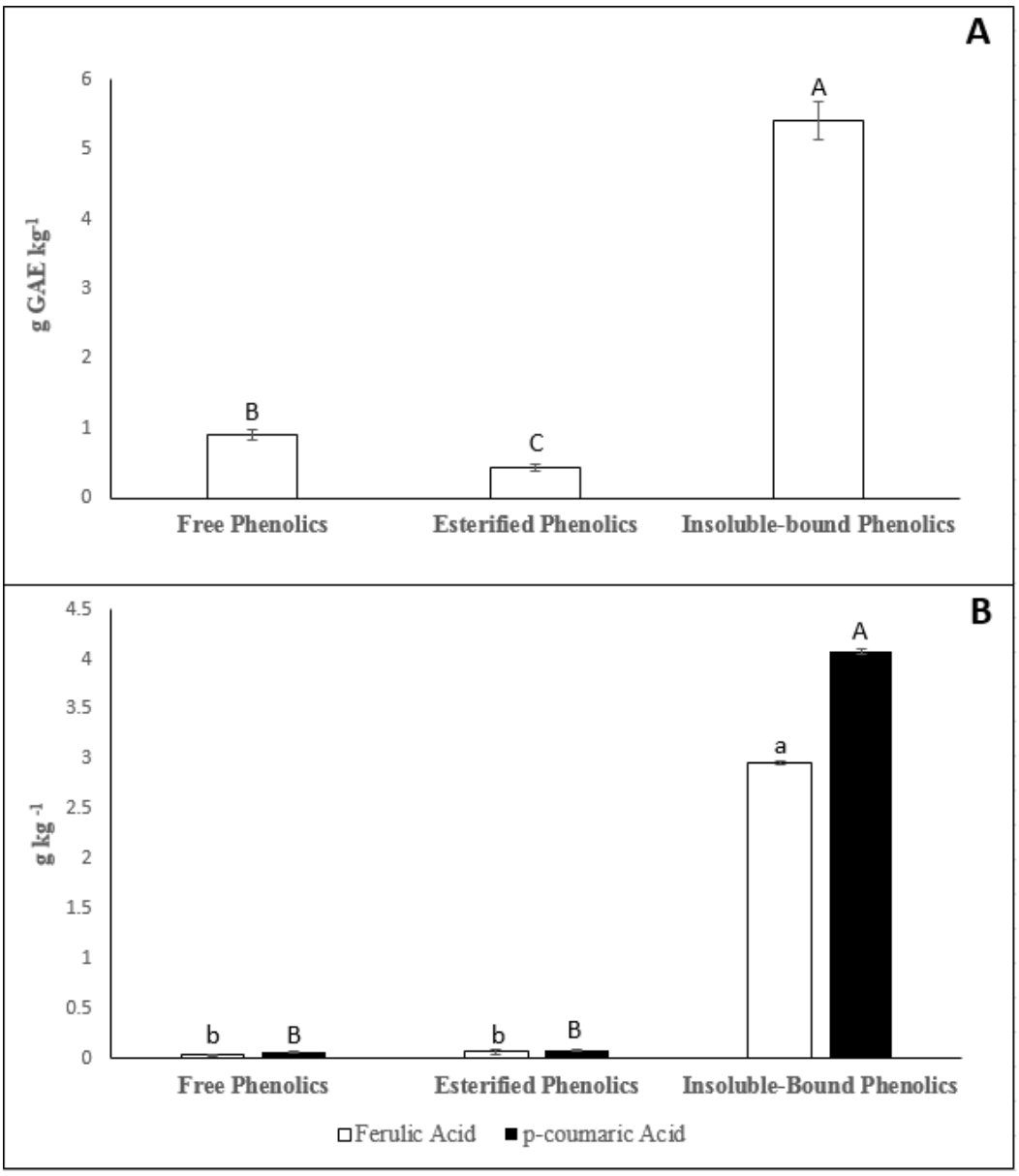

Figure 2.2: Total phenolic content (A) and amount of ferulic and p-coumaric acid (B) in free, esterified and insoluble-bound fractions of sweet corn cob.

Values are presented in means ± standard deviation of triplicate samples; fractions with different letters within the same phenolic compound are significantly different ($p<0.05$).

2.3.3 Extraction and characterization of carotenoid compounds

In this study, lutein, zeaxanthin and β-carotene were the three carotenoids identified and quantified in SCC (**Table 2.4**). β-carotene was the main carotenoid in SCC followed by zeaxanthin and lutein. Supercritical fluid extraction (SFE) was able to extract high amounts of carotenoids, as compared to conventional extraction. No correlation was found between carotenoid content and antioxidant activity in all three assays (data not shown) thus the contribution of carotenoid content in SCC towards antioxidant activity was assumed negligible. Similarly, Gil et al. (2002) found no correlation between carotenoid and antioxidant activity of nectarines, peaches and plum.

Previous studies carried out by Kurilich and Juvik (1999) reported that the carotenoids present in sweet corn kernels included zeaxanthin (2.16 mg kg^{-1}), lutein (5.95 mg kg^{-1}), and β-carotene (0.68mg kg^{-1}). Our results showed that there were higher levels of zeaxanthin and β-carotene content in SCC, compared to those reported for sweet corn kernels. Therefore, SCC could potentially be a relatively good source of carotenoid compounds as compared to sweet corn.

In this study, two different extraction methods were compared (**Table 2.4**), the SFE and conventional method. SFE resulted in significantly higher levels of carotenoids than conventional extraction. This confirms the effectiveness of the SFE techniques in the extraction of carotenoid compounds in SCC. It is well-known that carotenoids are highly sensitive to light, air, heat and pH (Panfili et al., 2004). SFE allowed the extraction of carotenoid compounds without exposure to lights and air, as the extraction is carried out in an air-tight and closed chamber. The content of lutein and zeaxanthin in SCC was twice as high in SFE extraction, as compared to conventional extraction. Furthermore, the content of β-carotene was three times higher in SFE extraction. This could be due to the higher sensitivity of β-carotene towards oxidation (Vanhasselt, 1972), as compared to lutein and zeaxanthin. In addition, the

combination of low viscosity and high diffusivity of supercritical fluid can enhance the penetration into porous solid material and consequently, result in faster and more effective extraction (Lang and Wai, 2001).

Table 2.4: Concentration of individual carotenoid compound present in sweet corn cob extracted by conventional extraction and supercritical fluid extraction (mg kg^{-1} DW).

Extraction Method	Lutein	Zeaxanthin	β-carotene
Conventional	1.67 ± 0.11^{b}	3.98 ± 0.43^{b}	49.35 ± 3.60^{b}
Supercritical fluid	3.81 ± 0.02^{a}	8.47 ± 0.09^{a}	177.29 ± 4.35^{a}

**Values are presented in means ± standard deviation of duplicate samples; mean values within the same carotenoid compound with different letters are significantly different ($p<0.05$).*

In conclusion, this research has shown that SCC appears to be a promising source of natural colorant (carotenoids), antioxidants (phenolics) and nutritional supplements (proteins and phytochemicals). The knowledge generated from this study may be useful to explore the use of agricultural waste as a source of functional food or value added products.

Chapter 3

Optimisation of enzyme assisted extraction of ferulic acid from sweet corn cob by response surface methodology

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The results from this chapter have been presented at:

(i) Nursten Symposium 2017, Belfast, Northern Ireland.

Award: Commendation award

(ii) Valorisation of Grain, Cereal and Bakery Waste organized by FoodWasteNet, Reading, UK.

(iii) Nursten Symposium 2018, University of Nottingham

Award: Commendation award

This chapter is written in paper publication format and intended to submit to *Process Biochemistry* for publication.

Abstract

Sweet corn cob (SCC), an agricultural by-product of the corn processing industry, contains more than 80% of insoluble-bound ferulic acid (FA). Extraction of these bound phenolics can be achieved through chemical or enzymatic hydrolysis. The shift towards greener chemistry has raised awareness towards the use of enzymatic hydrolysis, which offers several advantages on the environmental impact such as low energy and solvent consumption. The ability of ferulic acid esterase (FAE) and xylanase (XY) to catalyse the hydrolysis of FA from SCC was investigated in this study. Response surface methodology (RSM) based on a five-level, four-factor central composite rotatable design (CCRD) was used to establish the optimum conditions for enzymatic hydrolysis of FA from SCC. SCC was treated with the combination of FAE and XY at various concentrations (FAE: 0.00 to 0.04 U/g; XY: 0.00 to 18093.5 U/g), temperatures (45 to 65°C) and pH (pH 4.5 to 6.5). The optimum extraction conditions were: FAE concentration of 0.02U/g, XY concentration of 3475.3 U/g, extraction pH of 4.5 and extraction temperature of 45°C. Under these conditions, the experimental yield of FA was 1.69 ± 0.02mg/g of SCC, which is in agreement with the value predicted by the model.

Keywords

Sweet corn cob, response surface methodology, ferulic acid, ferulic acid esterase, xylanase

Highlight

- RSM was used to optimise the extraction of FA from SCC.
- Optimum extraction conditions were FAE concentration at 0.02U/g, XY concentration at 3475.4 U/g, pH 4.5 and 45°C.
- The yield of FA was 1.69 ± 0.02mg/g of SCC at optimum extraction condition.

3.1 Introduction

The annual world production of corn is about 520 Tg with most of the corn being used for animal feed or human consumption (64 and 19% of global production, respectively) (Kim and Dale, 2004). Sweet corn cobs (SCC) are an agricultural by-product of the corn-processing industry. Zheng et al. (2014) reported that the average yield of corn cob is about 14% of grain yield, which accounts for up to 16% of the total corn stover in a field. Utilization of corncob as animal beddings (Leys et al., 2012), biological substrate for the production of furfural (Sánchez et al., 2013), carbon adsorbents (Tsai et al., 2001) and forage protein (Perotti and Molina, 1988) have been widely studied. In addition, research on corn cob as a source of ferulic acid (FA) has notably increased in recent years (Kumar and Pruthi, 2014). Based on our previous findings using alkaline hydrolysis, 97% of FA was present as insoluble bound (Chapter 2). The insoluble bound FA is covalently bound to the polysaccharide components of plants through ester linkages and these crosslinks significantly limit the degradation of the cell wall by rumen microorganism, thus limiting the digestibility by ruminants (Yu et al., 2002).

Extraction of FA has been carried out via alkaline (Buranov and Mazza, 2009), acidic (Xu et al., 2005), pressurised solvents (Li et al., 2006), ultrasonic (Sun and Wang, 2008), supercritical CO_2 (Sun et al., 2006), microwave-assisted (Liu et al., 2006) and enzymatic (Mussatto et al., 2007) extraction. Commonly, the release of FA from SCC has been carried out using alkali hydrolysis (Torre et al., 2008, Ares et al., 2016), however, this conventional method of hydrolysis has several disadvantages including the usage of large amount of solvent and subsequent solvent disposal problems (Alinia et al., 2010), leading to an increase in environmental pollution. In this context, enzymatic hydrolysis has drawn great interest due to its lower environmental impact as the use of chemicals is negligible and requires low energy (Alvira et al., 2010). In Chapter 2, alkali hydrolysis of SCC showed that SCC contains 3.06mg g^{-1} of total FA. Recently, Pérez-Rodríguez et al. (2017) investigated the utilization of high

hydrostatic pressure along with feruloly esterase on the release of FA from corn cob. Ferulic acid esterase (FAE) was reported to break the ester linkage between FA and the attached sugar, thus releasing the FA from the complex cell wall (Yu et al., 2002). However, a specific cell wall degrading enzyme such as xylanase could be used to further improve the extraction by solubilizing part of the cell wall structure forming low molecular weight ferulolyted compounds, to allow FAE to act on these low relative molecular weight ferulolyted compounds releasing the FA (Faulds and Williamson, 1995b).

However, the main drawback of using enzymatic hydrolysis is the low hydrolysis rate compare to chemical hydrolysis. To overcome this, several physicochemical factors such as incubation temperature, incubation time, enzyme concentration and pH need to be considered prior to enzymatic hydrolysis (Yin et al., 2011). The conventional optimisation involves changing one independent variable at a time while keeping the rest of the factors constant. However, this conventional experimental design does not include interaction among the variables and therefore is often incapable of detecting the optimum conditions (Tanyildizi et al., 2005). In order to overcome this problem, response surface methodology (RSM) can be used to carry out optimization studies (Bezerra et al., 2008). RSM generates a mathematical model based on the linear, quadratic and interaction effect of variables, and it is then use to calculate the optimal response. RSM is less laborious and time-consuming than conventional optimisation method as it reduces the number of experimental trials needed to evaluate the effect of multiple parameters and their interaction (Yin et al., 2011).

This research aimed to investigate the effect of extraction parameters (enzyme concentration of FAE and XY, pH, and temperature) on the yield of FA from sweet corn cob. RSM optimisation by central composite rotatable design (CCRD) was used for model fitting and to predict the optimum value.

3.2 Materials and method

3.2.1 Materials

Sweet corn cob (SCC) used in this study was harvested in Senegal in December 2015 and was kindly provided by Barfoots of Botley Company Ltd (West Sussex, United Kingdom). Ferulic acid esterase (FAE) by *Clostridium thermocellum* and endo-1,4-β-xylanase (XY) by *Trichoderma viride* were purchased from Prozomix Limited (Northumberland, United Kingdom) and Megazyme International Ireland Limited (Bray, Ireland). All other chemicals used in this experiment were of analytical grade.

3.2.2 Sample preparation

The corn kernels were removed manually from the cob and discarded. The sweet corn cobs were then chopped into 5cm pieces in length, frozen in the blast freezer (-18°C) for an hour and then freeze dried (Christ Gamma 2-16) until constant weight was achieved. The dried samples were finely ground in a mill (Apex Comminuting Mill), sieved through a 150 mesh screen (particle size <0.1mm), thoroughly mixed and stored in the freezer (-80°C) until further analysis.

3.2.3 Enzyme activity test

Enzyme activity assays were performed at 45°C in sodium phosphate buffer at pH 4.5. One unit of enzyme was defined as the amount of enzyme used to release 1μmol of product per minute. Ferulic acid esterase was assayed with methyl ferulate as the substrate as previously described in Kroon et al. (2000). The amount of FA that was released was analysed by using HPLC as describe in Section 2.2.7. The activity of *Trichorderma viride* xylanase was assayed using beech wood arabinoxylan (1mg/mL) as the substrate. Xylanase activity was determined by measuring the release of reducing sugar by 3, 5-Dinitrosalicylic acid (DNS) reagent (Saqib and Whitney, 2011), and was expressed as xylose equivalent. Briefly, DNS reagent was

prepared using dinitrosalicylic acid (DNS), sodium potassium tartrate and sodium hydroxide. 4mL of DNS reagent was added to 1mL of test sample and placed in boiling water for 5 minutes, before measuring the absorbance at 540nm.

3.2.4 Preliminary work: determination of independent variables and their levels

Preliminary experiments were conducted to select a suitable range of temperature, pH and time of FAE and XY for the design of the experimental RSM run. First, the concentration of FAE was determined by hydrolysing the freeze dried SCC (5%) using various concentrations of FAE (0.02, 0.05, 0.19U/g of SCC) for 4 hours at 37°C and pH 6.5 (optimum temperature and pH from manufacturer). FAE concentration at 0.02U/g of SCC showed the highest amount of FA being released (**Appendix I, Figure A1**). The concentration of enzyme used in this research is lower than the amount reported by Pérez-Rodríguez et al. (2017) (0.044U of FAE per gram of dry milled corn cob). Then, hydrolysis of ferulic acid from freeze dried SCC (0.1g) using FAE (0.02U/g) was carried out at different pH (pH 4, 5, 6, 7) and temperature (20, 35, 40, 50, 60°C) for an hour to obtain the optimum pH and temperature of FAE. FA content was then quantified using HPLC (section 2.2.7). Similarly, the end product of XY hydrolysis (878.9 U/g of SCC) at different pH and temperature, xylose, was analysed using the DNS method as describe in Section 3.2.3.

Adopting the best working temperature (55°C) and pH (5.5) for both FAE and XY, the combination of FAE:XY at different ratios (1:0, 1:1, 1:10, 1:100, 1:1000, 1:10000, 2:0, 2:1, 2:10, 2:100, 2:1000 and 2:10000 U/U) was used to determine the best concentration for the maximum release of FA from SCC. The combination of FAE:XY at 1:10000 was found to release the maximum amount of FA (**Appendix I, Figure A2**). Finally, FAE at 0.02U/g with XY at (9048.5U/g) was used to hydrolyse 0.1g of SCC at 55°C and pH 5.5 at various extraction time (1 to 24 hours) to determine the best extraction time for the release of FA from SCC.

Based on the results, the three levels (lower, middle, upper) of each variable were determined and selected for RSM.

3.2.5 Enzymatic hydrolysis

Five grams of freeze dried SCC powder were defatted in a Soxhlet apparatus with hexane for six hours before the hydrolysis. For each experiment, a mixture of defatted SCC (0.1g) with varying amounts of phosphate citrate buffer, FAE (0.00 to 0.04U/g) and XY enzymes (0.00 to 18093.50 U/g) was used as shown in **Table 3.1**. The mixtures were stirred in a shaking water bath at different reaction temperatures (45 to 65°C), for three hours. The pH of the mixtures was varied from pH 4.5 to 6.5. The range of enzymes, pH and incubation temperature were determined based on the preliminary experiments. After the reaction was completed, the enzyme was inactivated by placing the mixture in a water bath at 90°C for five minutes. The suspension was centrifuged at 12,500 rpm for ten minutes and the supernatant was collected. FA in the supernatant was extracted 6 times using diethyl ether at a supernatant-to-solvent ratio of 1:1 and was evaporated to dryness. The extract containing the FA was then re-dissolved in methanol prior to HPLC analysis.

3.2.6 HPLC analysis of FA

The quantification of FA was carried out according to the method as described in Chapter 2 (Section 2.2.7). Detection at 280nm was used for the quantification of FA using an external calibration curve (concentration from 0.01 to 0.2 mg/g FA; $R_2 = 0.9998$).

3.2.7 Experimental design

RSM was used to determine the optimum conditions for the enzymatic hydrolysis of FA from SCC powder. After determining the preliminary range of the extraction variables, a five-level-four-factor central composite rotatable design (CCRD) with 31 experiments was employed in this study (**Table 3.2**). The experimental design and statistical analysis were performed using

Minitab® software 17.1.0. The variables optimised were concentration of ferulic acid esterase (X_1), concentration of xylanase (X_2), pH (X_3) and temperature (X_4). The design consisted of sixteen factorial points, eight axial points and seven replicates of the centre point. The 31 experiments were randomised and the response (yield of FA) was recorded in **Table 3.2**. Data from the CCRD was analysed by multiple regression to fit the quadratic polynomial model. The analysis of variance and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. *p*-values of less than 0.05 were considered to be statistically significant.

Verification and validation of the model were conducted by running three additional confirmation experiments using the optimum conditions generated by the RSM. The experimental and predicted values were compared and tested for statistical differences.

Table 3.1: Variables and their levels for central composite rotatable design

Variables	**Levels**				
	-α	**-1**	**0**	**+1**	**+α**
Ferulic acid esterase concentration/X_1 (U/g)	0.00	0.01	0.02	0.03	0.04
Xylanase concentration/ X_2 (U/g)	0.00	4526.00	9048.20	13571.00	18093.50
pH/ X_3	4.5	5.0	5.5	6.0	6.5
Temperature/ X_4 (°C)	45	50	55	60	65

Table 3.2: Central composite rotatable design and response values for the yield of ferulic acid (mg/g)

Standard Order	Concentration of Ferulic acid esterase/X_1 (U/g)	Concentration of Xylanase/X_2 (U/g)	Extraction pH/X_3	Extraction Temperature/X_4 (^oC)	Ferulic acid yield (mg/g)	
					Experimental	Predicted
1	0.01	4526.00	5.00	50.00	1.20	1.14
2	0.03	4526.00	5.00	50.00	1.45	1.42
3	0.01	13571.00	5.00	50.00	0.85	0.78
4	0.03	13571.00	5.00	50.00	0.84	0.92
5	0.01	4526.00	6.00	50.00	1.02	0.94
6	0.03	4526.00	6.00	50.00	1.23	1.28
7	0.01	13571.00	6.00	50.00	0.85	0.83
8	0.03	13571.00	6.00	50.00	0.98	1.02
9	0.01	4526.00	5.00	60.00	0.91	0.83
10	0.03	4526.00	5.00	60.00	1.11	1.15
11	0.01	13571.00	5.00	60.00	0.73	0.73
12	0.03	13571.00	5.00	60.00	0.81	0.90
13	0.01	4526.00	6.00	60.00	0.62	0.57
14	0.03	4526.00	6.00	60.00	0.93	0.95
15	0.01	13571.00	6.00	60.00	0.79	0.73
16	0.03	13571.00	6.00	60.00	0.91	0.95

17	0.00	9048.50	5.50	55.00	0.00	0.46
18	0.04	9048.50	5.50	55.00	1.00	0.97
19	0.02	0.00	5.50	55.00	0.61	0.83
20	0.02	18093.50	5.50	55.00	0.52	0.48
21	0.02	9048.50	4.50	55.00	1.17	1.25
22	0.02	9048.50	6.50	55.00	1.03	1.10
23	0.02	9048.50	5.50	45.00	1.37	1.43
24	0.02	9048.50	5.50	65.00	1.03	1.05
25	0.02	9048.50	5.50	55.00	1.28	1.24
26	0.02	9048.50	5.50	55.00	1.19	1.24
27	0.02	9048.50	5.50	55.00	1.20	1.24
28	0.02	9048.50	5.50	55.00	1.27	1.24
29	0.02	9048.50	5.50	55.00	1.14	1.24
30	0.02	9048.50	5.50	55.00	1.28	1.24
31	0.02	9048.50	5.50	55.00	1.30	1.24

3.3 Results and Discussion

3.3.1 Preliminary determination of range of temperature, pH, FAE and XY in RSM

The efficiency of FAE and XY is indicated by the increasing yield of FA and xylose, respectively. For FAE, the extraction yield of FA increased with the increase of pH value (pH 4 to pH 6.5) and reached the maximum value (0.28 ± 0.00mg FA/g) at pH 6 (**Figure 3.1A**). Furthermore, the amount of FA released by FAE increased when the extraction temperature increased from 20 to 50^{0}C (**Figure 3.1B**), reaching the maximum yield (0.20 ± 0.03 mg FA/g) at 50^{0}C. Topakas et al. (2007) reported that microbial FAEs have a wide range of temperature and pH dependences, with optimal activities occurring between 30 to 60°C and pH 4-8 . Therefore, the FAE used in this experiment was working within its best temperature and pH range.

The yield of xylose by XY increased as pH increased from pH 3 to pH 5, and decreased as pH increased further to pH 7.5 (**Figure 3.1A**). In addition, the yield of xylose increased as temperature increased from 20°C to 60°C, with maximum yield of 13.61 ± 0.13mg XE/g (**Figure 3.1B**). The yield of xylose decreased rapidly as temperature increased to 70°C. Results from this research are in agreement with Polizeli et al. (2005), where they reported that the peak activity of endoxylanases generally falls between 40 and 80°C and between pH 4.0 and 6.5. Iyer and Ananthanarayan (2008) reported that several phenomenons are known to promote changes of the activity and spatial configuration of an enzyme such as pH, ionic strength, temperature, autolysis or chemical agents. They further reported that these physical denaturants can disrupt the hydrogen bond in the enzyme and results in aggregation or formation of highly disordered structure. Therefore, it was crucial to determine the best working pH and temperature for both FAE and XY used in the experiment.

Figure 3.1C shows that the yield of FA was markedly affected during the first 6 hours of hydrolysis. An increase in the incubation time for up to 24 hours did not increase the yield of FA. No significant differences were found between 3 hours (1.05 ± 0.00mg FA/g) and 6 hours (1.02 ± 0.03mg FA/g) of extraction, which might be due to the accumulation of products inhibiting the enzyme activity or the depletion of the substrates. In a study conducted by Frieden and Walter (1963) on product inhibition of enzyme, they reported that the products of almost all enzyme-catalysed reactions may act as suppressants when present in high enough concentrations relative to the enzyme and substrate. Consequently, a period of 3 hours was chosen and used throughout the experiments.

Therefore, when taking into consideration the best working temperature and pH conditions for both FAE and XY, 55°C and pH 5.5 were chosen as the middle point for RSM, along with 50°C and pH 5 for lower point, and 60°C and pH 5 for high point (**Table 3.1**).

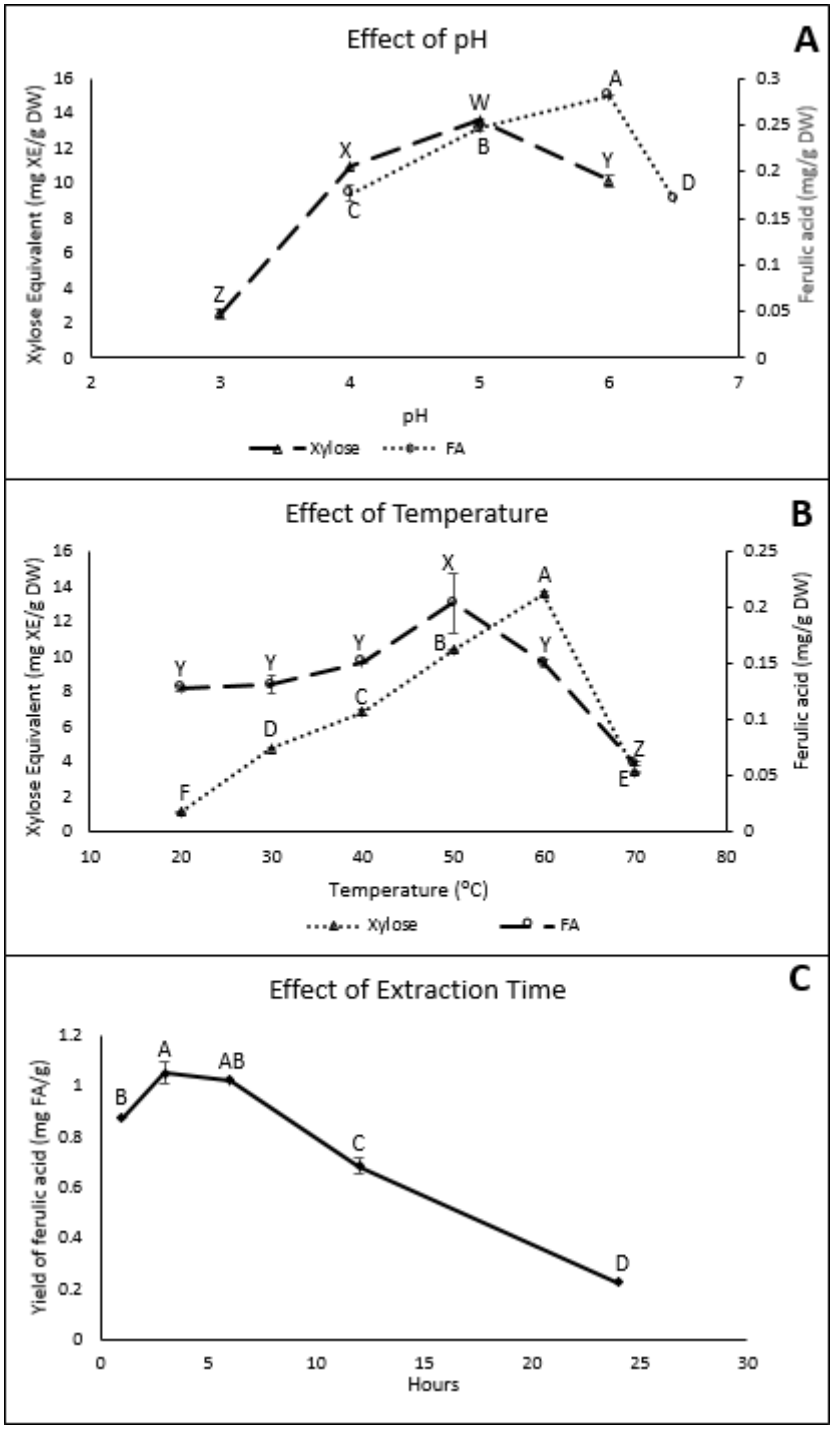

Figure 3.1: Effect of pH, temperature and time on enzymatic hydrolysis of ferulic acid from sweet corn cob using ferulic acid esterase (FAE) and xylanase (XY).

Different letters showed significant difference ($p<0.05$) between treatments.

3.3.2 Statistical Analysis and the model fitting

In this study, there were a total of 31 runs for optimizing the four individual parameters in the CCRD. The yield of FA along with the experimental conditions are shown in **Table 3.2**. Results showed that the yield of FA ranged from 0.00 to 1.45mg/g FA. The maximum amount of FA (1.45mg/g) was found in conditions of X_1=0.0285 U/g, X_2=4526U/g, X_3=5, X_4 = 50°C. The results were fitted with a second order polynomial equation:

$$\text{mg FA} = 3.53 + 65.1\,X_1 - 0.0002\,X_2 + 0.32\,X_3 - 0.082\,X_4 - 1838\,X_1^2 - 0.00X_2^2 - 0.0625\,X_3^2 + 0.000351\,X_4^2 - 0.001\,X_1X_2 + 3.34\,X_1X_3 + 0.173\,X_1X_4 + 0.000032\,X_2X_3 + 0.000003\,X_2X_4 - 0.001\,X_3X_4$$

The statistical significance of the regression model was evaluated by *p*-value and F-test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in **Table 3.3**. The determination coefficient (R^2=0.893) indicates that the model was adequate for prediction within the range of experimental variables. Table 3 showed that the linear coefficient (X_1) and the quadratic coefficient (X_1^2 and X_2^2) were found significant at p<0.001. Linear coefficient (X_2 and X_4) and interaction coefficient (X_2X_3) was found significant at p<0.01 and p<0.05. The other term coefficients (X_3, X_3^2, X_4^2, X_1X_3, X_1X_4, X_2X_4 and X_3X_4) were found not significant (p>0.05). Three-dimensional and contour plots were used to predict the relationships between the dependent and independent variables (**Figure 3.2** and **3.3**).

Table 3.3: Estimated regression model of relationship between response variables (yield of ferulic acid) and independent variables ferulic acid esterase (X_1), Xylanase (X_2), pH (X_3), and temperature (X_4).

Factor	SS	df	MS	F	*p*
X_1	0.45	1	0.45	25.36	*
X_1^2	0.79	1	0.79	44.17	*
X_2	0.15	1	0.15	8.37	**
X_2^2	0.64	1	0.64	35.83	*
X_3	0.03	1	0.03	1.57	NS
X_3^2	0.01	1	0.01	0.39	NS
X_4	0.22	1	0.22	12.50	**
X_4^2	0.00	1	0.00	0.12	NS
X_1X_2	0.03	1	0.03	1.53	NS
X_1X_3	0.00	1	0.00	0.23	NS
X_1X_4	0.00	1	0.00	0.06	NS
X_2X_3	0.08	1	0.08	4.65	***
X_2X_4	0.07	1	0.07	3.80	NS
X_3X_4	0.00	1	0.00	0.01	NS

**Significance at p≤0.001*

***Significance at p≤0.01*

****Significance at p≤0.05*

NS = not significant

3.3.3 Effect of ferulic acid esterase (X_1), xylanase (X_2), pH (X_3) and temperature (X_4) on the yield of FA

The effect of ferulic acid esterase (X_1) concentration, xylanase (X_2) concentration, pH (X_3), temperature (X_4) and their interactions on the extraction efficiency of FA from SCC are reported in **Table 3.3**. The yield of FA was positively correlated to the linear effect of FAE concentration ($p \leq 0.001$), XY concentration ($p \leq 0.01$) and temperature ($p \leq 0.01$). Concentration of FAE was highly significant ($p < 0.001$) in the release of FA from SCC. Similarly, concentration of xylanase (X_2) was found to be significant in this study. The presence of xylanase was reported to contribute towards the degradation of arabinoxylan, and thus enhance the release of FA. These endoxylanases attack the arabinoxylan backbone in an irregular manner, causing a decrease in the degree of polymerisation of the substrate and thus liberating the xylose, xylobiose and oligomers while retaining their configuration (Courtin and Delcour, 2002). This is in agreement with Yu et al. (2002) who also reported the release of FA from oat hulls using combinations of FAE and XY.

Response surface was plotted by using Minitab 17.0.1 to study the effects of parameters of interest and their interactions on the yield of FA. To visualise the effect of independent parameters and their interaction, three dimensional (**Figure 3.2**) and contour plot (**Figure 3.3**) were plotted to show the effects of two factors on the response at a time while keeping the other two factors at level zero. The three dimensional and contour plots in **Figure 3.2** and **3.3a**, which gives the yield of FA as a function of FAE and XY concentration at a fixed extraction pH (pH 5.5) and temperature (50°C), indicated that the extraction yield of FA increased as the concentration of FAE increased from 0.00 to 0.03U/g, followed by a decrease in the extraction yield of FA at FAE concentrations higher than 0.03U/g. Similarly, the yield of FA increased as the concentration of XY increased to 11,000U/g, and decreased as concentration of XY increased.

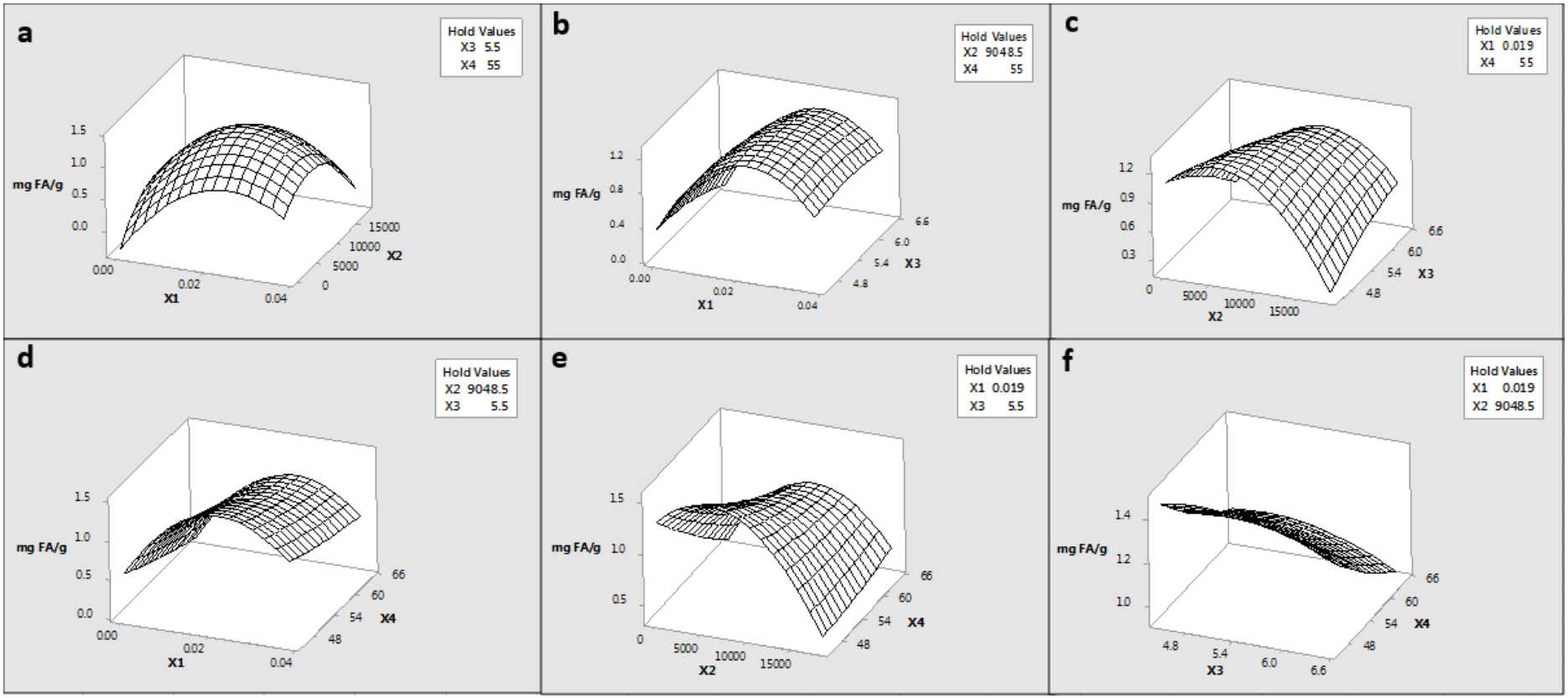

Figure 3.2: Response surface (3-D) showing the effect of FAE (X1), XY concentration (X2), pH (X3) and temperature (X4) on yield of ferulic acid.

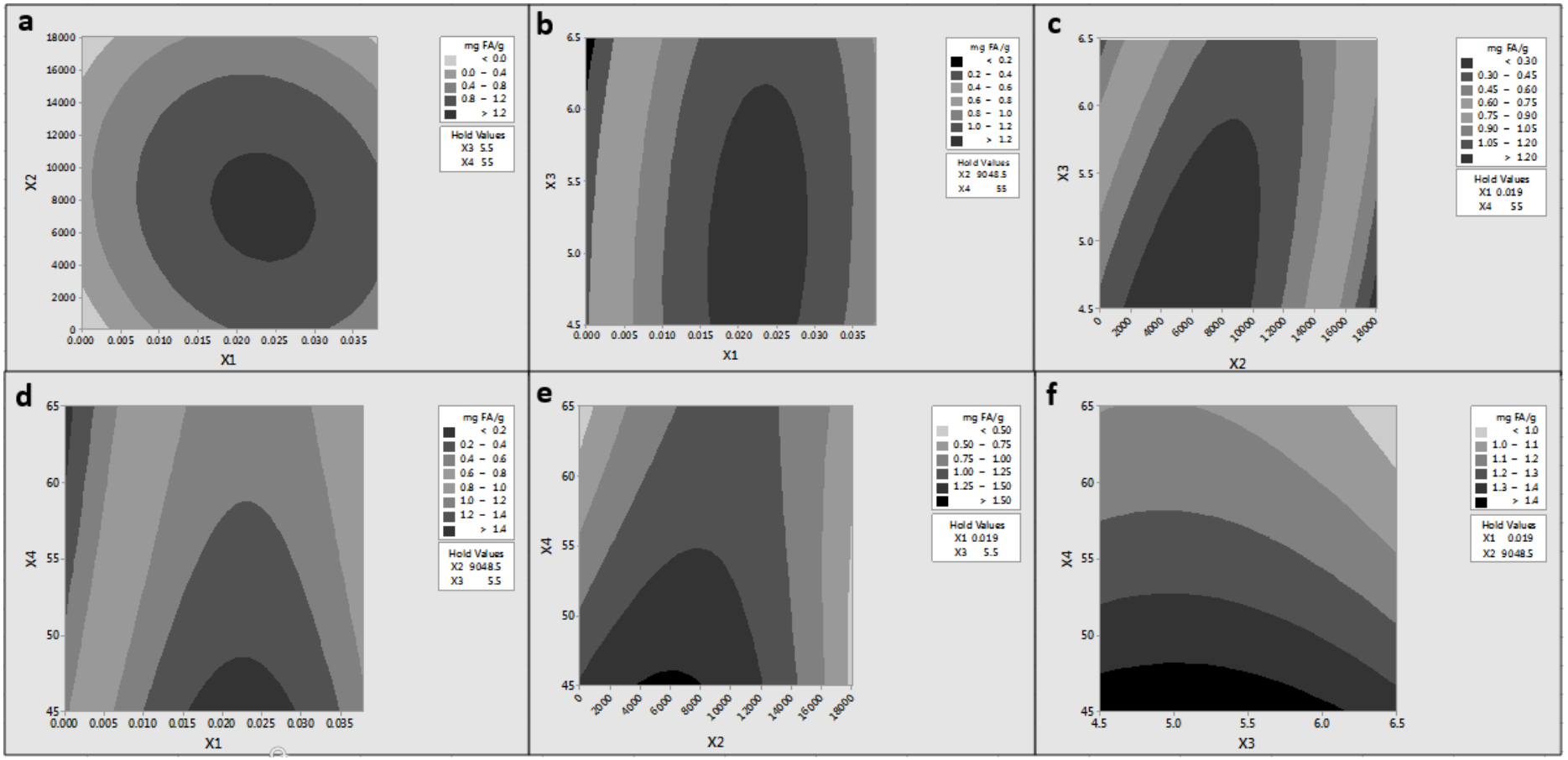

Figure 3.3: Contour plots showing the effect of FAE (X1), XY concentration (X2), pH (X3) and temperature (X4) on yield of ferulic acid

Figure 3.2 and 3.3b showed the three dimensional response and the contour plots at varying FAE concentration over a range of pH at a fixed XY concentration and temperature. It can be observed that the yield of FA increased as FAE increased to 0.08U/g, however, as pH increased from pH 6.0 to 6.5, the yield of FA decreased. In **Figure 3.2 and 3.3c**, the three dimensional response surface and the contour plots were developed for the extraction yield of FA with varying pH and XY concentration at a fixed FAE concentration and temperature. The plots indicated that the maximum extraction yield of FA can be achieved when concentration of XY increases to 10,000U, and decreases further at higher concentration of XY. It also showed that yield of ferulic acid decreases as pH increases from pH 6.0 to 6.5.

Figure 3.2 and 3.3d showed the three dimensional response surface plot and contour plot at varying temperature and FAE concentration at fixed extraction conditions of pH 5.5 and xylanase concentration. It can be seen that increasing FAE concentration increases the yield of FA, however, as temperature increases, the yield of FA decreases. It can be observed that the yield of FA by xylanase decreases as temperature increases above 45^0C (**Figure 3.2 and 3.3e**). However, no interactions were found between temperature and pH at a fixed amount of FAE and XY concentration (**Figure 3.2 and 3.3f)**

3.3.4 Verification of predictive model

The accuracy of the model equation for predicting the optimum response value was carried out under the condition: FAE concentration (0.02U/g), XY concentration (3472 U/g), pH (4.5) and temperature (45^{o}C). This set of optimum conditions was determined by the RSM optimization (**Table 3.4**) and was used to validate the experimental and predicted yields of the responses using the model equation. A mean value of 1.69 ± 0.02mg/g (n=3) was obtained from the experiment. This further validates the RSM model, showing that the model was adequate for the optimization of FA extraction from SCC.

Table 3.4: Predicted and experimental values of the responses at optimum condition

	FAE Concentration (U/g)	**XY Concentration (U/g)**	**pH**	**Temperature (°C)**	**Yield of ferulic acid (mg/g)**
Predicted	0.02	3475.32	4.50	45.00	1.70
Experimental	0.02	3475.32	4.50	45.00	1.69 ± 0.02

Pérez-Rodríguez et al. (2017) reported that the enzymatic hydrolysis of corn cob using Ultraflo®, in combination with thermal pre-treatment released a higher amount of FA (226mg/L) than the raw sample (177 mg/L). In this study, when comparing the FA content obtained by alkali hydrolysis (3.06mg/g of SCC, Chapter 2), enzymatic hydrolysis only extracted about half of the amount (55%) of FA in SCC (1.69mg/g of SCC). This result was lower than oat hull (69%) (Yu et al., 2002) and wheat bran (95%) (Faulds and Williamson, 1995b) but higher than that from maize bran (0.6%) (Faulds et al., 1995) and barley spent grain (30%) (Bartolome and Gomez-Cordoves, 1999). The discrepancy in the release of FA might be due to the complexity of the cell wall material (lignification), and also the physical and steric factors caused by branching of the arabinoxylan backbone (Yu et al., 2002). Faulds et al. (1995) reported that the highly branched xylose in the side chain of heteroxylan backbone of maize bran may hinder the action of endoxylanases, thus FAE can only act on those easily accessible regions. Therefore, less-substituted xylan substrate such as barley spent grain and wheat bran are better substrates for the release of FA by FAE, as compared to more substituted substrates such as maize bran (Bartolome and Gomez-Cordoves, 1999). Furthermore, wheat bran containing nonlignified cell walls is more susceptible to enzymatic degradation Yu et al. (2002) as compared to the highly ligninfied corn cob (Pastell et al., 2009) that is less susceptible to the esterase.

RSM is a useful tool in the optimization of the enzymatic hydrolysis of FA from sweet corn cob. The concentration of FAE, XY and temperature markedly affects the extraction efficiency of FA from sweet corn cob and thus, optimisation of these parameters is crucial to obtain the maximum yield of ferulic acid. Under the optimum condition, the yield of FA (1.69 ± 0.02mg/g) agreed closely with the predicted yield obtained from the model. However, enzymatic hydrolysis of SCC with the combination of FAE and XY does not release a high amount of FA as compared to alkali hydrolysis. Therefore, the combination of novel technologies with enzymatic hydrolysis may be explored to increase the yield of extraction for FA in SCC.

Chapter 4
Influence of sweet corn cob on gut microbiota ecology

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Abstract

The beneficial effects on the overall gut health following the consumption of phenolic-rich foods are potentially due to their modulation of gut microbiota. Hence, the effect of sweet corn cob (SCC) and sweet corn cob extract (SCCE) on several bacterial groups including *Lactobacillus, Bifidobacterium, Eubacterium* and *Clostridium* spp. were evaluated. The free and bound phenolic composition was determined by high performance liquid chromatography (HPLC) and results showed that SCC comprised mainly of insoluble bound phenolic acids (Chapter 2, ferulic and *p*-coumaric acid) while SCCE comprised mainly of free ferulic and *p*-coumaric acid, along with xylooligosaccharide (xylobiose and xylotriose). The SCCE showed a trend of an increase in the beneficial bifidobacteria, and a decrease in the pathogenic *Clostridium spp.*, although the results were not significant. On the other hand, SCC showed a significant increase in the production of short chain fatty acids (acetate, propionate and butyrate). This chapter provides information on the effect of free and bound ferulic acid on the population of gut microbiota and the potential health benefits of SCC and SCCE associated with improvement in gut health.

4.1 Introduction

Gut microbiota, a complex ecological community composed of trillions of microbes in the human intestine, plays an important role in health and physiology of the host. Malnutrition (Kau et al., 2011), obesity (Ley et al., 2006), inflammatory bowel diseases (IBD) (Frank et al., 2007) and cancer (Lupton, 2004) have been associated with an imbalance between gut microbiota and the host. The composition of the gut microbiota is greatly influenced by different factors including lifestyle, diet and use of antibiotics (Nicholson et al., 2012). Among all these factors, dietary habit is one of the main factors contributing to the diversity of the gut microbiota that can affect the functional relationship with the host (Laparra and Sanz, 2010).

There is an increasing interest in the health promoting effects of functional food components. Diplock et al. (1999) defined functional food as food components that are beyond adequate nutritional effect and showed beneficial effects towards one or more target functions in the body. Gibson et al. (2007) reported that some functional food components can influence the growth, composition, and function of gut microbiota. The functional food concept has moved towards the development of dietary supplementation that may affect the composition and activities of the gut microbiota (Ziemer and Gibson, 1998). Additionally, there has been increased interest in developing dietary supplements containing polyphenols or polyphenol-rich food products due to their potential to prevent or mitigate the occurrence of chronic diseases (Martin and Appel, 2010). Several studies have reported that polyphenols can also inhibit the growth of pathogenic bacteria and stimulate the growth of beneficial and commensal microbiota. For example, Lee et al. (2006a) reported that tea phenolics and their derivatives were able to suppress the growth of potentially pathogenic bacteria such as *Clostridium difficile*, *C. perfringens* and *Bacteroides* spp. while probiotics such as *Lactobacillus* spp. and commensal anaerobes including *Bifidobacterium* spp. were less affected.

Sweet corn cob (SCC), a by-product of corn processing industry, is comprised mainly of hemicellulose and cellulose (Chapter 2). Our findings have also showed that SCC contains considerable amount of phytochemicals including ferulic and *p*-coumaric acid. These phytochemicals are present mainly as insoluble-bound form and are covalently bound to cell wall structural components such as hemicellose, cellulose, pectins and lignins (Wong, 2006). Our study showed that more than 80% of ferulic and *p*-coumaric acid in SCC was present as insoluble bound (Chapter 2). It has been shown that the health benefits of insoluble bound phenolic compounds are more effective in the colon while the free and conjugated phenolics are more readily absorbed and distributed throughout the body for health benefits such as inhibition activities against oxidation of liposomes and LDL cholesterol (Acosta-Estrada et al., 2014). This is due to the low bioavailability of bound phenolic acids that can only be absorbed after being released by digestive enzymes in the intestinal lumen (Anson et al., 2009a), as compared to the free and conjugated phenolic acids, which are more readily available to be absorbed in the human small and large intestine (Zhao et al., 2004). The bran matrix of these bound phenolics hinders their access to enzymes such as ferulate esterases and xylanase, thus limiting its release in the human gastrointestinal tract (Zhao et al., 2005).

Although there are many studies on the effect of dietary polyphenols on the gut microbiota, limited studies have been carried out to compare the effect of free and bound phenolics, particularly ferulic acid, on gut health. Gálvez Ranilla et al. (2017) reported that the free and bound phenolic compounds of Peruvian purple corn did not inhibit the growth of beneficial probiotic such as *L. helveticus* and *B. longum* and the pathogenic *Helicobacter pylori*. Batch culture fermentation is designed to simulate the environmental conditions in different parts of the colon (Macfarlane and Macfarlane, 2007). Batch cultures have been used to assess the effect of polyphenols on the gut microbiota and also the effect of the gut microbiota on polyphenol stability (Dueñas et al., 2015). Furthermore, batch cultures have

aided evaluation of environmental conditions that favour or limits polyphenolic bioconversion (Dueñas et al., 2015). The present study was aimed at evaluating the possible prebiotic effect of SCC (containing fibres and insoluble phenolic compounds) and SCC extract (SCCE; containing free phenolic compounds and xylooligosaccharides) on the human gut microbiota by *in vitro* faecal batch culture fermentation.

4.2 Material and methods

4.2.1 Quantification of ferulic acid in SCC and SCCE

The SCC used in this research was harvested in Senegal in December 2015 and was provided by Barfoots of Botley Company Ltd. The preparation of SCC was described in Chapter 2 (Section 2.2.4). The SCCE was simulated using standards (ferulic acid, *p*-coumaric acid, xylobiose and xylotriose) and the concentration was estimated according to the SCC extract obtained by enzymatic hydrolysis using ferulic acid esterase and xylanase (Chapter 3). The concentration of ferulic acid, *p*-coumaric acid, xylobiose and xylotriose of the SCC and SCC extract were determined by HPLC (Chapter 2, Section 2.2.7) and recorded in **Table 4.1**.

Table 4.1: Concentration of ferulic acid, p-coumaric acid, xylobiose and xylotriose in sweet corn cob (SCC) and extract (SCCE).

Compounds	SCC (mg/g)	SCC extract (mg/L)
Total ferulic acid	3.06	168.87 ± 2.04
Total *p*-coumaric acid	4.22	44.59 ± 0.14
Glucose	-	2174.25 ± 58.87
Xylose	-	73.52 ± 9.11
Xylobiose	-	20.38 ± 2.44
Xylotriose	-	70.95 ± 0.93

4.2.2 Substrate dose determination and pre-digestion

Six different substrates including SCC, SCCE, ferulic acid, *p*-coumaric acid, xylooligosacharride (XOS) and fructooligosaccharide (FOS) were used in the batch culture fermentation. The substrate dose was determined in accordance with the research of Tzounis et al. (2011). The concentration of ferulic acid was selected to reflect the approximate gastrointestinal concentrations of ferulic acid achieved from the daily dietary intake of 150 mg ferulic acid/d (Zhao and Moghadasian, 2008). With the assumption of a stomach volume of 1–1.5 L, this equated to 0.1–0.15 mg total ferulic acid/mL reaching the gastrointestinal tract. The concentration of substrate used in this research was listed is **Table 4.2**.

Table 4.2: Substrate dosage used for batch culture fermentation.

Vessel	SCC[a] (mg)	FA[b] (mg)	pCA [c](mg)	XOS [d*](mg)	FOS[e] (mg)
NC	-	-	-	-	-
SCC	1000	-	-	-	-
SCCE	-	3	0.78	1.62	-
FA	-	3	-	-	-
pCA	-	-	0.78	-	-
XOS	-	-	-	1.62	
FOS	-	-	-	-	1.62

**xylooligosaccharide combination of xylobiose and xylotriose*

a: sweet corn cob powder, b: Ferulic acid; c: p-coumaric acid; d: xylooligosaccharide; e: Fructooligosaccharide

4.2.3 Faecal sample preparation

Batch culture fermentations were carried out using fresh faecal samples provided by three healthy volunteers (one male, two females; age 28-33 years, omnivores). Donors were free of known metabolic and gastrointestinal diseases and they did not receive antibiotic or probiotic treatment for at least 6 months prior to the experimentation. Faecal samples were collected in sterile plastic containers which were stored in anaerobic jars containing AnaeroGen sachets (Oxoid, Basingstoke, UK). Stool samples were used within 2 hours of collection. Faecal samples were diluted 1/10 (w/w) in sterile phosphate-buffered saline (PBS) and homogenised in filter bags using a stomacher (Stomacher 400, Seward) for 4 min (460 paddle/min) to create a homogenous faecal slurry. Resulting faecal slurries were used to inoculate the batch-culture systems. A different faecal sample was used for each of the triplicate experiments.

4.2.4 *In vitro* batch cultures fermentation

Sterile mini batch fermentation vessels (20 mL working volume) were aseptically filled with 19 mL of sterile basal nutrient medium and sparged with O_2 - free N_2 (15 mL min^{-1}) overnight to establish anaerobic conditions. The basal medium (per litre) consisted of: 2g peptone water, 2g yeast extract, 0.1g NaCl, 0.04g K_2HPO_4, 0.04g KH_2PO_4, 0.01g $MgSO_4.7H_2O$, 0.01g $CaCl.6H_2O$, 2g $NaHCO_3$, 2mL Tween 80, 0.05g hemin, 0.01mL vitamin K1, 0.5g L-cysteine-HCl, 0.5g bile salt and 4mL resazurin solution (0.25g/L). The substrates were added (1% w/v, Table 4.2) to the respective fermentation vessels just before the addition of the faecal slurry. Vessels were kept at 37° C using a circulating water bath and the pH was controlled between 6.7 and 6.9 with the aid of 0.5M HCl or NaOH, using an automated pH controller (Fermac 260, Electrolab.). Each vessel was inoculated with 1 ml of fresh faecal slurry (1:10 w/w). For each donor, 7 vessels were prepared for 7 treatments: SCC, SCC extract, ferulic acid, *p*-coumaric acid, xylobiose + xylotriose, FOS, and negative control. Batch cultures were

conducted for 24 hours, and samples of 3.7mL were collected from each vessel at 0, 6, 12 and 24 hours for counting of bacterial populations and short chain fatty acid (SCFA) analysis.

4.2.5 Short chain fatty acid (SCFA) analysis by gas chromatography

Samples (1 mL) from each fermentation time point were centrifuged at 13,000xg for 10 minutes. Supernatants were filtered through a 0.22µm Millipore syringe filter. The extraction of SCFA was done according to Richardson et al. (1989) with slight modification. Briefly, 600µL of sample was transferred to a labelled glass tube with 30µL of 2-ethylbutyrc acid (0.1M, internal standard). 300µL of concentrated hydrochloric acid and 1.8mL of diethyl ether were added to each glass tube and vortexed for 1 minute. Samples were then centrifuged at 2000xg for 10 minutes. 400µL of ether extract (upper layer) were added to a tube containing 50µL of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). The tube was capped and the mixture was left at room temperature for 72 hours to allow complete derivatisation of lactic acid.

An Agilent/HP 6890 Gas Chromatograph (Hewlett Packard, UK) using an HP-5MS 30m×0.25mm column with a 0.25µm coating (Crosslinked (5%-Phenyl)-methylpolysiloxane, Hewlett Packard, UK) was used for the analysis of SCFA. Temperatures of injector and detector were 275°C, with the column programmed from 63°C for 0 minutes to 190°C at 15°C min^{-1} and held at 190°C for 3 minutes. Helium was the carrier gas (flow rate 1.7 ml min^{-1}, head pressure 133 KPa). A split ratio of 100:1 was used. Quantification of the samples was obtained through external calibration curves of acetic (R^2=0.999), propionic (R^2=0.9998), and butyric (R^2=0.9998) acid in concentrations between 12.5 and 100 mM. The mean metabolite concentrations were expressed as mM.

4.2.6 Enumeration of faecal microbial populations by flow cytometry-fluorescence *in situ* hybridisation (FISH)

Collected samples (750 µl) were centrifuged at 13,000xg for 5 min at room temperature. Pellets were fixed for further fluorescence *in situ* hybridisation and kept at -20°C. Briefly, after centrifugation pellets were resuspended in 375µl of phosphate buffer saline (PBS; 0.1M) and 1,125.5 µl of cold 4 % paraformaldehyde. Suspension was mixed and stored at 4 ºC for 4–6 h. After incubation time, samples were washed twice with 1 mL of PBS. Finally, samples were centrifuged at 13,000xg for 5 minutes, supernatant was discarded and the pellet was resuspended in 300 µl of PBS and 300 µl of ethanol. Samples were vortexed and stored at –20 ºC for further analysis. For Flow-FISH cytometry, the 16S ribosomal RNA molecule labelled with the fluorescent was used for the enumeration of bacterial groups (**Table 4.3**).

75 µl of the fixed samples were collected from the fix cell solution stored at -20C. The fixed cells were washed twice with PBS and pre-treated for 10 min with lysozyme at 1mg/ml. Cells were resuspended in 1 mL of hybridisation buffer (HB; **Table 4.4**). All hybridisations were performed in the dark at 35° C overnight in the hybridisation solution containing the appropriate labelled probe (**Table 4.3**). One hundred and fifty microliter of HB (without probe) was added to stop the reaction. Cells were centrifuged at 13,000xg for 3 minutes, resuspended in pre-warmed washing buffer (**Table 4.4**) and incubated at 37 °C for 20 min to remove non-specific binding of the probe. Finally, cells were centrifuged at 10,000xg for 3 min and resuspended in PBS for flow cytometry analysis. Quantification of bacterial populations was performed on an Accuri C6 flow cytometer and Cflow software (BD Biosciences, USA).

Table 4.3: Name, sequence, and target group of oligonucleotide probes used for fluorescence in situ hybridization analysis for bacterial enumeration.

Name of Probe	Sequence (5' to 3')	Targeted group	References
Non Eub	ACTCCTACGGGAGGCAGC	Control probe	Wallner et al. (1993)
Eub338	GCTGCCTCCCGTAGGAGT	Total bacteria	Amann et al. (1990)
Eub338II	GCAGCCACCCGTAGGTGT	Total bacteria	Daims et al. (1999)
Eub338III	GCTGCCACCCGTAGGTGT	Total bacteria	Daims et al. (1999)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	Langendijk et al. (1995)
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	Manz et al. (1996)
Erec482	GCTTCTTAGTCARGTACCG	*Eubacterium rectale*/*Clostridium cocoides* (*Clostridium* cluster XIVa and XIVb)	Franks et al. (1998)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* spp.	Walker et al. (2005)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii*	Suau et al. (2001)
Chis150	TTATGCGGTATTAATCTYCCTTT	*Clostridium histolyticum* (*Clostridium* cluster I and II)	Franks et al. (1998)

Table 4.4: Components of hydridization and washing buffer used.

Components	**Hybridization Buffer (µL)**	**Washing Buffer(µL)**
Sodium chloride (5M)	180	12.8
Trizma base/Hydrochloric acid (1M)	20	20
Formamide	300	10
Distilled water	499	956.2
10% Sodium dodecyl sulphate	1	1

4.2.7 Statistical analysis

Statistical analyses were performed using Minitab version 17.1.0. Analysis of variance (ANOVA) and Fisher test were used to determine significant changes in the microbiota populations and concentration of SCFA at inoculation and sampling points. The effect of different substrates at the same time point were compared and differences were considered to be significant when $p<0.05$.

4.3 Results and discussion

4.3.1 Effect of phenolic acids on human faecal bacteria

To assess the impact of free and bound phenolic acids on the intestinal microbiota composition, pH-controlled, anaerobic, faecal batch cultures were conducted and bacteria were enumerated by FISH. Samples were taken after 6, 12 and 24 hours of fermentation. The changes in bacterial groups for each fermentation experiment are presented in **Figure 4.1**. All vessels showed a decrease in total bacteria from 0 to 24 hours of fermentation (**Appendix II, Figure A3**), although this decrease was not significant. Among the bacterial groups studied (*Bifidobacterium* spp., *Lactobacillus/Enterococcus* spp., *Bacteroides* spp., *Eubacterium rectale - Clostridium coccoides*, *Roseburia* and *Clostridium histolyticum* group), addition of SCCE containing free phenolic compounds and XOS to the faecal fermentation mixtures promoted the growth of *Bifidobacterium* at 6 and 12 hours (**Figure 4.1A**), although it was not statistically significant. At the end of the fermentation, all vessels showed higher amounts of *Bifidobacterium* as compared to the negative control, except vessels containing SCC. This might be due to the compounds in SCC not being readily available for the bacterial uptake, due to the complex structure of SCC composed mainly of insoluble fibers (Chapter 2). Davin et al. (2009) reported that under anaerobic conditions, it is considered unlikely for the degradation of these highly polymerized lignins, which are composed mainly of phenolic compounds. This showed that the gut microbiota was unable to release the bound phenolic acids from SCC and thus, showed no increase in the growth of *bifidobacterium*. *Bacteroides/Provotella* spp. showed an increase across all vessels from 6 to 12 hours, with the highest increase being observed in vessels containing SCC followed by SCCE. A decrease in *Eubacterium/Clostridium* spp. was observed in all vessels and significantly lower amounts were found in vessels containing SCC as compared to FOS (**Figure 4.1B**). Overall, no significant differences were observed between the negative control and all the substrates being studied over the 24 hours period and this might

be due to the inter-individual variation in faecal bacteria. Microbial diversity among individuals might be due to the presence of different bacterial populations present in the faecal microbial community with different enzymatic capacities (Zoetendal et al., 2008). In addition, the results further suggested that it is possible that a daily intake of 150mg FA (Zhao and Moghadasian, 2008) might be insufficient to significantly promote the growth of gut microbiota .

Lactobacillus and *Bifidobacterium* have been widely studied due to health promoting effects including stimulation of the immune system (Gill et al., 2007) , inhibition of the growth of harmful bacteria (Gagnon et al., 2004), increase in synthesis of vitamin B (LeBlanc et al., 2011) as well as absorption of certain ions (López-Molina et al., 2005). Results obtained in this study showed a possible bifidogenic activity of SCCE containing free phenolic acid and XOS. The combination of free phenolics with XOS were more readily available for the uptake by the bacteria and thus promoted growth. Previously, the bifidogenic effect of phenolic compounds has been well proven by several authors. Yuan et al. (2005) reported that feruloyl oligosaccharide released from wheat bran promoted the growth of *Bifidobacterium bifidum*. In addition, feruloylated arabino-oligosaccharide extracted from sugar beet pectin was reported to selectively stimulate the growth of bifidobacteria (Holck et al., 2011).

Vessels containing SCC showed no increase in *Lactobacillus* (**Appendix II, Figure A4**). Similarly, Gálvez Ranilla et al. (2017) reported that the bound phenolic fraction rich in hydroxycinnamic acid derivatives (ferulic acid at 0.016mg/mL) of purple Peruvian corn did not affect the growth of probiotic lactic acid bacteria. Furthermore, in an research carried out by Puupponen-Pimiä et al. (2001) using agar diffusion technique, they reported that pure phenolic acids such as *p*-coumaric acid, transcinnamic, chlorogenic, cafffeic and ferulic acid did not affect the growth of *L. rhamnosus, L. reuteri, L. paracasei, L johnsonii, L.crispatus* and *L,plantarum* at a dosage of 0.05 to 0.5mg/well. This showed that ferulic acid might not have any impact on the growth of *Lactobacillus*.

The fermentation of SCC also showed an increase in the populations of *Bacteroides* spp. (**Figure 4.1C**). The increase might be due to the presence of polysaccharides in SCC (Chapter 2). *Bacteroides* are reported to be able to utilise many types of plant polysaccharides as substrates (Salyers et al., 1981). Although the increase of this group may exert a detrimental effect on the health of the colon due to the metabolites, these groups also contain saccharolytic species which may result in the production of beneficial SCFAs (Guergoletto et al., 2016). Similarly, Hughes et al. (2007) observed an increase in *bacteroides* following fermentation of arabinoxylan fractions of wheat. SCC showed a trend of decrease in *Clostridium* Cluster I and II (**Figure 4.1D**) and *Eubacterium rectale* (**Figure 4.1B**) at the end of the fermentation, as compared to the negative control. The growth of these pathogenic *Clostridium* group can potentially be associated with diseases. For example, the growth of the proteolytic bacteria from *C. histolyticum* group can potentially be related to negative effects such as the progression towards inflammatory bowel disease and colorectal cancer (Gibson, 2008). In addition, *C. perfringens* has been reported to potentially associate with gangrene and gastrointestinal diseases (Petit et al., 1999). This study showed that SCC may have the ability to inhibit the colonisation of potential pathogenic *Clostridium* spp. in the colon.

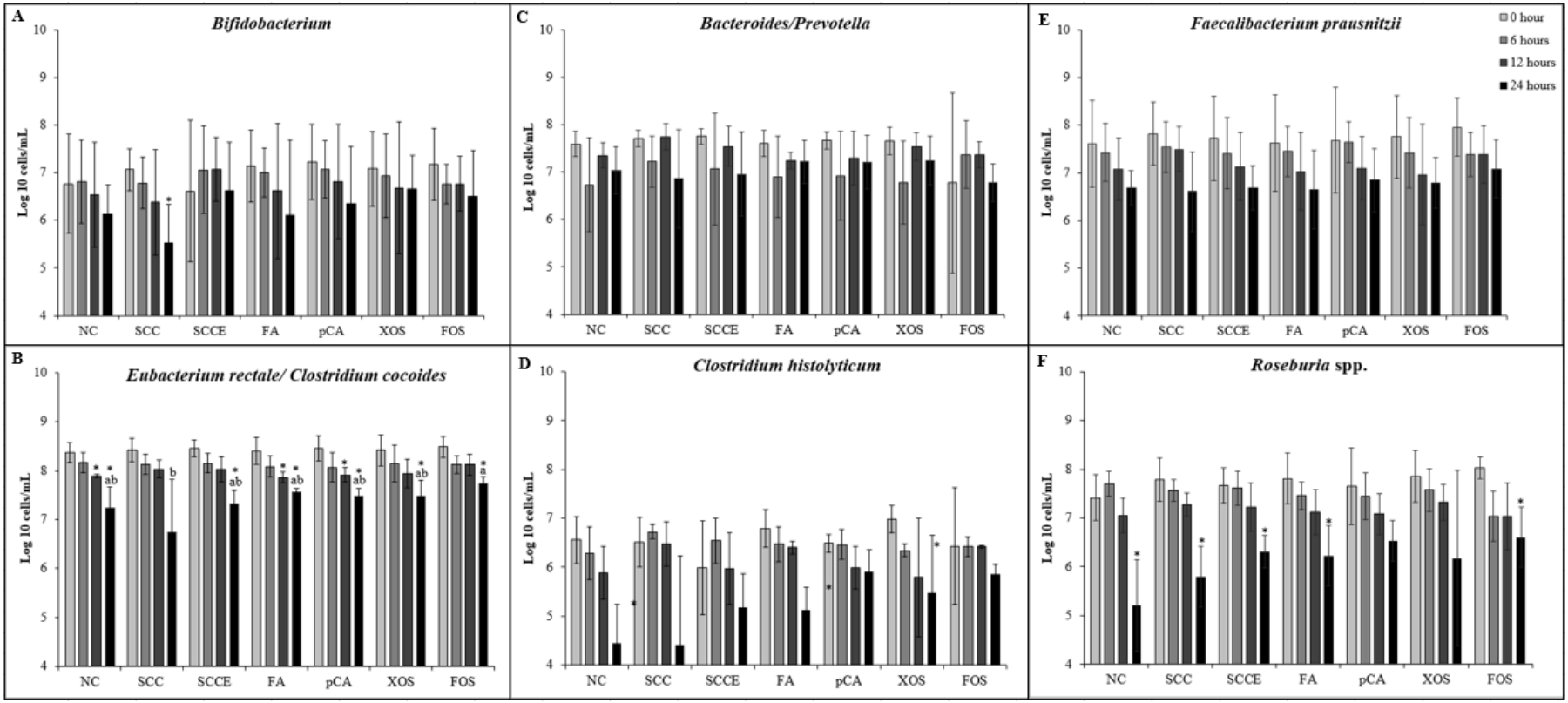

Figure 4.1: Bacterial populations (*Bifidobacterium, Eubacterium/Clostridium, Bacteroids/Prevotella, Clostridium histolyticum, Faecalibacterium prausnitzii and Roseburia spp*.) analysed by Flow-FISH in pH controlled batch cultures containing different substrates.

*(NC= negative control; SCC= Sweet corn cob; SCCE: Sweet corn cob extract; FA: ferulic acid, pCA: p-coumaric acid; XOS: Xylooligosaccharide and FOS: Fructooligosaccharides). Error bars indicate SD (n = 3). Significant differences between substrates at the same time point are indicated with letters (P < 0.05). * indicates significantly different compared to 0 h within the same substrate (P < 0.05).*

4.3.2 Effect of polyphenol on bacterial production of the major SCFAs

SCC incubation significantly increased (p<0.05) the production of all major SCFAs including acetate, butyrate and propionate, in relation to the control (**Figure 4.2**). All substrates showed an increase in the production of SCFA throughout the duration of fermentation from 0 to 6 hours. Significantly higher (p<0.05) concentrations of acetate (**Figure 4.2A**) was detected at 6 hours collection time in the vessel containing SCC (16.21±2.85mM) as compared to the negative control (11.09±1.47mM). Furthermore, the production of both propionate (**Figure 4.2B**) and butyrate (**Figure 4.2C**) showed significantly higher amounts at 6 and 12 hours in vessels containing SCC. This is in agreement with Salvador et al. (2007) where increases in butyric acid were observed from xylose rich substrates. In addition, Adam et al. (2001) also reported an increase in butyric acid in arabinoxylan-rich wheat flour as the substrate. All the other substrates showed no significant difference as compared to the negative control.

The saccharolytic metabolism of gut microbiota in the large intestine results in the production of SCFA (Gibson, 2004). The significant increase in the production of SCFA in the vessel containing SCC might be due to the presence of fibers. McIntyre et al. (1993) reported that the fermentation of fiber by anaerobic bacteria produces hydrogen, methane, carbon dioxide and SCFA, predominantly propionate, acetate and butyrate. In addition, they reported that the fermentation of wheat bran (consisting of cellulose, hemicellulose and lignin) resulted in an increase in butyrate production, as compared to oat bran (rich in beta-glucan) and guar gum (pure galactomannan). The production of SCFA by gut microbiota in the colon has an important impact on human health especially butyrate, and is often associated as the major energy source for colonocytes (Donohoe et al., 2011). In addition, acetate enters the systemic circulation and is used in lipogenesis, while propionate is transported to the liver for its role in gluconeogenesis (Scott et al., 2013). The presence of butyrate in the colon is reported to be important for reducing risk factors associated with ulcerative colitis (Simpson et al., 2000) or

colon cancer (Weaver et al., 1988). Previous research by Louis and Flint (2009) has reported that the two most important groups of butyrate-producing bacteria in the human intestine appear to be *Faecalibacterium prausnitzii* and *Eubacterium rectale/Roseburia* spp.. This is in agreement with the results of FISH hydridisation, where an increase in *Faecalibacterium prausnitzii* (**Figure 4.1E**) and *Roseburia* spp. (**Figure 4.1F**) were observed at 12 hours in the vessel containing SCC, as compared to the control vessel.

In conclusion, SCCE showed a trend of enhancing the growth of *Bifidobacterium* and inhibiting the growth of pathogenic *Clostridium histolyticum* while SCC showed significant production of short chain fatty acids in batch culture fermentation with human faecal microbiota. The effect of SCCE on the growth of bifidobacteria might be due to the readily available free phenolic compounds and XOS for bacterial uptake and utilisation for growth as compared to the bound phenolic compounds in SCC. However, the fermentation of fiber present predominantly in SCC lead to the increase in beneficial SCFA including acetate, propionate, and butyrate. Therefore, fermentation of SCC led to a positive impact on the production of SCFA, which could have a systemic effect on the host. The result also suggests that a daily intake of 150mg of FA per day might not be sufficient for the growth of gut microbiota. This research presented a solution to the effective utilisation of agricultural waste, SCC, whether as a powder with considerable amount of fibre or extract with free phenolic compounds and xylooligosaccharides, where both can exert beneficial effect towards human health.

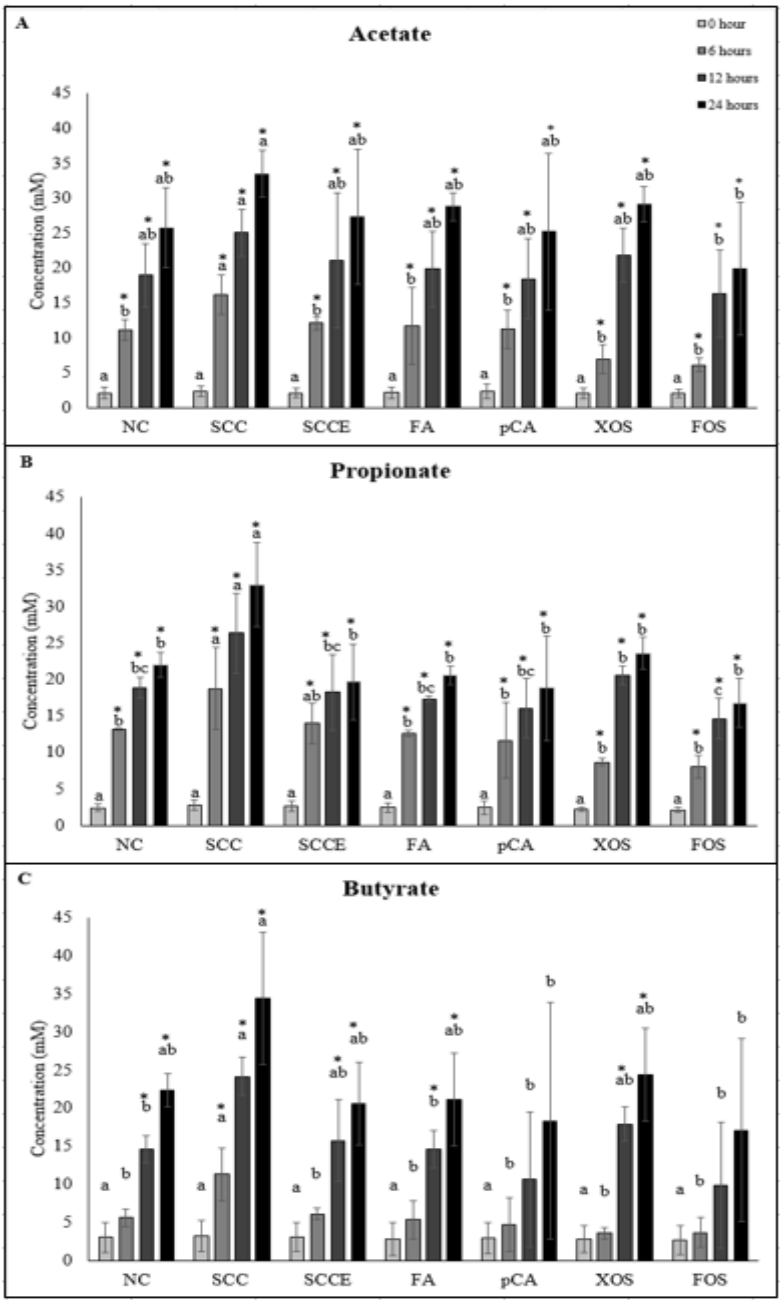

Figure 4.2: Acetate, propionate and butyrate concentrations (mM) obtained in pH controlled batch cultures at 0, 6, 12 and 24 h of fermentation from different substrates.

*(NC= negative control; SCC= Sweet corn cob; SCCE: Sweet corn cob extract; FA: ferulic acid, pCA: p-coumaric acid; XOS: Xylooligosaccharide and FOS: Fructooligosaccharides). Error bars indicate SD (n = 3). Significant differences between substrates at the same time point are indicated with letters. * indicates significantly different compared to 0 h within the same substrate (P < 0.05).*

Chapter 5

Sweet corn cob as a functional ingredient in bakery products

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Abstract

Sweet corn cob (SCC), a rich source of ferulic acid, is a by-product of sweet corn processing. The effect of baking on ferulic acid content, colour, texture and physical characteristics on muffin incorporated with SCC flour as a value added food ingredient was investigated using a model system. The freeze-dried SCC flour containing 6.02mg g^{-1} of ferulic acid was used to replace the rice flour at varying level of 10, 20, and 30%. The increase in the amount of SCC flour to 20% showed an increase in terms of the height of the muffin and number of bubbles, and decreases in the specific volume, as well as hardness of muffins. These effects were not observed in other flour combinations. The yellowness of the muffin crumb increases as SCC flour increases due to the presence of carotenoid compounds in SCC flour. In general, baking can increase the free ferulic acid content compounds. Incorporating SCC flour up to 20% into the formulation can improve the quality of gluten-free rice flour muffin.

5.1 Introduction

Recently, there has been an increase in consumer demand towards value-added food incorporated with functional ingredients such as fibre and natural antioxidants. Incorporating by-products as a food ingredient does not just provide health benefits, but also offers alternative solutions for environmental concerns associated with disposal (Rupasinghe et al., 2009). Sweet corn cob (SCC), an under-utilised agricultural by-product of the corn processing industry, is high in dietary fibre, minerals and phytochemicals including ferulic acid (refer to Chapter 2). Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a phenolic compound present in many foods and offers beneficial effects against cardiovascular diseases, hypertension, inflammatory diseases, cancer, Alzheimer and diabetes (Zhao and Moghadasian, 2008). These health benefits can be achieved by consumption of either free or bound phenolics. Consumption of bound phenolics will contribute towards the chemopreventive activity against colon cancer (Acosta-Estrada et al., 2014). In addition, free and soluble conjugated form are more rapidly absorbed in the small intestine and stomach, therefore it can be distributed throughout the body offering health benefits such as inhibition against oxidation of liposomes and LDL cholesterol .

Muffins are very popular among the consumer as snack food due to their good taste and soft and spongy texture (Matos et al., 2014). Traditionally, wheat flour, oil, sugar, milk and egg are used in the recipe for muffin baking. However, those suffering from coeliac disease are unable to consume this type of product as they contain wheat flour. As a result, demand over gluten-free (GF) products has increased for people suffering from gluten intolerance and sensitivity, as well as for the consumer demands for wheat free products (Nachay, 2010). Generally, GF products are made from starches or flour with low dietary fibre content (Singh et al., 2016) and they are often of poor colour, crumbling crumb, low quality and exhibiting low volume (Matos Segura and Rosell, 2011). Rice is one of the most suitable cereals for the preparation of GF products due to its hypoallergenicity, low fat and sodium, high digestibility,

white colour and bland taste (Marcoa and Rosell, 2008). However, the use of rice flour in the preparation of muffin causes issues such as lower volume, poor texture, colour and crumb structure (Matos et al., 2014). Therefore, previous authors have tried to improve the quality of GF muffins by incorporating protein isolates (Shevkani et al., 2015), flour replacement (Herranz et al., 2016), xanthan gum and fibre (Singh et al., 2016).

Rice contains very low level of ferulic acid and it ranged from 0.18 ± 0.05 to 5.26 ± 0.14mg/100g (Hager et al., 2012, Zhou et al., 2004, Tian et al., 2004). In contrast, our previous study showed that SCC contained 306mg/100g of ferulic acid (Chapter 2), and thus, the incorporation of SCC flour into the formulations can be used to improve the ferulic acid content in rice flour muffin. However, there have been limited studies reporting the use of SCC flour in bakery products. Therefore, the present study was undertaken to produce ferulic acid-rich GF rice muffin incorporated with varying levels of SCC flour and to evaluate the physicochemical properties of these muffins.

5.2 Materials and Method

5.2.1 Materials

The sweet corn used in the experiments was harvested in Senegal in December 2017 and was provided by Barfoots of Botley Company Ltd (UK). The corn kernels were removed manually from the cobs and discarded. The SCC flour was prepared as described in Chapter 2 (Section 2.2.2).

5.2.2 Extraction and quantification of ferulic acid content in rice and SCC flour

The extraction and quantification of free, esterified and insoluble-bound phenolic compound in rice and SCC flour was carried out according to Chapter 2 (Section 2.2.4, 2.2.5 and 2.2.7).

5.2.3 Water holding capacity (WHC) and oil holding capacity (OHC) of rice and SCC flour

The WHC and OHC of rice flour and SCC flour were determined using the method as described by Mateos-Aparicio et al. (2010). Briefly, 0.5g of sample was hydrated in 30mL of water (for WHC) or oil (for OHC) at room temperature for 18 hours. The samples were then centrifuged and the supernatants were decanted. The weight of the residue was recorded and the WHC and OHC were calculated as the amount of water or oil retained by the pellet (g of water or oil/ g of sample dry weight).

5.2.4 Batter and muffin preparation

The muffins were prepared according to the recipe by Shevkani et al. (2015) with slight modification. The control muffin was 100% rice flour and the new formulations contained 10, 20, and 30% of SCC flour. 150g of flour, 90g of white granulated sugar, 5g of baking powder, 75g of egg, 75g of whole milk and 75g of sunflower oil were mixed for 5 minutes in an electric mixer (Model A901, Kenwood Chef). 65g of batter was then dispensed into 7 muffin paper cups (65mm diameter). The muffins were arranged in a muffin baking tray and baked for 23 minutes at 180°C in a rotary electric oven that had been preheated for 10 minutes. After baking, the muffins were left to cool to room temperature for an hour before packing them into polypropylene bags and stored at 20°C for 1 day, after which analysis were conducted. The muffins from each formulation were prepared in three replicates, and baked on three separate days.

5.2.5 Ferulic acid content in batter and muffin

The batter and muffin were frozen at -18°C and then freeze dried (Christ Gamma 2-16) until constant weight was achieved. The freeze dried samples were ground by pestle and mortar at room temperature for 10 minutes and stored in the freezer (-18°C) until analysis. The samples

were defatted by hexane and subjected to alkali hydrolysis (as described in Chapter 2, Sections 2.2.4, 2.2.5 and 2.2.7) in order to determine the amount of ferulic acid.

5.2.6 Physical characteristic of baked muffin

5.2.6.1 Weight loss, height and volume of muffin

The weight loss, height and volume of muffin were measured according to the method as described by Matos et al. (2014). The weight loss upon baking was calculated by the difference of the weight of muffin before and after baking. Height was measured using a digital calliper from the highest point of the muffin to the bottom of the paper cup. The volume of muffin was determined by rapeseed displacement. The specific volume of the individual muffin was calculated by dividing the volume by weight.

5.2.6.2 Air bubbles quantification

The muffins were cut horizontally at the height of the muffin paper cup and a flatbed scanner (HP Scanjet G2710, Hewlette-Packard) was used to capture the images of the muffins. For the image analysis, the number and average bubble size were analysed using Image J software (Rodríguez-García et al., 2014). The images were cropped to 5cm x 5cm section (**Figure 5.1**) and the image was split into colour channels. Then, the contrast was enhanced and binarised after grayscale threshold. Bubble count and average bubble size within the crumb were calculated. The values were the mean of three replicates for each formulation.

5.2.6.3 Colour measurements

A colorimeter (Chroma meter CR400, Konica Minolta) was used to measure the crumb and crust colour parameters (*L*, a*, b**) of the muffin. The muffins were cut horizontally at the height of the cup and colour of the crumb was measured. The crumb and crust colour was measured at several points **(Figure 5.2**) on the surface of the muffin. Hue angle and chroma

values were calculated according to Ahn and Lee (2008) and recorded in **Table 5.3**. Data from three muffins per formulations were averaged.

5.2.6.4 Textural characteristic of the muffins

The instrumental texture measurement of the muffins were carried out according to Matos et al. (2014). Texture profile analysis (TPA) was performed on crumb cubes (12.5 mm^3) using a texture analyzer (TAXT2, Stable Micro System) equipped with a 5 kg load cell. A double compression test was performed with a 75 mm diameter flat-ended cylindrical probe (P/75) and compressed to 50% of the initial height at a speed of 1 mm/s with 5 seconds waiting time between the two cycles. The results of firmness, springiness, cohesiveness, chewiness and resilience were obtained. The mean of at least three replicates for each formulation baked on different days was recorded.

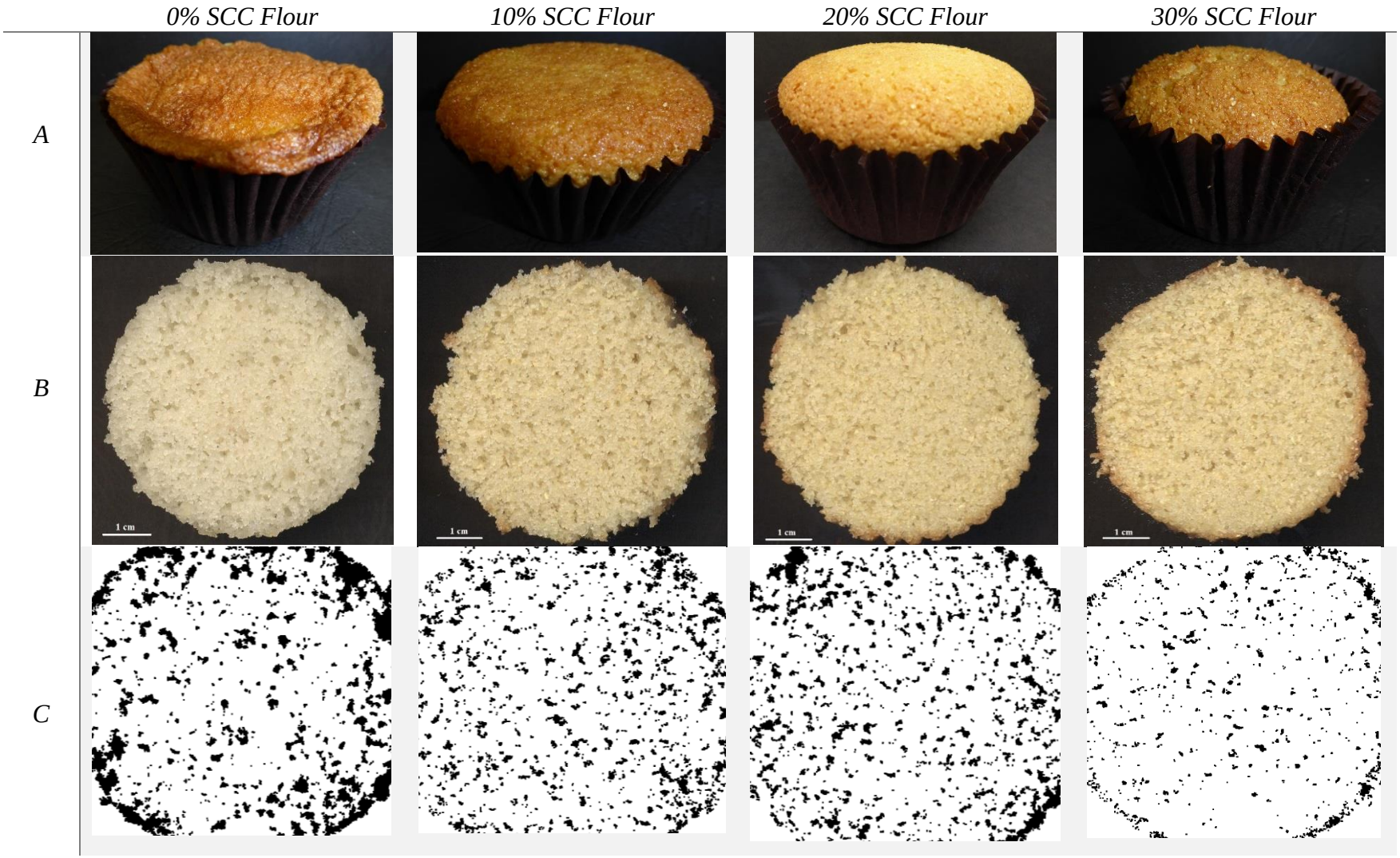

Figure 5.1: Photographs (A), scan images of transversal section (B), and image of bubble size distribution (C) of muffin baked with different levels of sweet corn cob flour.

5.2.7 Statistical analysis

For each parameter measured, one way analysis of variance (ANOVA) was carried out using Minitab statistical software (State College, USA). Fisher test was used to assess the significant differences ($p<0.05$) among samples.

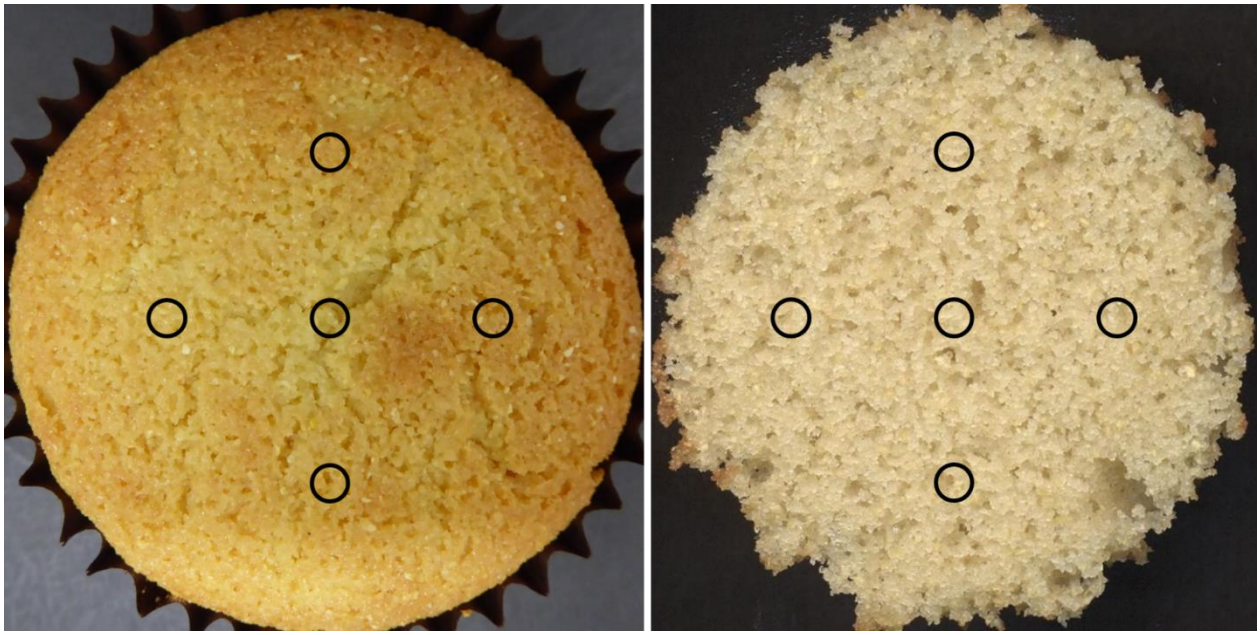

Figure 5.2: Points at which colours of crust and crumb were taken.

5.3 Results and Discussion

5.3.1 Ferulic acid content of rice and SCC Flour

The ferulic acid content in rice and SCC flour is presented in **Table 5.1**. The total amount of ferulic acid was higher in SCC flour (6.03 mg/g) as compared to rice flour (0.02 mg/g). The ferulic acid content in the SCC flour used in this chapter are of different batch (harvested in different year) and showed higher content of ferulic acid compared to the SCC flour used in the previous chapters (Chapter 2,3, and 4). It has been reported previously that the phytochemicals in rice are more abundant in the bran layer and only a small amount is present in the milled rice (Shen et al., 2009). In this research, ferulic acid was present in rice

flour as insoluble-bound form only. Similarly, Qiu et al. (2010) reported that insoluble ferulic acid is the most abundant phenolic acid present in rice. SCC flour contains significantly higher insoluble bound ferulic acid (5.82 ± 0.41mg/g DW) than rice flour (0.02mg/g). In addition, SCC flour also contains free and esterified ferulic acid.

5.3.2 WHC and OHC of rice and SCC Flour

WHC and OHC measures the interaction of water or oil with proteins and this is important for the flavour and texture in food (Yu et al., 2007). SCC flour had significantly higher values of WHC and OHC (7.73g of water and 4.51g of oil/g sample, respectively) than rice flour (1.19g of water and 0.81g of oil/g of sample, respectively) (**Table 5.1**). The WHC and OHC of rice flour reported in this study was slightly lower than the value reported by Joshi et al. (2015) (1.54g water/g of sample and 1.10g oil/g of defatted rice flour). The WHC and OHC of SCC was higher as compared to other gluten free flour such as sorghum (1.31g water/g flour; 0.90g oil/g flour) (Elkhalifa and Bernhardt, 2010), chickpea (2.54g water/g flour; 1.19g oil/g flour), soybean (3.53g of water/g flour; 1.61g oil/g flour), and almond (2.20g water/g sample; 2.32g oil/g flour) flour (Joshi et al., 2015).

The higher WHC and OHC of SCC might be due to higher content of dietary fibre in SCC as compared to rice flour. Our studies showed that more than 60% of SCC was composed of insoluble dietary fibre (Chapter 2). On the other hand, Fernando et al. (2012) reported that polished white rice flour contained 1.27% and 0.58% of insoluble and soluble dietary fiber. Dietary fibre can affect some functional properties of food including increase in OHC, WHC, formation of gel and emulsification (Elleuch et al., 2011). Sudha et al. (2007) reported higher WHC in apple pomace (8.39g water/g solid) as compared to wheat flour (1.01g water/g solid) due to the presence of fibre. In addition, Esposito et al. (2005) reported that WHC increases with the increasing amount of fibre in durum wheat debranned by-products. The high WHC and OHC of SCC flour suggests that it can help to avoid syneresis of the formulated product

and can be used to stabilise foods with a high percentage of emulsion and fat (Elleuch et al., 2011). SCC flour with high WHC could have possible application in products such as baked goods that require viscosity development, hydration and conservation of freshness (Alfredo et al., 2009). Furthermore, the high OHC of SCC flour can have potential to improve the palatability and flavour retention of a food product (El Nasri and El Tinay, 2007), and can be incorporated in many food applications including ground meat formulation, bakery products and meat substitutes (Zielińska et al., 2018).

Table 5.1: Ferulic acid content, water holding capacity (WHC) and oil holding capacity (OHC) of rice and sweet corn cob (SCC) flour.

Sample	Ferulic acid fraction			WHC (g of water/g DW)	OHC (g of oil/g DW)
	Free (mg/g)	Esterified (mg/g)	Insoluble-bound (mg/g)		
Rice Flour	ND	ND	0.02 ± 0.01^{b}	1.19 ± 0.07^{b}	0.81 ± 0.02^{b}
SCC Flour	0.01 ± 0.00^{a}	0.20 ± 0.01^{a}	5.82 ± 0.41^{a}	7.73 ± 0.19^{a}	4.51 ± 0.04^{a}

Means ± standard deviation (n=3) with different letters within a column indicate significant differences at p<0.05.

Table 5.2: Physical characteristics of muffin prepared with different level of sweet corn cob flour.

SCC Flour (% by weight)	Height (mm)	Weight loss (g)	Moisture Content (%)	Specific volume (mL/g)	Bubble count	Average bubble size (cm^2)
0	37.11 ± 0.74^{c}	4.9 ± 1.12^{a}	20.40 ± 0.89^{a}	2.49 ± 0.04^{b}	464 ± 34^{d}	0.007^{a}
10	43.07 ± 1.12^{b}	5.7 ± 0.01^{a}	22.03 ± 2.48^{a}	1.44 ± 0.01^{c}	738 ± 55^{a}	0.006^{a}
20	43.44 ± 0.79^{b}	4.9 ± 0.20^{a}	21.87 ± 0.88^{a}	1.49 ± 0.05^{c}	656 ± 45^{b}	0.006^{a}
30	45.66 ± 0.86^{a}	5.1 ± 0.01^{a}	21.85 ± 1.45^{a}	2.70 ± 0.06^{a}	513 ± 35^{c}	0.003^{b}

Means ± standard deviation (n=3). Different letters within a column indicate significant differences at p<0.05.

5.3.3 Physical characteristic of muffins

5.3.3.1 Weight loss, height and volume of muffin

There was no significant difference between any of the samples in terms of their weight loss and moisture content after baking. In general, a similar behaviour was found between both samples incorporated with 10 and 20% of SCC flour (**Table 5.2**). The muffin with 30% SCC flour showed the highest specific volume (2.70±0.06mL/g) while a significant reduction ($p<0.05$) in the specific volume in muffin incorporated with 10% (1.44 ± 0.01mL/g) and 20% (1.49±0.05mL/g) of SCC flour as compared to rice flour muffin (2.49±0.04mL/g) was observed. The low specific volume of rice flour, 10% SCC flour muffin and 20% SCC flour muffin could be due to the lower fiber content, preventing the holding of water and entrapment of air bubbles and thus resulting in insufficient specific volume (Demirkesen et al., 2010). Sabanis et al. (2009) reported that the incorporation of maize fibre into gluten free breads significantly increased the loaf volume as compared to non-fibre gluten free bread due to the increase in the dough viscosity associated with fibre contents.

Muffin height was significantly ($p<0.05$) affected by the level of SCC flour incorporated. The largest effect on height was found with muffin incorporated with 30% of SCC flour. The increase of height as amount of SCC flour increases might be due to the increase of fibre present in the muffin, contributed by the SCC flour. Fibre has a great capacity to form gels, increase viscosity and act as emulsifiers (Elleuch et al., 2011). This can then strengthen the network of muffin during baking, preventing them from collapse. Gularte et al. (2012) reported that during thermal treatment, oat fibres were able to give some strength to the network to counteract the collapse of cake. Another possible reason is that the components in SCC flour (e.g. fibre, protein and minerals) may increase the gelatinisation temperature of starch by reduction of the water available for starch gelatinization (Majzoobi et al., 2016) and thus promote expansion of muffin. The same author reported that gluten free cake baked with rice

and corn flour have the lowest consistency and viscosity in the batter, causing the collapse of the cake due to the escape of air bubble.

5.3.3.2 Muffin air bubbles

The spongy texture of muffin is characterised by its high volume and porous structure contributed by tiny air bubbles (Martínez-Cervera et al., 2012). **Table 5.2** shows the bubble count and average bubble size measured using Image J analysis from the binarized images of the different muffin crumb. Our results showed that muffins with 10% SCC flour incorporated showed the highest number of air bubbles, followed by muffin with 20%, 30% and 0% of SCC flour incorporation. Muffin with 30% SCC flour incorporation showed significantly smallest bubble size as compared to the rest of the muffin.

The inclusion of baking powder in the recipe generates carbon dioxide and leads to the growth of air bubbles and thus helps in leavening of the muffin during baking. When batter consistency and viscosity are too low, the air bubbles will rise to the surface due to buoyancy and are lost during baking. However, when the batter has a very high consistency and viscosity, this can restrict the expansion of bubbles during baking (Gomez et al., 2010). The increase in fibre content as SCC flour increases can increase the batter consistency and viscosity, and thus retain the bubbles during baking. However, the further increase in consistency and viscosity of the batter incorporated with 30% SCC flour restricts the expansion of bubbles, thus resulting in smaller bubble size.

5.3.3.3 Muffin colour

Incorporation of SCC flour in the development of high ferulic acid content muffin also had an impact on the colour of the crust and crumb of the baked muffins. **Table 5.3** shows the colour value (*L*, *a*, *b*), hue angle (*h*°) and chroma (*C*) of the crust colour of muffins. For all the muffins, the crust colour was found to be darker (lower *L* value) than the crumb colour due to

the occurrence of Maillard reaction during baking. Similar effects were observed by Martínez-Cervera et al. (2012) when baking muffins made of wheat flour. During baking, reactions between proteins and reducing sugars as a result of the Maillard reaction are important for the development of the brown colour as well as flavour and texture (Michalska et al., 2008). The increase in temperature during baking causes the water content of the crust layer to reduce rapidly, causing the degradation of sugar and thus favouring the occurrence of Maillard reaction. However, water losses in the interior of the muffin (crumb) are lower causing slower progress of Maillard reaction, therefore, crumb is only slightly coloured (González-Mateo et al., 2009).

5.3.3.3.1 Crust colour

Muffin incorporated with 20% and 30% SCC flour showed significantly lower redness (*a* value) on the crust colour as compared to rice flour and 10% SCC flour muffin. Lightness and yellowness (*L* and *b* values) of the crust colour increased with the increase of SCC flour in the formulation. The increase in *b* values of muffin prepared with the increased amount of SCC flour may be attributed to the natural yellowish colour of SCC flour due to its carotenoid content. In relation to hue angle (*h*°) and chroma (*C*), slight variation was found between samples. The yellow-orange hue of these muffins were further confirmed by having positive *h*° values (71.08 - 81.63°) in all muffins, with an increase in *h*° values as SCC flour increases. Our previous findings (Chapter 2) showed that SCC flour contains β-carotene (177.29µg/g), lutein (3.81µg/g) and zeaxanthin (8.47µg/g). This is in agreement with Nasar-Abbas and Jayasena (2012) when baking muffin incorporated with lupin flour which contained natural yellow pigment. They observed a decrease in *a* value and an increase in *b* value as percentage of lupin flour increased in the muffin formulation. The increase in lightness (*L*) might be due to the decrease in protein content as the SCC flour increases. The protein content in rice and SCC flour is 8.75 (Ju et al., 2001) and 6.7% (Chapter 2), respectively. The decrease in protein

content as the amount of SCC flour increases reduced the browning reaction in the muffins by Maillard reaction. Hence, the muffin became lighter in colour and showed high *L* values. In addition, increases in chroma as the amount of SCC flour increased revealed the higher intensity of the yellow component contributed by the carotenoid content in SCC flour.

5.3.3.3.2 Crumb colour

Results from the crumb colour parameters are presented in **Table 5.3**. A slight decrease in the lightness was observed in the muffins with SCC flour (significant decrease only in muffin with 10% SCC flour) probably due to the reduction in the proportion of rice flour in the formulation, resulting in a loss of the characteristic white colour of rice flour. As the percentage of SCC flour increased, greenness (-*a*) and yellowness (*b*) increased. The crumb colour of muffins showed positive *h*° values of 91.73 to 99.00° further confirm their yellow-orange hue. Similar to crust colour, this could be due to the fact that SCC flour, which contains naturally occurring yellow pigments due to its carotenoid content (Chapter 2), impart yellowness to the product when mixed with rice flour. Similarly, the increase in chroma was observed with the increase of SCC flour in the formulation due to the increase in the intensity of yellow colour (*b* value), contributed by the pigmented carotenoid compounds in SCC flour.

Table 5.3: Crust and crumb colour parameters of muffin incorporated with sweet corn cob flour.

Crust Colour					
SCC Flour (% by weight)	***L***	***a***	***b***	***C****	***h (°)***
0	53.44 ± 4.82^{b}	12.60 ± 2.10^{a}	36.96 ± 3.53^{b}	39.10 ± 3.43^{cb}	71.08 ± 3.30^{ab}
10	53.35 ± 1.75^{b}	12.36 ± 1.86^{a}	39.42 ± 3.93^{ab}	41.37 ± 3.69^{ab}	72.42 ± 3.21^{b}
20	63.04 ± 1.93^{a}	6.73 ± 1.56^{c}	40.68 ± 1.69^{ab}	41.26 ± 1.79^{ab}	80.63 ± 2.05^{a}
30	61.77 ± 2.58^{a}	9.22 ± 1.95^{b}	42.73 ± 1.56^{a}	43.74 ± 1.74^{a}	77.85 ± 2.34^{a}
Crumb Colour					
0	73.52 ± 3.55^{a}	-2.92 ± 0.71^{b}	18.35 ± 1.85^{c}	18.58 ± 1.90^{c}	99.00 ± 1.69^{a}
10	69.77 ± 2.94^{b}	-2.35 ± 0.37^{b}	28.09 ± 0.79^{b}	28.19 ± 0.81^{b}	94.78 ± 0.67^{b}
20	72.43 ± 1.45^{ab}	-1.88 ± 0.42^{ab}	31.16 ± 1.91^{b}	31.21 ± 1.92^{b}	93.45 ± 0.67^{bc}
30	72.65 ± 1.67^{ab}	-1.10 ± 0.77^{a}	36.01 ± 1.93^{a}	36.03 ± 1.94^{a}	91.73 ± 1.18^{c}

Means ± standard deviation (n=3). Different letters within a column indicate significant differences at $p<0.05$. Also, L value is a measure of the lightness (0=black; 100= white). a measures redness (positive value) or greenness (negative value) while b measures yellowness (positive value) or blueness (negative value)

5.3.3.4 Muffin texture

The effect of incorporation of SCC flour on the texture of the muffins is shown in **Table 5.4**. The muffins incorporated with 10 and 20% of SCC flour showed significantly lower crumb hardness as compared to control muffin with 100% rice flour. Products made of rice flour are known to become hard and decline in taste and texture over time (Wu et al., 2010). This is due to the starch retrogradation in rice flour, where the re-association of gelatinized starch forming crystallites upon cooling. Kadan et al. (2001) also reported that rice bread was more prone to retrogradation and have harder texture than whole wheat bread.

Muffin prepared with 10% SCC flour showed the lowest crumb hardness of 237.04*g*, which increased to a value of 325.00*g* and 424.05*g*, respectively with the incorporation of 20 and 30% of SCC flour. One possible explanation could be that the high water binding capacity of SCC flour can avoid water loss and possibly delay the starch retrogradation by forming hydrogen bonds between fibre and starch (Elleuch et al., 2011). These authors reported a softer crumb when fibre was added to bread when compared to the control bread. In this study, the extent of the effect depended on the level of inclusions of SCC flour. Further incorporation of 30% of SCC flour into the muffin formulation significantly increased the hardness. The increase in hardness might be due to the large amount of insoluble dietary fibre in the muffins contributed by SCC flour which increased the force needed for compression. Nasar-Abbas and Jayasena (2012) reported that incorporation of lupin flour causes hardness, due to the higher dietary fibre content of lupin flour. Similarly, Martínez-Cervera et al. (2011) reported a decrease in hardness (*N*) in wheat flour muffin incorporated with increasing cocoa fibre (11.5 and 23.0g/100g flour) as compared to control muffin. However, a further increase of cocoa fiber (34.5g/100g flour) increases the hardness of the muffins. In addition, Gómez et al. (2010) reported the hardness of wheat based cakes increases when incorporated with high levels of insoluble fibers (20%).

Table 5.4: Texture parameters of muffin incorporated with different level of sweet corn cob flour.

SCC Flour (% by weight)	Hardness (g)	Springiness	Cohesiveness	Chewiness (g)	Resilience
0	376.00 ± 64.11^{b}	0.73 ± 0.05^{a}	0.30 ± 0.03^{a}	82.47 ± 22.76^{ab}	0.13 ± 0.02^{ab}
10	237.04 ± 48.95^{d}	0.76 ± 0.05^{a}	0.37 ± 0.03^{a}	66.54 ± 12.37^{b}	0.15 ± 0.01^{a}
20	325.00 ± 36.37^{c}	0.68 ± 0.03^{b}	0.34 ± 0.04^{a}	75.57 ± 13.51^{ab}	0.13 ± 0.02^{ab}
30	424.05 ± 53.06^{a}	0.63 ± 0.06^{c}	0.34 ± 0.06^{a}	93.93 ± 28.73^{a}	0.12 ± 0.02^{b}

Means ± standard deviation (n=3). Different letters within a column indicate significant differences at $p<0.05$.

Regarding the springiness and resilience, both parameters decreased as the level of SCC flour incorporation was increased. The decrease in resilience value showed that the muffin is denser with lower numbers of bubbles as SCC flour increases (Martínez-Cervera et al., 2012), thus a longer time is needed for the structure of the muffins to recover after compression. This is in line with the results reported in Section 5.3.3.2 where muffin with 30% SCC flour showed lowest number of bubbles, as compared to muffin with 10 and 20% SCC flour. Similarly, Gómez et al. (2010) reported reduction in resilience as fiber increases in gluten-free cakes. Muffins incorporated with 10% SCC flour showed an increase in springiness and cohesiveness (0.76 and 0.37, respectively), as compared to muffin prepared with rice flour only (0.73 and 0.30, respectively). With the percentage of SCC flour increase from 10 to 30%, the chewiness increased from 66.54 to 93.93g. Similarly, Grigelmo-Miguel et al. (2001) reported low level (2%) of peach dietary fibre showed similar textural characteristics to the control and a further increase of peach dietary fiber (>3%) increase the chewiness of muffins. In addition, Gómez et al. (2010) reported that an increase in fibre causes an increase in chewiness, springiness and cohesiveness in fiber-enriched layer cakes.

The results indicated that less crumbly and spongier GF muffins could be prepared by incorporating SCC flour at level of ≤ 20%. As most of the GF baked products in the market have a crumbling texture (Shevkani et al., 2015), our results showed that the textural profile of GF rice muffin can be improved by incorporating SCC flour.

5.3.4 Effect of baking on ferulic acid content in the muffin

The effect of baking on free, esterified and bound ferulic acid in muffin incorporated with different levels of SCC flour is presented in **Figure 5.3**. Baking significantly increased (p<0.05) the level of free ferulic acid in all muffin recipes except for the control muffin baked with rice flour. After baking, 0.29, 0.32 and 0.40mg/muffin of free ferulic acid was detected in muffins incorporated with 10, 20 and 30% of SCC flour. The significant increase in free ferulic

acid could be due to the release of bound ferulic acid, which may be responsible for the reduction in bound ferulic acid. This effect was similar to that observed by Abdel-Aal and Rabalski (2013), where free ferulic acid increased (70.5%) in wholegrain muffin baked with a mixture of einkorn and corn flour. Previously, Cheng et al. (2006) had reported that the degradation of conjugated polyphenolic compounds due to heat stress caused an increase in free phenolic acids in wheat.

A decrease of esterified ferulic acid was found in all muffins with the highest decrease in the muffin incorporated with 10% SCC flour (-37%), followed by muffin with 20% SCC flour (-29%), muffin with 30% SCC flour (-25%) and control muffin (-6%). Bound ferulic acid showed a different pattern depending on the muffin formulation. Significant increase in bound ferulic acid was found in control muffin. On the other hand, muffin incorporated with 30% SCC flour showed a significant decrease in esterified (from 2.20 to 1.64mg/muffin) and bound (from 35.68 to 21.53mg/muffin) ferulic acid before and after baking were observed. This suggest that the discrepancy might be due to the difference in the nature and source of flour used. Holtekjølen et al. (2008) reported an increased in bound phenolic acids in bread containing barley flour after baking. This might be due to the release of bound phenolics from the matrix during baking or thermal processing (Duodu, 2011). In contrast, Abdel-Aal and Rabalski (2013) reported that baking resulted in a slight decrease in bound phenolic acids in wheat muffins. Phenolics are very reactive and unstable (Cheynier, 2005), and degradation of phenolics will occur due to oxidation and heat treatment during the baking process. Cinnamic acids such as ferulic acid can decarboxylate under heat treatment to form ring-substituted styrenes (Maga and Katz, 1978). During baking, various mechanism such as thermal degradation, polymerization and oxidation of phenolics, depolymerisation of high molecular weight phenolics such as condensed tannins, products of Maillard reaction and release of bound phenolics from food matrix can influence the change in the phenolic acid profile (Duodu, 2011)

of a given food. In this study, the bound ferulic acid in SCC flour might be more sensitive to heat treatment as compared to the ferulic acid in rice flour leading to a decrease in bound phenolics after baking. In this study, the highest amount of total ferulic acid was observed in muffin incorporated with 30% (23.58mg/muffin) and 20% of SCC flour (20.15mg/muffin), followed by muffin with 10% SCC flour (10.08mg/muffin) and control muffin with 0% SCC flour (1.76mg/muffin). A significant decrease in total ferulic acid was found in muffins incorporated with 30% SCC flour before and after baking. The decrease in total ferulic acid content is due to the significant decrease in bound phenolics after baking. Although muffin incorporated with 20% and 30% of SCC flour showed a decrease in the total ferulic acid content after baking, the level of ferulic acid still remained high (20.15 and 23.58mg FA/muffin, respectively) as compared to control muffin (1.76mg FA/muffin).

The increasing replacement of rice flour with SCC flour in the making of muffin increased the total ferulic acid content and impacted the texture profile of the muffin. Results showed that substituting rice flour with SCC flour at a level of 10 to 20% produced muffins with better textural properties than control muffin with 100% rice flour. Moreover, the nutritional value of the GF rice muffin was improved since the enrichment with SCC flour results in a progressively higher fibre and ferulic acid content. This study showed that GF rice muffins with SCC flour up to 20% can improve height, colour and texture as well as increasing the amount of total ferulic acid in the muffin. The use of SCC, a by-product from the corn processing industry can offer alternative solutions towards environmental concerns regarding disposal. The results indicate that SCC could be considered as an alternative GF flour or value-added food ingredient for bakery products, or functional foods and nutraceuticals.

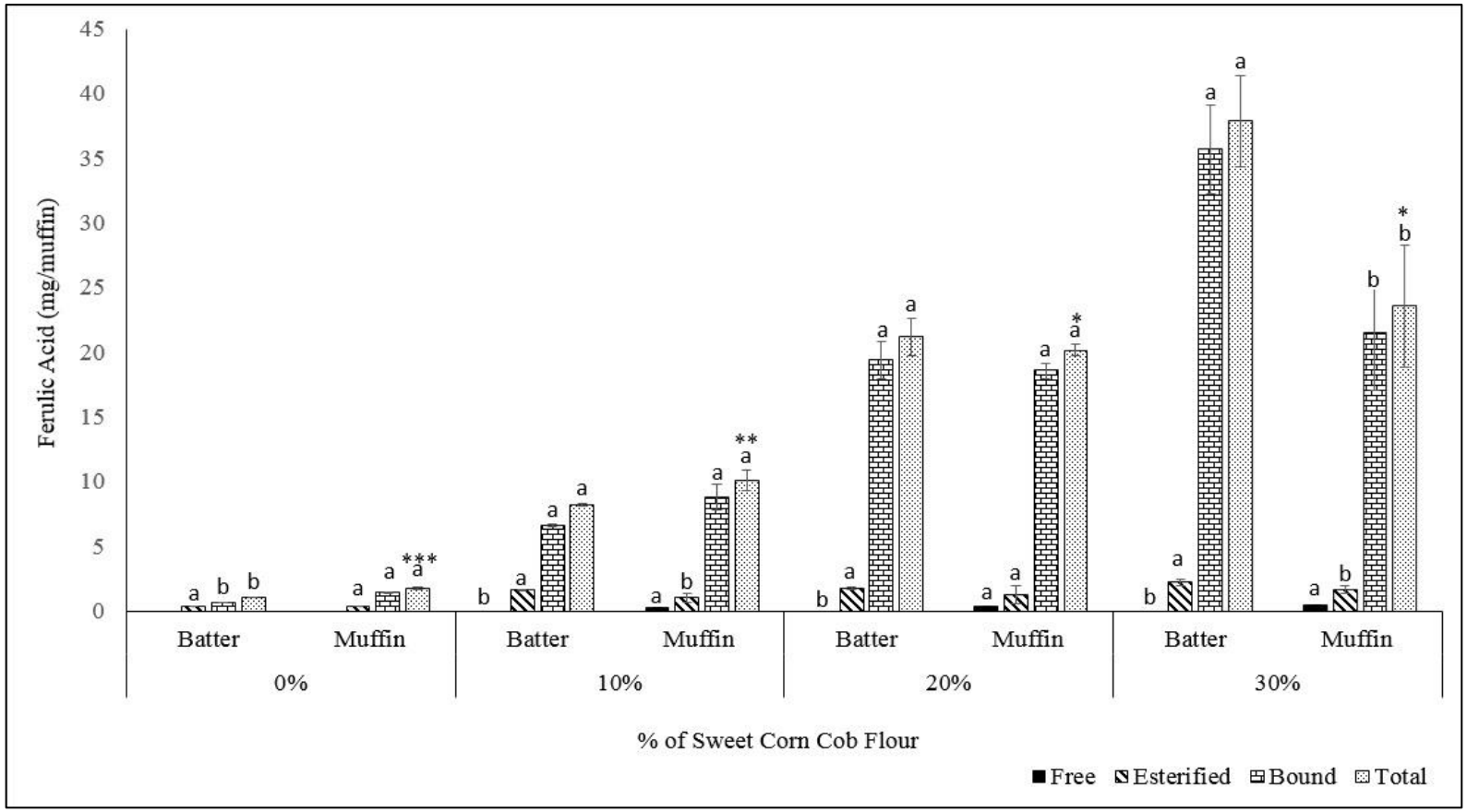

Figure 5.3: Changes in ferulic acid content (free, esterified, bound and total) in batter and muffin incorporated with different levels of sweet corn cob flour (10, 20, and 30%).

Results are expressed as mg/muffin (n=3). Different letters within the same fraction of batter and muffin indicate significant difference at p<0.05.

** showed significant difference in total ferulic acid content in muffin prepared with different formulations.*

Chapter 6

Concluding remark and future work

6.1 General Discussion

Sweet corn cob (SCC) generated from the corn milling industry has limited use in the food industry. Therefore, the main goals of the present study were to investigate the bioactive compounds present in sweet corn cob and secondly, to explore its potential in the food industry by using it as a flour for baking.

Compositional analysis of SCC showed that cellulose was present at the highest amount (40.40%) followed by hemicellulose (19.12%), lipid (7.18%), protein (6.70%), starch (3.21%) and ash (3.04%). In addition, phosphorus (10.12mg g^{-1}), potassium (9.62mg g^{-1}) and magnesium (1.67mg g^{-1}) also present in SCC, followed by manganese (0.08 mg g^{-1}), lead (0.07mg g^{-1}), zinc (0.04mg g^{-1}), iron (0.01mg g^{-1}) and copper (0.01mg g^{-1}). Non-essential amino acids are present at a higher amount, with the highest in serine (1.36mg g^{-1}) and glutamine (1.16mg g^{-1}), as compared to the essential amino acids. This showed that SCC has the potential to be a source of insoluble dietary fibre, minerals and non-essential amino acids. Then, phenolic compounds in SCC were fractionated via alkali hydrolysis into free, esterified and insoluble-bound fractions. The insoluble bound fraction showed the highest in total phenolic content and antioxidant assays (TEAC, DPPPH and FRAP). The HPLC analysis showed that the insoluble-bound fraction contained the highest amount of ferulic and *p*-coumaric acid, as compared to free and esterified phenolics. Pearson correlation analysis found a high correlation between total phenolic content and antioxidant assay, indicating that phenolic content is responsible for the antioxidative effect of SCC. Furthermore, ferulic and *p*-coumaric acid showed a highly positive correlation with the antioxidant assays and total phenolic content, which further confirmed its antioxidant effect. The carotenoid content in SCC was extracted

via SFE and compared against conventional solvent extraction. Results showed that SFE was more effective in the extraction of carotenoid, and β-carotene (177.29 mg kg^{-1}) was found at the highest level, followed by zeaxanthin (8.47mg kg^{-1}) and lutein (3.81mg kg^{-1}). For centuries, carotenoids have been used as natural food colorants and can be extracted from rich natural sources such as saffron, annatto, tomato and marigold (Rodriguez-Amaya, 2018). The results showed that SCC can be a potential source of ferulic and *p*-coumaric acid, as well as carotenoid (β-carotene, zeaxanthin and lutein).

Over the years, there has been an increase attention towards the use of a greener and more sustainable approach in the release of the bound phenolics due to the environmental impact of using large amount of solvents. In Chapter 2, alkali hydrolysis was used to hydrolyse SCC, liberating its bound phenolic content. The amount of alkali-hydrolysed phenolic compounds obtained was used to compare the effectiveness of enzyme hydrolysis of SCC (Chapter 3). In this research, ferulic acid esterase (FAE) was used to break the linkage between ferulic acid and the arabinoxylan backbone, releasing the bound ferulic acid. However, the complex nature of the arabinoxylan structure of SCC hindered the action of FAE and hence, endoxylanase was used in combination with FAE to release the ferulic acid. Xylanase (XY) breaks the glycosidic linkages of the arabinoxylan to ease the action of FAE. Optimisation of pH, temperature, enzyme concentration is crucial to maximise the yield of ferulic acid. Response surface methodology was used to optimize these parameters and to study the interactions between the parameters. Under optimised conditions, only half the amount of ferulic acid (1.62mg g^{-1}) was released, as compared to alkali hydrolysis (3.06mg g^{-1}). Enzymatic hydrolysis of SCC using the combination of FAE and XY successfully released the bound ferulic acid in SCC, however, it is not as effective as alkali hydrolysis.

The biological activity of dietary polyphenol depends on their bioavailability, where it is modulated by gut microbiota. Most of the dietary phenolics were poorly absorbed in the

small intestine, and when they reached the large intestine, they were metabolized extensively by the gut microbiota. SCC and SCC extract (extracted enzymatically) were subjected to batch culture fermentation to examine their effect on the ecology of the gut microbiota and production of beneficial short chain fatty acids. The composition of SCC was reported previously and is comprised mainly of insoluble dietary fibre and bound phenolic compounds. On the other hand, SCC extract contains free phenolic compounds (including ferulic and *p*-coumaric acid) and xylooligosaccharide (xylobiose and xylotriose). The bacterial populations were enumerated by fluorescence *in-situ* hydridization (FISH). All results in batch culture fermentation were shown to be not statistically significant. However, a trend in increasing *bifidobacteria* and a decrease in pathogenic *Clostridium spp.* was found in vessels containing SCC extract. This showed the possible bifidigenic effect of SCC extract. On the other hand, gas chromatography analysis of short chain fatty acid in SCC showed a significant increase in production of acetate, propionate and butyrate, possibly due to the content of dietary fibre.

Research on the application of SCC in the food industry is limited. SCC flour was incorporated into the formulation of rice flour muffin to examine its effect on the quality and texture of muffins. The incorporation of SCC flour (≤20%) produces muffin with increased height and number of bubbles, and a decrease in specific volume and hardness as compared to the controlled muffin baked with 100% rice flour. The higher water and oil holding capacity of SCC due to the higher content of dietary fibre as compared to rice flour increases the viscosity of the batter and thus, increased the number of bubbles and decreased the firmness of the muffins. In addition, the carotenoid content of SCC flour gives the characteristic yellowness in the muffin crust and crumb. Baking results in an increase in the amount of free ferulic acid in the baked muffin. The effect of baking on bound ferulic depends on the nature of the flour, where increased bound ferulic acid was observed in the control muffin baked with rice flour. On the other hand, muffin incorporated with 30% of SCC flour showed a decreased amount of

bound ferulic acid after baking. This might be due to the different heat sensitivity of the ferulic acid in rice and SCC flour. Although decreases in bound ferulic acid were observed in muffin incorporated with SCC flour, the muffins (20 and 30% of SCC flour) still contained a higher amount of total ferulic acid after baking, as compared to the control muffin.

Based on the results of this research, SCC contained considerable amounts of bioactive compounds such as carotenoid and phenolic compounds. These compounds can be extracted for further application as natural antioxidants or colorants. Furthermore, SCC can also be used as a source of insoluble dietary fibre and minerals including potassium, phosphorus and magnesium. The extraction of carotenoids via supercritical fluid extraction and bound phenolics via enzymatic hydrolysis provides an alternative towards green extraction technology. Besides extraction of the bioactive compounds, the SCC can also be incorporated into the baking of muffin to improve the texture, quality and nutrition of rice flour muffin. This research provides an insight on the valorisation of SCC into functional food and thus, reducing the environmental impact associated with the disposal of this lignocellulosic waste.

6.2 Contribution to knowledge

Whilst there is abundant literature regarding the use of SCC as biomasses, there have been limited studies exploring the application of SCC as a functional food. The results from this research provide useful information to the community, food scientists and agricultural waste valorisation industries, which can be used to further develop SCC as a functional food. The contribution to the scientific knowledge are summarised as follows:

(i) The compositional analysis of SCC including phenolic and carotenoid content was carried out.

(ii) The extraction efficiency of bound ferulic acid from SCC using the enzymes ferulic esterase and xylanase was investigated and compared with alkali hydrolysis.

(iii) The effect of free and bound ferulic acid, as well as fibre of SCC on the ecology of gut microbiota was carried out.

(iv) The effect on texture, quality and ferulic acid content of gluten-free rice flour muffin incorporated with SCC flour was studied and compared with those made with rice flour.

6.3 Limitation and future work

During this research, there were several limitations needed to be addressed. Although there were several interesting findings being discovered, however not all were fully explored due to the limited time frame and scope of the study. Further investigations can be explored to fill the gap of knowledge and provide extra information regarding the valorisation of SCC.

- Due to the large amount of SCC being used in this study, the SCC used in Chapter 4 was obtained from a different batch of SCC as compared to the rest of the chapters. Although both batches were harvested in the same country, Senegal, the second batch (harvested in December 2017) showed double the amount of total ferulic acid as compared to the first batch (harvested in December 2015). The phenolic composition has been reported to depend on different factors such as climatic conditions, variety, fruit ripeness, and storage conditions (Škevin et al., 2003). Therefore, further investigation is needed to fully understand the effect of these conditions on the accumulation of ferulic acid in SCC.
- The combination of enzyme ferulic acid esterase and xylanase extracted only half the amount of ferulic acid as compared to alkali hydrolysis. This might be due to the complex structure of SCC hindering the action of these enzymes to release the bound ferulic acid. Novel technology such as pulsed electric field or

ultrasonic can be used prior/in conjunction with enzymatic hydrolysis to increase the enzyme accessibility and thus increase the yield of ferulic acid.

- Large variation between the gut microbiota ecology among the donors caused no significant differences among the results. Therefore, an increase in the number of replicates might help to improve the results. Furthermore, the amount of ferulic acid used in Chapter 4 for batch culture fermentation was based on the daily intake of ferulic acid (150mg), however, there was no significant difference among the substrates being investigated. Hence, the minimum amount of ferulic acid required to increase the amount of beneficial gut microbiota can be further investigated.
- Due to time constraints, the antioxidant activity of muffins with SCC flour was not evaluated. Therefore, it would be of interest to investigate the effect of heat treatment on antioxidant activity of muffin incorporated with SCC flour. The shelf life study of these muffin incorporated with SCC flour can be further investigated to evaluate the effectiveness of the antioxidative effect of SCC flour in shelf life extension. Further consumer research studies would be of great importance in order to truly understand the consumer perceptions and to evaluate the consumer's acceptability of SCC flour in gluten free rice muffin.

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Appendix I

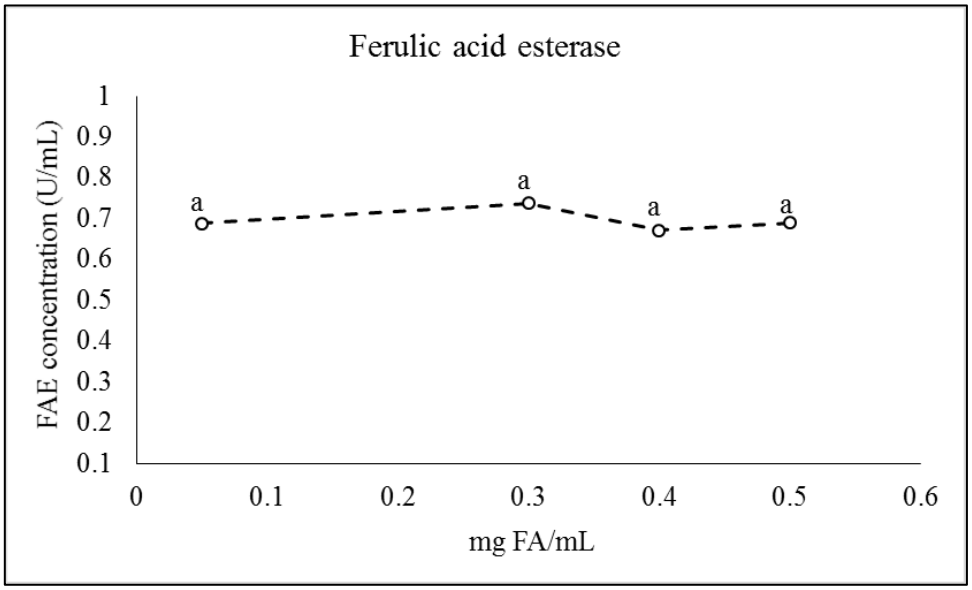

Figure A1: Yield of ferulic acid (mg/g) after enzymatic hydrolysis of sweet corn cob by ferulic acid esterase at different concentration (U/mL).

Significant differences between substrates at the same time point are indicated with letters ($p < 0.05$).

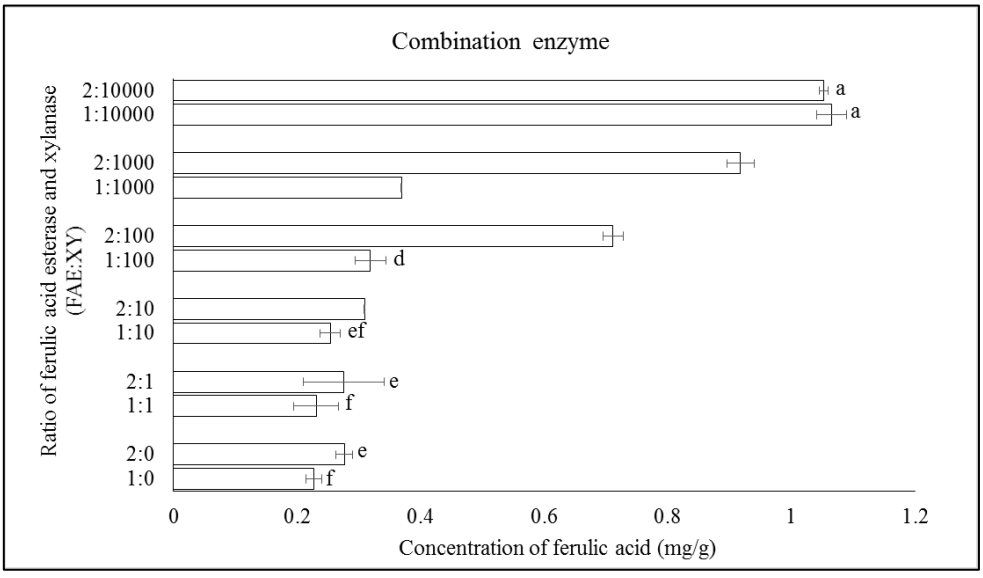

Figure A2: Yield of ferulic acid (mg/g) after enzymatic hydrolysis of sweet corn cob by combination of ferulic acid esterase and xylanase at different concentration (U/mL).

Significant differences between substrates at the same time point are indicated with letters ($P < 0.05$).

Appendix II

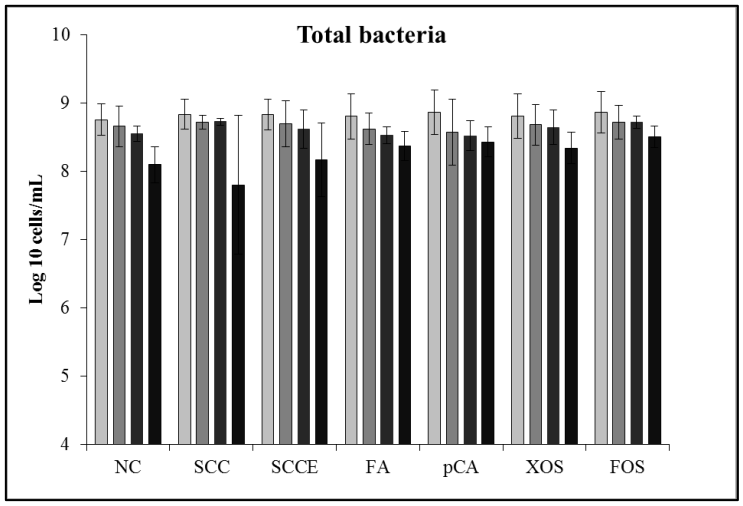

Figure A3: Total bacteria population analysed by Flow-FISH in pH controlled batch cultures containing different substrates.

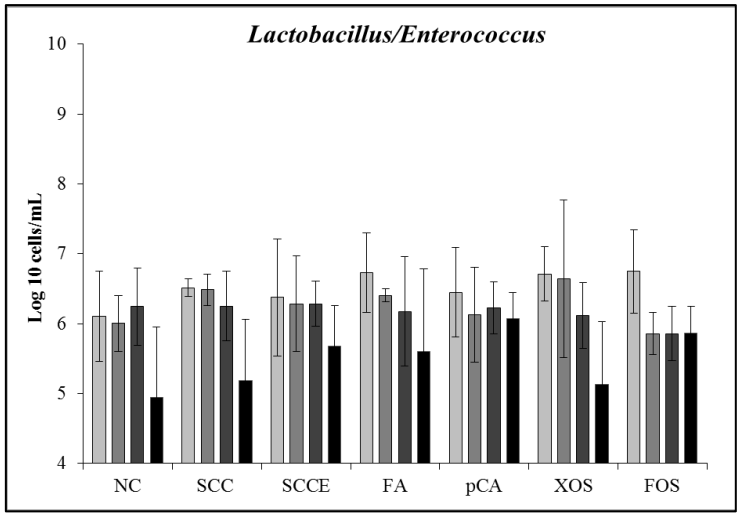

Figure A4: *Lactobacillus/Enterococcus* population analysed by Flow-FISH in pH controlled batch cultures containing different substrates.

*(NC= negative control; SCC= Sweet corn cob; SCCE: Sweet corn cob extract; FA: ferulic acid, pCA: p-coumaric acid; XOS: Xylooligosaccharide and FOS: Fructooligosaccharides). Error bars indicate SD (n = 3). Significant differences between substrates at the same time point are indicated with letters (P < 0.05). * indicates significantly different compared to 0 h within the same substrate (P < 0.05).*